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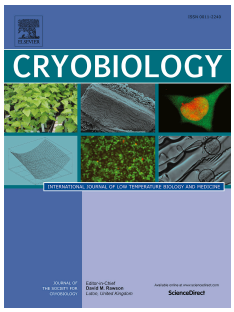

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Title

Rapid cooling of rabbit embryos in a synthetic medium

Magda Teixeira[1,*], Loris Commin[1], Lucie Gavin-Plagne[1], Pierre Bruyère[1], Samuel Buff[1], Thierry Joly[1,2]

1: Université de Lyon, VetAgro Sup, Interaction cellule environnement, Marcy l'Etoile, France

2: Université de Lyon, ISARA-Lyon, Lyon, France

[*]corresponding author: magda.guedes-teixeira@vetagro-sup.fr

Abstract

Embryo cryopreservation media usually contain animal-derived products, such as bovine serum albumin (BSA). These products present two major disadvantages: an undefined variable composition and a risk of pathogen transmission. We aimed to evaluate the effect of replacing BSA in rabbit embryo rapid cooling “freezing” and warming media with a chemically defined medium with no animal-derived products: STEM ALPHA.Cryo3 (“Cryo3”).

A total of 1540 rabbit morulae were divided into three cryopreservation groups (group 1: BSA, group 2: 20% Cryo3 and group 3: 100% Cryo3) and a fresh controls group. After rapid cooling, embryos were cultured (*in vitro* approach), or transferred into synchronized does (*in vivo* approach). In the *in vitro* approach, post-warm survival rates obtained with 100% Cryo3 (94.9 %) were superior to BSA (90.8%) and 20% Cryo3 (85.6 %). The blastocyst formation rate was similar between BSA, 20% Cryo3 and 100% Cryo3 groups (85.1, 77.9 and 83.3 %, respectively), as was the expansion / hatching rate (63.1, 63.4 and 58.0%, respectively) and embryo mitochondrial activity. In the *in vivo* approach, pregnancy (80.0, 68.0 and 95.2 %, respectively), implantation (40.5, 45.9 and 44.8%, respectively), and live-foetus rates (35.6, 35.5 and 38.1 %, respectively) were similar between the three groups. To conclude, Cryo3 can replace BSA in rabbit embryo rapid cooling “freezing” and warming media.

1. Introduction

Over the past few decades, embryo cryopreservation has become crucial to the long-term preservation of genetic material in biobanks. Along with embryo transfer (ET), this technology has contributed to the distribution of genetic materials worldwide, replacing animal exchange [36]. The World Organisation for Animal Health assembled recommendations on risk management procedures concerning embryo collection and processing [74]. Even if these guidelines are the best way to reduce infectious disease transmission, embryo contamination is still of concern to health authorities [36].

Animal-derived products, such as bovine serum albumin (BSA) or foetal calf serum, also referred to as foetal bovine serum, are commonly added to animal embryo cryopreservation media [5,49,66,68,77]. Serum-derived product composition is poorly known. Media containing BSA or serum are classified as semi-defined or non-defined, respectively [72]. These products contain growth factors, cell attachment and spreading factors, hormones, carbohydrates, amino acids, proteins (such as albumin), vitamins and various undefined molecules [9,72].

Serum-derived products promote embryonic viability and development [4,9,60,69,71,72] and have numerous advantageous properties in cryopreservation media, such as metal chelating activity, oncotic pressure regulation, pH regulation [22] and toxin-scavenging [11]. Additionally, animal sera have surfactant properties, which reduce the surface tension in the media, preventing embryos from floating or sticking to glass and plastic surfaces [22,73], and avoiding the adsorption of some media compounds (as hormones, growth factors and carrier proteins) to the material surfaces [53]. Moreover, the addition of serum-derived products to the cryopreservation media seems to protect embryos from possible toxic effects of cryoprotectants during the cryopreservation process [23,55].

