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Comparison Between an Animal-Derived Product Medium and a Chemically Defined Medium for Ram Sperm Cryopreservation

Lucie Gavin-Plagne,^{1,*} Loris Commin,^{1,*} Pierre Bruyère,¹ Samuel Buff,¹ and Thierry Joly²

Animal-derived products are widely used in sperm cryopreservation for their cryoprotective properties. These components, however, tend to be replaced because of sanitary risks. STEMALPHA.CRYO3 (Ref. 5617; Stem Alpha, Saint-Genis-l'Argentière, France), called "CRYO3," is a chemically defined preservation medium currently used for freezing human tissue and adult stem cells. The aim of this study was to evaluate the effect of a CRYO3-based medium on ram sperm freezing regarding *in vitro* parameters and *in vivo* fertility. Semen from nine Charolais rams was collected using an artificial vagina, then split and frozen using two media: a CRYO3-based medium or a control medium containing egg yolk (10%) and milk (45%). Sperm membrane integrity (propidium iodide [PI]/SYBR-14 and calcein AM/ethidium homodimer-1), acrosome integrity (FITC-PNA/PI), and mitochondrial membrane potential (JC-1) were assessed using flow cytometry, while functional membrane integrity was assessed using a hypo-osmotic swelling test and motility parameters, evaluated by computer-assisted sperm analysis. Pregnancy rates, prolificacy, and the average daily weight gain (DWG) of lambs were evaluated after performing 195 laparoscopic inseminations. The control medium showed significantly higher results than CRYO3-based medium for all *in vitro* parameters, except for linearity and straightness (motions parameters). Conversely, field trials showed no significant difference between the control medium and the CRYO3-based medium for pregnancy rates (72.2% and 67.9%, respectively), prolificacy (1.8 and 1.6, respectively), and the DWG (0.34 and 0.35 kg/d, respectively). This preliminary study showed that CRYO3 cannot replace egg yolk and milk in freezing extenders for commercial purposes. However, as laparoscopic inseminations allowed a 67% pregnancy rate, CRYO3-based medium remains an option for international transport or long-term storage of genetic diversity.

Keywords: semen freezing, ram sperm, protein-free, animal-derived products, pregnancy rate, hyaluronic acid

Introduction

ANIMAL-DERIVED PRODUCTS (egg yolk, milk) have long been used to preserve frozen sperm in many species,¹ especially rams.^{2,3} Since 1939,⁴ egg yolk has been routinely added to the sperm freezing media to minimize cryoinjuries. Indeed, egg yolk provides protection against cold shock^{4,5} and has a protective effect on the plasma membrane.² Milk, as skimmed milk or whole milk, has also been used as a component of freezing media.²

Milk and egg yolk appear to reduce the deleterious effects of the freezing process on sperm.⁶⁻⁸ Indeed, caseins micelles, major proteins of milk, are believed to protect the sperm by reducing the binding of Binder of Sperm (BSP) proteins to sperm and then lipid loss.^{9,10} With egg yolk, low-density lipoproteins (LDL) are known to be responsible

for sperm protection during cryopreservation.^{9,10} Lusignan et al.¹⁰ demonstrated that BSP proteins in seminal plasma bind with LDL of egg yolk instead of the choline phospholipid proteins of the sperm membrane. These findings show that the components of milk and egg yolk, sequestering BSP proteins, improve sperm cryo survival by decreasing lipid efflux from the sperm membrane.⁷

Nevertheless, the use of animal-derived products presents numerous drawbacks. Indeed, the first major problem of using egg yolk and milk is the sanitary risk. Potential risks of contamination by pathogenic agents have been reported by Bousseau et al.¹¹ Moreover, the egg yolk biochemical composition varies depending of the source and then could give heterogeneous results. Likewise, the presence of debris and the greater viscosity of these components render standardizing microscopic assessment of frozen thawed semen more

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difficult.¹² Additionally, egg yolk provides aromatic amino acids and therefore contributes to the production of reactive oxygen species by dead spermatozoa to the detriment of live spermatozoa.^{6,13} Finally, because of the international trade in milk and egg products, there is a risk to introduce exotic diseases via semen media containing egg yolk and milk.

Today, alternatives to animal-derived products for sperm freezing are available. Indeed, soya lecithin and liposome-based media have been used extensively in many species to replace media containing animal proteins.^{3,11,12,14–17} Their protective effect could be called into question, however, because of their unstable composition and variable properties.¹⁸ In French ovine insemination centers, sperm is cryopreserved according to Colas,¹⁹ a conventional freezing procedure using egg yolk and milk in extenders. For these reasons, to overcome new biobanking regulations in the coming years, it might be necessary to cryopreserve semen in a stable, synthetic, and chemically defined medium.