Despite the numerous beneficial effects of serum on embryos during and after cryopreservation, negative effects have also been suspected. Ruminant embryos cultured with serum before blastocyst formation may present increased incidence of unusual development, accompanied by "large offspring syndrome": high birth weight, prolonged gestation, frequent dystocia, elevated abortion rates and organ defects [35,76].

Sera can be contaminated with pathogenic agents such as bacteria, viruses [20,58], yeast, fungi, and mollicutes such as mycoplasmas [14], or prions [44], even if the risk of prion contamination seems to be low [75]. Although commercial sera are usually declared to be pathogen-free, treatments like heat inactivation and gamma irradiation don't always seem to be efficient [58].

The advantages of using synthetic medium, in cryopreservation media are widely recognized as providing more defined, more consistent and more reproducible conditions, in addition to avoiding animal welfare and ethical concerns.

Numerous studies have aimed to replace animal products in cryopreservation media with media free of animal-derived products, such as silk protein sericin [24], vegetal peptones [18], HA [25,52], and non-organic macromolecules such as polyvinyl alcohol [21,38,50,62], polyvinylpyrrolidone [21,32,65] and Ficoll [21,32]. Hyaluronic acid (HA) is a glycosaminoglycan that can be synthetized in its pure form [16] and can be found in follicular, oviduct and uterine fluids [37] and its concentration increases in the uterus by the time of implantation [78]. After successfully replacing albumin in embryo culture [17,42], HA became an interesting candidate to replace animal products during cryopreservation.

Animal derived sera composition not only changes between batches but is also extremely variable [9]. This variation can occur as a result of physiological and biochemical differences between donors [40], and more generally with gender [2], age [29], diet [41], photoperiod [64] and preparation methods [33]. Regarding embryotrophic properties of BSA, some authors observed considerable variations between suppliers and even between distinct lots from the same supplier [4,27,45].

STEM ALPHA.Cryo3 (referred to as "Cryo3", Stem-Alpha, Saint-Genis-l'Argentière, France), is a patented serum-free, protein-free and dextran-free medium (manufactured according to good manufacturing practices [cGMP-annex 1] in compliance with 2001/83/EC). CRYO3 is composed of synthetic HA of high molecular weight (> 106 D), glucose, carbohydrates, amino acids, mineral salts, vitamins, fatty acids esters and buffers. This product was originally created for clinical applications, as a serum substituent in somatic and human adult stem cell freezing medium.

Bruyère (2013) investigated foetal calf serum thermodynamic properties of three different suppliers and compared them to the synthetic medium Cryo3 (used at 18% v/v). All the solutions presented similar thermodynamic characteristics, but media containing foetal bovine serum presented more variable results, as well as aberrant values, unlike 18% Cryo3 medium, whose results appeared to be more stable.

The impossibility of characterizing animal-derived product composition and its variability lead to unpredictable development rates and to experimental results that might not be reproducible.

Consequently, all serum-derived products seem to be unsuitable when the goal of a study is to obtain defined media and standardized cryopreservation methods.

Bruyère observed that Cryo3 can successfully replace animal products in rabbit embryo and bovine embryo slow-cooling “freezing” media [7,8].
Rapid-cooling “freezing” procedures comprise the use of higher solute concentrations than slow-cooling “freezing”. These solutions, combined with a rapid cooling technique (such as direct plunging in liquid nitrogen), allow the formation of an amorphous state during cooling, avoiding the danger of ice crystal formation that occurs during slow-cooling “freezing”. However, unlike vitrification media, the formation of ice crystals during rapid-cooling “freezing” procedures is possible, especially during warming, if (i) warming rates are not quick enough [70], (ii) insufficient high total solute concentration or (iii) exposure to cryopreservation solution was too brief.

The aim of our study was to evaluate the effect of replacing BSA with Cryo3 in rapid cooling “freezing” and warming solutions on the *in vitro* and *in vivo* development of rabbit morulae.

2. Materials and methods

The Ethical and Animal Welfare Committee of VetAgro Sup approved this study (Permit Number: 05/26). All animals were handled according to the EU Directive 2010/63/EU for animal experiment guidelines. Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.1 Embryo production and recovery

A total of 62 rabbit New Zealand does (SARL HYCOLE, Marcoing, France) were housed in groups of five and fed a commercial diet. Does received five doses of a pFSH:LH (ratio 5:1, 31.5 µg total, Stimufol, Reprobiol, Belgium) preparation (administered twice-daily, subcutaneously). Eight hours after the last injection, does were inseminated with sperm from multiple males (pooled ejaculates), and an intramuscular injection of buserelin (2.0 µg Receptal, MSD Animal Health, Beaucouzé, France) was administered.
Rabbit does were euthanized 65 to 68 h after the buserelin administration by cervical dislocation. The oviducts and uteri were flushed using Euroflush (IMV Technologies, L’Aigle, France) at room temperature. Embryos were recovered at the morula stage and classified according to the International Embryo Transfer Society (IETS) manual, [6][5][48]Bó and Mapletoft 2013) and quality 1 embryos [6] were pooled. Embryos (n = 1540) were randomly divided into three cryopreservation groups and two control groups.
A group of embryos (n = 40) was cultured without cryopreservation (*in vitro* fresh control), and a group of embryos (n = 59) was transferred without cryopreservation (*in vivo* fresh control).

2.2 Embryo rapid cooling

Unless specified otherwise, all percentages are expressed as volume/volume.

Prior to rapid cooling, embryos (n = 1441) were randomly divided into three cryopreservation groups. All media contained the same cryoprotectant composition and the following base media: group 1 cryopreservation medium: IMV Embryo holding medium (IMV Technologies, L'Aigle, France), containing 0.4 % (w/v) BSA (n = 543); group 2 cryopreservation medium: D-PBS supplemented with 20% of Cryo3 (n = 423); group 3 cryopreservation medium: 100% Cryo3 medium (n = 475). Embryos were transferred into a first equilibration solution composed of 5 % Me_2SO and 5 % ethylene glycol (EG) (5 min), and a second equilibration solution composed of 10% Me_2SO and 10% EG (2 to 3 min). Embryos were then exposed to the cryopreservation solution of the correspondent group (30 sec) containing 20% Me_2SO (approx. 2.8 M) and 20% EG (approx. 3.6 M), before being loaded to a Fibreplug (CVM kit, Cryologic) and cryopreserved by solid surface vitrification (three to four embryos per Fibreplug). Warming was performed by immersing the end of the Fibreplug directly into a thawing solution (0.5 M sucrose in group 1, group 2, or group 3 base medium, respectively) at 38.5 ℃ for 5 min, followed by three successive dilution baths (0.3 M, 0.1 M and 0.0 M sucrose).

2.3 *In vitro* embryo culture and morphology assessment

Embryos (n = 40) from the *in vitro in vitro* fresh control group were cultured (38.5 ℃, 5 % CO_2) to the expanded blastocyst stage in Medium 199 (without glutamate) supplemented with 10% foetal calf serum and antibiotics (67 UI/mL penicillin and 67 µg/mL streptomycin, Dutscher, Brumath, France). *In vitro* development was assessed after 24 h and 48 h of culture and classified according to their development stage as morula, blastocyst, expanded and hatching embryos. Slightly expanded blastocysts with herniation of embryonic cells (Figure 1) were included in the expanded / hatching embryo group.

2.4 *In vivo* embryo transfer

Fresh embryos (n = 59) and warmed vitrified (total = 905; group 1 n = 358, group 2 n = 270, group 3 n = 277) embryos were transferred to synchronized New Zealand recipient does (n = 84), according to the protocol described by Salvetti [61]. Briefly, recipient does were synchronized with a buserelin injection (0.8 µg, intramuscular, Receptal), 50 to 60 h before transfer. After anaesthesia, a midventral laparotomy was performed, and 4 to 7 embryos (mean = 5.4) were transferred to each uterine horn. Pregnancy diagnosis was realized by palpation 20 days after embryo transfer.