STEMALPHA.CRYO3 (Ref. 5617; Stem Alpha, Saint-Genis-l'Argentière, France), called “CRYO3,” is a current good manufacturing practice (cGMP) serum-free, protein-free, and dextran-free solution. CRYO3 is composed of high molecular weight synthetic hyaluronic acid (HA), glucose, carbohydrates, amino acids, mineral salts, vitamins, fatty acids esters, and buffers (personal communication from Daniel Licari, March 2018). It was initially designed for clinical applications, to replace serum in somatic and human adult stem cells freezing medium.^{20–23}

Recently, it has been found that CRYO3 could be a potential additive to cryopreserve reproductive cells. Indeed, CRYO3 can be used for rabbit²⁴ and bovine embryo cryopreservation.²⁵ No significant difference was found for the rabbit embryo *in vitro* survival rate between CRYO3 and fetal calf serum (FCS). However, CRYO3 was found to be better for bovine embryos. Moreover, they observed better *in vivo* embryo viability, after embryo transfers when CRYO3 was used compared to FCS in the rabbit. Despite good performance of soya lecithin and liposomes on sperm quality, the objective of this study was to avoid all forms of derived (plant or animal origin), or unstable and variable products.

The aim of this study was to evaluate the effect of a chemically defined cryopreservation medium (CRYO3-based medium) on ram sperm regarding *in vitro* parameters and *in vivo* fertility.

Materials and Methods

The design of this veterinary clinical study was approved by the animal research Ethics Committee of VetAgro Sup. All animal procedures conformed to the European Regulations [Regulation (EU) 2016/1012 related to zoo technical and genealogical conditions for breeding, trade in, and entry into the Union of purebred breeding animals, hybrid breeding pigs, and the germinal products thereof].

Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Semen collection

Sexually mature Charolais rams ($n=9$; 3–5 years old) with proven fertility were used for this study. The rams were kept in a semen collection and storage center (agreement No. FRCO871; INSEM OVIN, Limoges, France) under

uniform conditions. All males were housed in groups of four to six rams. They were fed with straw and hay and had free access to water. Animals were maintained under natural light and were subjected to semen collection once a day from Monday to Friday during breeding season.

For the study, rams were subjected to semen collection once in the morning with an artificial vagina during the breeding season. Briefly, the artificial vagina was filled with warm water at a temperature ($\sim 37^\circ\text{C}$) that mimics the natural condition of the female tract. A teaser ewe was presented to the ram and semen was recovered in a graduated glass tube attached to the artificial vagina.

Directly after collection, the volume, concentration (ACCUREAD photometer; IMV Technologies, L'Aigle, France), and subjective motility ($10\times$ negative phase contrast objective) of each ejaculate (one per animal) were evaluated. The volume and the concentration of ejaculates varied between 0.6 and 1.3 mL and 1.9 and 3.3×10^9 spermatozoa/mL (spz/mL), respectively. Only ejaculates that presented a white color and a visual motility higher than 70% were used and held at 37°C in a water bath before dilution and freezing procedure.

Semen freezing

Each ejaculate ($n=9$) was split into two equal aliquots that were cryopreserved in a CRYO3-based medium composed of a chemically defined product, CRYO3 (pH=6.8–7.6; osmolarity = 305–390 mOsm; viscosity = 1–7 cps) or in medium containing egg yolk and milk (control medium). Each sample was processed using a two-step dilution procedure currently used in French ovine insemination centers according to Colas.¹⁹ Each freezing medium (CRYO3-based medium and control medium) were then composed of two extenders. Briefly, the first step consisted of diluting semen at 30°C in a glycerol-free medium at a concentration of 800×10^6 spz/mL.

For the control medium, the first extender was composed of lactose (102.96 g/L), 20% (v:v) hen egg yolk, and gentamicin (10 mg/mL; Gibco) in sterile water. For the CRYO3-based medium, the first extender of the first step contained 20% (v:v) CRYO3 in a Tris buffer supplemented with citric acid, glucose (TCG), and antibiotics (tris-hydroxymethyl-aminomethane, 30.26 g/L; citric acid, 17 g/L; glucose, 12.5 g/L; mix of sodium benzylpenicillinate 200 UI/mL, procaine benzylpenicillinate 300 UI/mL, and dihydrostreptomycine, 0.5 mg/L; pH=7.0).