2.5 Mitochondrial activity assessment with JC-1

The cationic dye JC-1 (5,5'6,6'-tetrachloro-1,1,3,3'tetraethylbenzimidazolycarbocyanine iodide; Thermofisher Scientific, Illkirch, France) exhibits different fluorescent properties, based on its accumulation within mitochondria. J-aggregates accumulate in mitochondria with high mitochondrial membrane potential (MMP), showing red fluorescence, while J monomers accumulate in low MMP mitochondria, presenting green fluorescence [54]. Consequently, embryos with more active mitochondria exhibit higher red to green ratios than less active or inactive

embryos. At the end of embryo culture, living embryos (n = 89) at the expanded / hatching stage from the three cryopreservation groups were subjected to a pretreatment of pronase (a protease, from *Streptomyces griseus*, 5 mg/mL) in Dulbecco's Phosphate-Buffered Saline medium (D-PBS) supplemented with D-glucose (5.56 mM), sodium pyruvate (0.33 mM) and bovine serum albumin (3 mg/mL), at 38.5 ℃, until the mucin coat began to d issolve. Embryos were then washed in six drops of modified D-PBS. Embryos were incubated with JC-1 for 75 min (1.5 µM, 38.5 ℃, 5 % CO2) and observed using an Olympus IX71 epifluorescence microscope, with an excitation wavelength of 488 nm. JC-1 aggregates were detected with a red filter (590 nm wavelength), whereas JC-1 monomers were detected with a green filter (530 nm wavelength). To evaluate embryo mitochondrial activity, the staining intensity (by pixel) was measured, from both channels, in two randomly defined areas on each embryo, using the Fiji package [63] of ImageJ software (National Institute of Health, Bethesda, Maryland, USA), and the red to green ratio was quantified.
An MMP disruptor (CCCP, carbonyl cyanide 3-chlorophenylhydrazone; Thermofisher scientific, Illkirch, France) was used as a control to confirm that directional changes in the dye signal were correctly interpreted.

2.6 Statistical analysis

In vitro and *in vivo* development rates were analysed with the chi-square test, whereas JC-1 red/green ratios were analysed by one-way analysis of variance. All tests were performed with R-Studio software [57]. Groups were considered significantly different at $p < 0.05$.

3. Results

In vitro and *in vivo* embryo development after cryopreservation

The *in vitro* blastocyst formation and expansion/hatching rates and *in vivo* development rates (pregnancy rate, implantation live-birth rates) after rapid cooling with media containing animal products or chemically defined products (group 2 and group 3) are summarized in Table 1. *In vitro* fresh control embryos expressed significantly superior blastocyst and expansion / hatching rates. The group 3 medium appeared significantly superior in *in vitro* post-warm survival rates than group 1 and group 2 media. No significant differences were observed regarding the other *in vitro* and *in vivo* development rates.

Mitochondrial activity assessment with JC-1

Ratios of J-aggregate to J-monomer of cryopreserved expanded or hatching embryos, cryopreserved with media containing animal products (group 1) or chemically defined products (groups 2 and 3) are represented in Figure 2 and summarized in Figure 3. No significant differences were observed between the three groups. After incubation with the CCCP control, images showed no red fluorescence.

4. Discussion

Over the last few decades, there have been important efforts to replace animal serum with defined media containing no animal products for embryo cryopreservation. Numerous natural or synthetic molecules have been used in slow cooling, as in rapid cooling media, to replace the biological and the physical properties of animal albumin. Studies demonstrate that animal products can be successfully replaced with products such as the silk protein sericin [24] and vegetal peptones [18] during bovine embryo slow-freezing, or HA during murine [26,52], bovine [51], and ovine [26] slow freezing.

The non-organic macromolecule polyvinyl alcohol has been used to slow-freeze and vitrify embryos from different species, obtaining equivalent post-thaw development rates for murine [21,50,51] and porcine [62] embryos. However, inferior development rates were also obtained for murine [12], bovine [51,65,67] and ovine [38] embryos. Studies using polyvinylpyrrolidone tend to demonstrate a negative effect on cryopreservation media [65], as well as inferior surfactant properties [21,65] and toxicity to embryos [13,32].

Kuleshova *et al.* cryopreserved mouse embryos by rapid-cooling, using animal product free media containing 35 % polymers (dextran or Ficoll) and 25 % of penetrating cryoprotectants (EG), using a double straw arrangement to diminish contamination risk, obtaining *in vitro* development rates of 100 % blastocyst expansion and *in vivo* foetuses rates of 76 % [31]. One year later, these authors obtained development rates (96 - 100% blastocyst expansion and 62 - 76 % live foetuses) after vitrifying mouse embryos with 34 to 49 % (w/v) of macromolecules (Ficoll or dextran) and 11 to 27% (w/v) EG, in protein-free media [32]. However, these authors did not compare these protein-free media with media containing animal products [32]. Another author evaluated the substitution of foetal calf serum with Ficoll, on mouse embryo quick freezing, obtaining equivalent development rates [21]. These studies suggest that these two molecules may be good candidates for replacement of animal products.

In 1990, Palasz obtained equivalent post-thaw murine and bovine embryo development rates after embryo slow-frezing with synthetic HA and with NCS (n = 206) [52]. Joly observed equivalent post-thaw murine (n = 443) and ovine embryo (n = 120) *in vitro* development rates, after embryo slow-freezing in media containing HA and BSA [26].

Bioniche Life Sciences Inc. developed synthetic holding and freezing media (SYNGRO®) for bovine, equine, sheep and goat embryos, based on synthetic HA. However, few studies regarding cryopreservation were published with these commercial products [22]. Some authors used these media to slow-freeze equine embryos [3] and to slow-freeze and vitrify bovine embryos [30], but these studies didn't aim to compare with animal derived product based media.

In our previous work, we showed that animal products could be successfully replaced with 20 % Cryo3 in bovine [7] and rabbit embryo slow-freezing [8], where better *in vivo* development rates were obtained with 20% Cryo3, compared to foetal calf serum [8].

In the present study, we evaluated the effect of replacing BSA in rapid-cooling solutions and in warming solutions, using the same synthetic product as in our previous studies: Cryo3.

In *in vitro* experiments, significantly superior survival rates were observed in the 100% Cryo3 (group 3) compared to BSA (group 1) or 20% Cryo3 (group 2). No differences were found

regarding blastocyst formation, blastocyst expansion or blastocyst hatching development rates between groups. In the literature, quite variable post-warm *in vitro* development can be found (survival: 95.3 - 95.6 %, blastocyst formation: 56 – 91.7 %, hatching or expansion: 45 - 91.7 %) [39,43,47,56]. This variability may depend on several factors, such as donor genetics, the housing conditions of the animals and the embryo culture medium.

Our post-warm development rates were in the range of values found in the literature.

Embryos were not subjected to a pronase treatment to remove the mucin coat prior to culture. Kasai compared the *in vitro* development with and without mucin coat digestion and observed that approximately half of the non-treated embryos did not expand to a diameter more than twice that of the morula (46 % non-treated vs 92 % treated embryos) [28]. Fischer observed that uterine components are vital in the transformation of the extracellular coverings in the rabbit embryo. In rabbit culture media lacking uterine components, the *zona pellucida* does not dissolve and loses elasticity, leading to herniation of embryonic cells into the mucin coat, instead of expansion and hatching [15]. Indeed, we observed slightly expanded embryos with embryonic cell herniation (Figure 1) in cryopreserved and in non-cryopreserved groups. Considering these findings, we pooled slightly expanded herniated blastocysts with expanded and hatching blastocysts.

To evaluate mitochondrial activity between cryopreservation groups, we only used developed embryos. If we had randomly picked embryos from all developmental stages, the development rates would have influenced the total mitochondrial activity and, therefore, confound our results. We obtained equivalent mitochondrial activity between the three groups, suggesting that all the developed embryos had the same energetic capacity of continuing further development. The obtained JC-1 ratios are equivalent to ratios found in the literature for fresh mouse blastocysts (approx. 1.35) [1]. Images obtained with the CCCP control demonstrated the JC-1 ratio is dependent on mitochondrial potential variations.