After equilibration of the diluted semen at 4°C for 90 minutes, the second step was performed at 4°C . A second extender (equilibrated at 4°C) containing glycerol was added in two steps 20 minutes apart at a final ratio of 1:1 to obtain a final concentration of 400×10^6 spz/mL. For the control medium, the second extender contained 90% (w:v) of milk powder diluted in sterile water (40 g/L of semi-skimmed milk; Regilait), 10% (v:v) glycerol, and gentamicin (10 mg/mL; Gibco); pH was adjusted to 6.8. For the CRYO3-based medium, the second extender was composed of 20% (v:v) CRYO3, 0.2 M of trehalose, and 10% (v:v) glycerol in TCG (pH=7.0). The diluted sperm samples were cooled for 2 hours at 4°C .

After equilibration, sperm was loaded into 0.25 mL French straws (IMV Technologies). Straws were then suspended horizontally in liquid nitrogen vapor 20 cm above the liquid nitrogen surface (between -50°C and -70°C) for 8 minutes before being plunged into liquid nitrogen. Straws

were stored for at least 2 weeks in liquid nitrogen before thawing for assessment or for artificial inseminations (AI).

Thawing was performed by submerging the straws in a water bath at 37°C for 30 seconds before *in vitro* sperm evaluation and insemination.

Sperm evaluation after thawing

Post-thawed semen was diluted in a Tris-citrate buffer supplemented with fructose (TCF; tris-hydroxymethyl-aminomethane: 27 g/L, citric acid: 14 g/L, fructose: 10 g/L; pH = 7.24; osmolarity = 294 mOsm) to obtain 100×10^6 spz/mL for assessment.

Flow cytometry analyses

Semen was evaluated by flow cytometry to assess the plasma membrane integrity (fresh and frozen-thawed samples), acrosome integrity, and mitochondrial membrane potential. For all samples, the sperm population was identified (forward scatter/side scatter [SSC] dot plots) and 10,000 events among the sperm population were analyzed at a low rate (200 cells/s). Analyses were performed using Flowsight Amnis (EMD Millipore, Seattle, WA) equipped with blue-green (488 nm, 60 mW), red (642 nm, 100 mW), and SSC (785 nm, 12 mW) solid-state lasers. The Flowsight is equipped with a quantitative imaging upgrade that includes a 488-nm laserpower doubler and increased image resolution (40× magnification).

This innovative device, the Flowsight Amnis, allowed the visualization of each event acquired via a classical microscope objective. It permitted us to gate precisely the sperm population and thus to eliminate debris and other cell particles, leading to an accurate analysis of the sample. Post-acquisition analyses were performed on Amnis IDEAS software (version 6.2; Millipore-Amnis).

Plasma membrane integrity was evaluated with a Live/Dead Sperm Viability Kit (Invitrogen, Eugene, OR) using propidium iodide (PI) and SYBR-14 staining. Intact spermatozoa appeared green in SYBR-14, whereas membrane-damaged spermatozoa were stained in red by PI. For each sample, 10 μ L of semen (100×10^6 spz/mL) were incubated with SYBR-14 (working concentration 45 nM) and PI (working concentration 11 μ M) in a TCF extender (5 minutes, 37°C). Sperm membrane integrity was assessed with a 488-nm excitation laser and a 530 ± 30 nm bandpass emission filter for SYBR-14 and 650 ± 13 nm bandpass emission filter for PI. The percentages of intact (SYBR-14+) and membrane-damaged (PI+) spermatozoa were determined.

A second evaluation of the plasma membrane integrity was carried out with a calcein AM and an ethidium homodimer-1 probe (C/EH; Invitrogen). Briefly, for each sample, 10 μ L of semen (100×10^6 spz/mL) was incubated with calcein AM (working concentration 2 μ M) and ethidium homodimer (working concentration 4 μ M) in a TCF extender (15 minutes, 37°C). Sperm membrane integrity was assessed with a 488-nm excitation laser and a 530 ± 30 nm bandpass emission filter for calcein AM and 650 ± 13 nm bandpass emission filter for ethidium homodimer-1. The percentages of intact (Calcein AM+) and membrane-damaged (Ethidium homodimer-1+) spermatozoa were determined.