In our *in vivo* experiments, no statistically significant difference was observed between fresh and cryopreserved embryos. No difference was found regarding pregnancy rates of the three rapid cooling groups, even if group 3 rates tended to be superior. Equivalent implantation rates and live-birth rates were obtained with the three rapid cooling media groups. As in *in vitro* development studies, post-transfer *in vivo* development rates found in the literature can considerably vary (pregnancy: 56 - 100%, implantation: 8.7 – 65 %, live foetuses: 6.4 – 57.1 %) [25,28,43,48,59]. *In vivo* development rates may depend on the cryopreservation medium, cooling device and technique, transfer conditions (laparotomy / endoscopy, surgeon), and the housing conditions of the animals. The *in vivo* development rates obtained in our study were in the range and close to the superior limit for pregnancy and implantation rates.

Regarding cryopreservation effects on embryos, a difference was found between fresh (expansion or hatching rate 97.5 %) and cryopreserved embryos during *in vitro* development, but this difference was no longer observed after *in vivo* transfers. A possible explanation would be that cryopreservation partially impairs embryos, and this damage can be reversible if embryos return to physiological conditions after cryopreservation. Both *in vitro* and *in vivo* experiments in this study indicate that animal products can be replaced by both concentrations (20% and 100%) of Cryo3.

Ménézo and Khatchadourian observed that non-defined peptides could bond to albumin, with subsequent deleterious effects on embryo post-thaw survival [46]. When using cryopreservation media entirely composed of synthetic chemically defined products, such as Cryo3, these interactions are avoided.

Moreover, the use of a commercial synthetic medium for embryo cryopreservation, prepared industrially under rigorous quality control instead of laboratory-made media, avoids preparation variability, and increases reproducibility and standardization of the cryopreservation process.

5. Conclusion

The results from this study seem to demonstrate that a chemically defined substitute (STEM ALPHA.Cryo3) can successfully replace BSA in rabbit embryo rapid-cooling and warming media. The elimination of animal products of embryo cryopreservation media may improve procedure standardisation, by avoiding variability in media composition and, consequently, more variable results. Additionally, it would avoid sanitary concerns inherent to animal-derived products.

To improve sanitary conditions, we have replaced BSA with 100% Cryo3 medium in rabbit embryo rapid-cooling media in the French National CryoBank.

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Table 1. *In vitro* and *in vivo* rabbit embryo development rates after rapid cooling with media containing animal products (group 1) or chemically defined products (group 2 and group 3).

		Group 1 (0.4 % BSA)	Group 2 (20% Cryo3)	Group 3 (Cryo3)	Control (Fresh)
In vitro development	**% Survival**	90.8%[a] (168/185)	85.6 %[a] (131/153)	94.9 %[b] (188/198)	
	% Blastocyst	85.1 %[a] (143/168)	77.9 %[a] (102/131)	83.3 %[a] (156/188)	97.5 %[b] (39/40)
	% Expansion*, or Hatching	63.1 %[a] (106/168)	63.4 %[a] (83/131)	58.0%[a] (109/188)	97.5 %[b] (39/40)
In vivo development	**% Pregnancy rate**	80.0%[NS] (24/31)	68.0%[NS] (17/24)	95.2 %[NS] (20/23)	83.3 %[NS] (5/6)
	% Implantation	40.5 %[NS] (117/303)	45.9 %[NS] (84/183)	44.8%[NS] (94/234)	46.9 %[NS] (23/49)
	% Live birth	35.6 %[NS] (103/303)	35.5 %[NS] (65/183)	38.1 %[NS] (80/234)	40.8%[NS] (20/49)

Different letters in the same row indicate a statistically significant difference ($p < 0.05$). NS indicates no statistically significant difference was observed.

% Survival: number of morphologically intact embryos after freezing per frozen embryos.

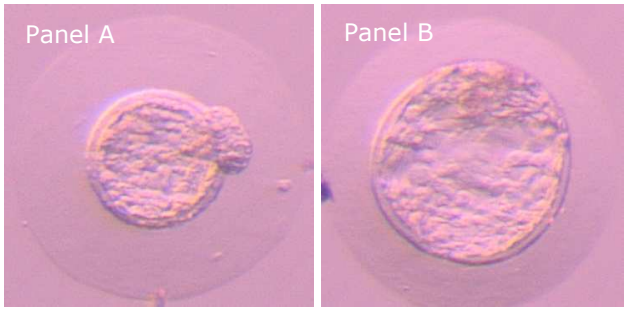
% Pregnancy rate: number of does positive to pregnancy diagnosis per recipient

% Implantation: number of born kits (alive and dead) per transferred embryos on pregnant females

% Live birth: number of live-born kits per transferred embryos on pregnant females

*Slightly expanded blastocysts with herniation of embryonic cells comprised in this group

Figure 1. Stereoscopic pictures of rabbit embryos.



Panel A) A slightly expanded blastocyst with embryonic cell herniation.

Panel B) An expanded blastocyst.

Figure 2. Epifluorescence photomicrographs of rabbit embryos stained with JC-1.

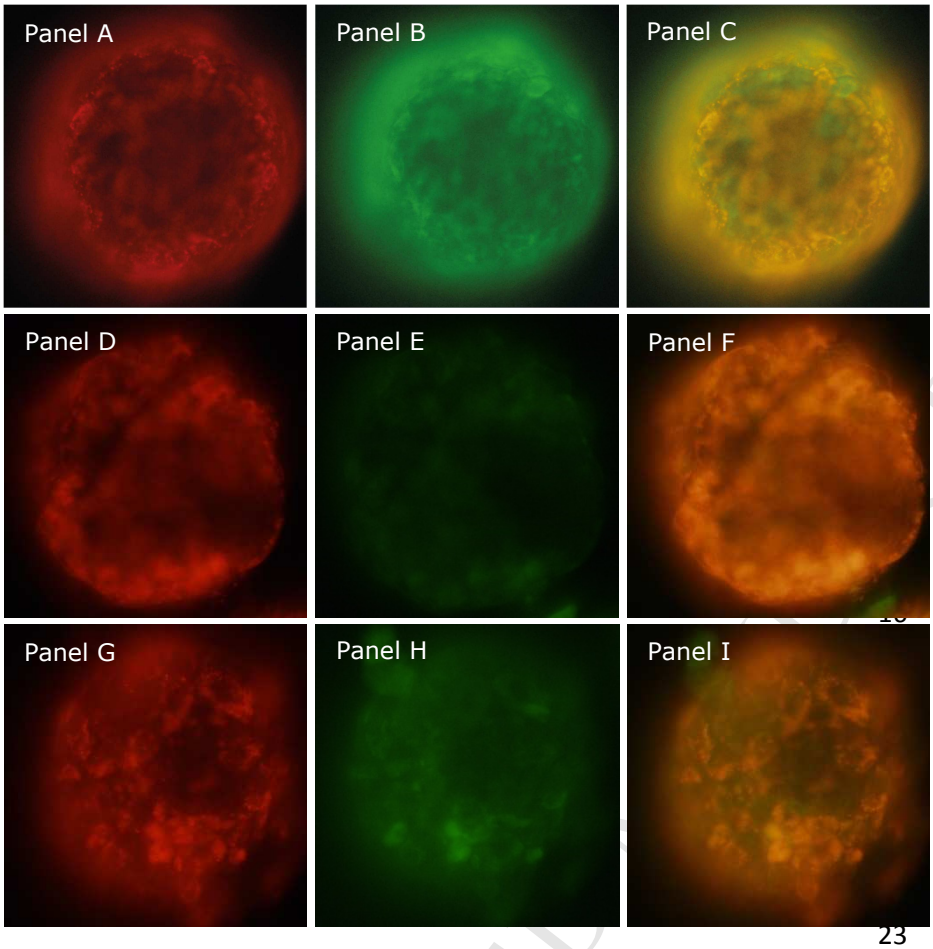
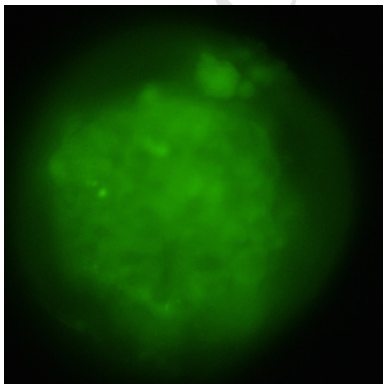