Acrosome integrity was assessed using FITC-PNA (1 mg/mL; Sigma-Aldrich). For each sample, 10 μ L of semen (100×10^6 spz/mL) were incubated with FITC-PNA (work-

ing concentration 10 μ g/mL) in a TCF extender (15 minutes, 37°C). PI (working concentration 11 μ M) was added 5 minutes before the end of the incubation. Fluorescence was collected with a 488 nm excitation laser, 530 ± 30 nm bandpass emission filter for FITC-PNA, and 650 ± 13 nm bandpass emission filter for PI. The percentages of cells with intact acrosome (PI-/FITC-PNA-) were recorded.

High mitochondrial membrane potential (hMMP) was assessed using the lipophilic cation JC-1 (1.5 mM; Invitrogen, Life Technologies, Eugene, Oregon USA). For each sample, 10 μ L of semen (100×10^6 spz/mL) were incubated with JC-1 (working concentration 0.75 mM) in TCF extender (15 minutes, 37°C). Fluorescence was analyzed with a 488-nm excitation laser, a 530 ± 30 nm bandpass emission filter and 610 ± 30 nm bandpass emission filter. The percentages of cells with hMMP staining in orange and with low mitochondrial membrane potential (green stained cells) were recorded.

Hypo-osmotic swelling test

The functional integrity of sperm membrane can be evaluated with the hypo-osmotic swelling test (HOST), as described by Jeyendran et al.²⁶ Briefly, 10 μ L of semen at a concentration of 100×10^6 sperm/mL were diluted in 100 μ L of a hypo-osmotic solution (100 mOsm; 4.9 g/L citrate sodium, 9 g/L fructose). A smear test was performed after 30 minutes of incubation at 37°C. A total of 200 spermatozoa were observed under a phase-contrast microscope at magnification 400×. Spermatozoa with functional membranes appeared with swollen and curved tails. The percentage of functional membrane integrity was calculated as the following equation:

$$\text{Functional membrane integrity} = \frac{\text{Number of spz with swollen and curved tails}}{200 \text{ spz counted}} \times 100$$

Motion characteristics

The post-thaw motility of the semen of the nine rams was evaluated using Sperm Class Analyzer software (SCA2013; Microptic S.L., Barcelona, Spain) with a 10× negative phase contrast objective. Each semen sample was diluted to a concentration of 20×10^6 spz/mL. Briefly, a 5 μ L sample of the diluted semen was loaded in a prewarmed analysis chamber with a depth of 20 μ M (Leja Products, Nieuw-Vennep, The Netherlands). For each sample, 10 fields were analyzed and percentages of motile (average path velocity [VAP] >10 μ m/s) and progressively motile (STR index >80%) spermatozoa, amplitude of lateral head displacement (ALH), beat cross frequency (BCF), curvilinear velocity (VCL), straight line velocity (VSL), VAP, linearity (LIN = VSL/VCL), wobble (WOB = VAP/VCL), and straightness (STR = VSL/VAP) were recorded. The camera setting was 50 frames/s.

Artificial insemination

Multiparous Charolais ewes ($n=195$) housed on a free pasture were used for laparoscopic insemination. Ewes were synchronized with an intravaginal fluorogestone acetate sponge (20 mg, Chronogest; MSD Animal Health, Angers, France) for 14 days. Fifty-eight hours before AI, the sponges

were removed and animals were given an intramuscular injection of 500–600 IU of equine chorionic gonadotropin (MSD Animal Health). Each ewe received one dose (one straw) of frozen-thawed semen. Briefly, the inseminating pipette was introduced into the lumen of each uterine horn. The straw was equally deposited (0.125 mL of frozen-thawed semen containing $\sim 100 \times 10^6$ sperm) in each horn (50×10^6 sperm/horn).

Ewes were randomly assigned to the control group ($n=97$ ewes inseminated with the control medium) and the CRYO3 group ($n=98$ ewes inseminated with the CRYO3-based medium). Frozen straws from the nine rams were randomly assigned to ewes and equally distributed for AI (~ 20 to 21 ewes inseminated per ram). AI procedures were performed by a certified operator (agreement No. 263 related to ovine species; CIA L'Aigle, L'Aigle, France). Also, animal welfare during and after laparoscopic procedures was of specific concern.