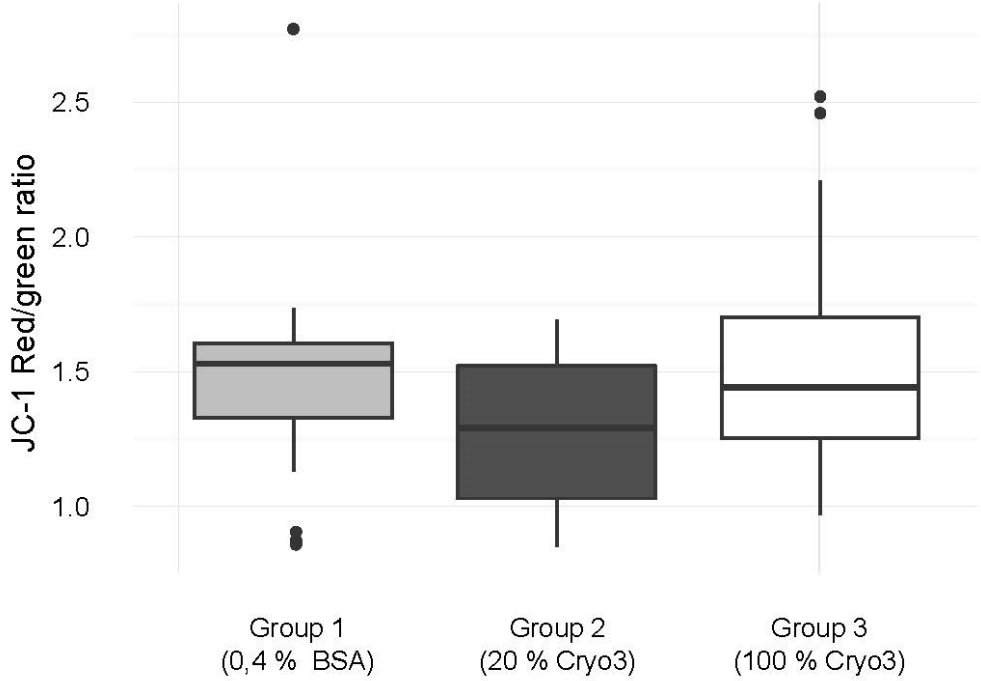
Figure 2a. A, D, G) Regions of high MMP are indicated by red fluorescence (emission ~590 nm).
B, E, H) Depolarized regions are indicated by green fluorescence (emission ~529 nm).
C, F, I) Merged images.
A, B, C) Embryo vitrified with a medium containing 0.4% BSA.
D, E, F) Embryo vitrified with a medium containing 20% CRYO3.
G, H, I) Embryo vitrified with a medium containing 100% CRYO3.



34 Figure 2b. After CCCP control (merged images). No regions of high MMP are visible.

35

Figure 3. JC-1 staining: red/green ratio of cryopreserved expanded or hatching blastocysts vitrified with media containing animal products (group 1) or chemically defined products (group 2 and group 3).



Red/green ratio of embryos vitrified with group 1 (n = 31), group 2 (n = 27), or group 3 (n = 31), obtained with epifluorescence microscopy. No significant difference was observed between groups.

Highlights

- Embryo cryopreservation media usually contain animal-derived products.
- These products present an undefined variable composition and a contamination risk.
- We aimed to replace BSA with a synthetic medium in rapid cooling “freezing” media.
- Cryo3 can replace BSA in rabbit embryo rapid cooling “freezing” and warming media.