Pregnancy rate, prolificacy rate, and average daily weight gain (DWG) of lambs were determined according the following equations:

$$\text{Pregnancy rate} = \frac{\text{Number of gravid ewes}}{\text{Total number of inseminated ewes}} \times 100$$

$$\text{Prolificacy rate} = \frac{\text{Mean number of lambs}}{\text{Delivered ewes}}$$

$$\text{Average daily weight gain of lambs} = \frac{\text{Weight at 70 days} - \text{Weight at 30 days}}{40 \text{ days}}$$

Statistical analysis

Statistical analyses were performed using R software.²⁷ The results were presented as the means \pm standard deviation. The percentage of membrane integrities (IP/SYBR-14 and calcein/ethidium), percentage of acrosome integrity, percentage of hMMP, percentage of functional membrane integrity (HOST), and sperm motility parameters were compared using a Wilcoxon test.

The average DWG of lambs exhibited a Gaussian distribution and were compared using a paired *t*-test. The Chi-square test was used to compare pregnancy rates. Prolificacy rates (assumed to follow a Poisson distribution) were compared using a general linear model including the freezing medium as a fixed effect. The correlation between *in vitro* parameters and reproductive performance was analyzed using a generalized linear model procedure. Differences with $p < 0.05$ were considered statistically significant.

Results

Before freezing, the percentage of membrane integrity (IP/SYBR-14) was $75.7\% \pm 9.0\%$. After thawing, the percentage of membrane integrity decreased radically for both media (Table 1). Significant differences were observed between media for all flow cytometry parameters (Table 1).

A significant difference between the control and CRYO3-based media was found after performing a HOST ($p < 0.05$). The control medium exhibited higher quality of spermatozoa in terms

TABLE 1. EFFECT OF THE FREEZING MEDIUM ON THE PLASMA MEMBRANE INTEGRITY, ACROSOME INTEGRITY, AND HIGH MITOCHONDRIAL MEMBRANE POTENTIAL OF THAWED SEMEN

Flow cytometry parameters	Control medium	CRYO3-based medium
Plasma membrane integrity (IP/SYBR-14), %	15.9 ± 4.2^a	4.2 ± 1.8^b
Plasma membrane integrity (C/EH), %	20.7 ± 6.4^a	7.7 ± 4.7^b
Acrosome integrity, %	20.2 ± 4.8^a	7.1 ± 3.5^b
High mitochondrial membrane potential, %	18.5 ± 4.3^a	5.1 ± 3.0^b

Results are presented as the means \pm standard deviation.
^{a,b}Values within a row with different superscripts differ significantly at $p < 0.05$.

of functional membrane integrity compared to the CRYO3-based medium ($39.9\% \pm 8.7\%$ and $16.9\% \pm 9.1\%$, respectively).

Significant differences between the control and CRYO3-based media were observed for the percentage of progressive and total motility, VSL, VCL, VAP, WOB, ALH, and BCF. These parameters were lower for the CRYO3-based medium compared with the control medium (Table 2). No significant difference was found between the control and CRYO3-based media for LIN and STR.

The reproductive parameters are shown in Table 3. No difference was found between the control and CRYO3-based media for pregnancy rate, prolificacy, or average DWG of lambs ($p > 0.05$).

No significant correlation between *in vitro* parameters (from flow cytometry, HOST, and computer-aided sperm analysis [CASA]) and reproductive parameters (pregnancy rate and prolificacy) was found.

Discussion

Semen cryopreservation has been developed in numerous mammalian species and is widely used in farm animal breeding, particularly in bovine.²⁸ Ram semen cryopreservation

TABLE 2. EFFECT OF THE FREEZING MEDIUM ON SPERM MOTILITY AFTER THAWING

Motion characteristics	Control medium	CRYO3-based medium
Progressive (%)	24.3 ± 5.1^a	8.4 ± 3.0^b
Motile (%)	45.0 ± 5.7^a	23.5 ± 5.3^b
VCL ($\mu\text{m}^2/\text{s}$)	118.2 ± 13.1^a	66.7 ± 12.4^b
VSL ($\mu\text{m}^2/\text{s}$)	41.9 ± 6.4^a	25.1 ± 6.4^b
VAP ($\mu\text{m}^2/\text{s}$)	61.9 ± 6.4^a	34.3 ± 7.1^b
LIN (%)	29.7 ± 3.0^a	26.1 ± 3.6^a
STR (%)	59.3 ± 4.1^a	57.8 ± 3.5^a
WOB (%)	48.8 ± 3.0^a	42.9 ± 4.0^b
ALH (μm)	2.8 ± 0.4^a	1.7 ± 0.3^b
BCF (Hz)	12.04 ± 1.7^a	8.2 ± 2.0^b

Results are presented as the means \pm standard deviation.
^{a,b}Values within a row with different superscripts differ significantly at $p < 0.05$.

ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, VSL/VCL (linearity); STR, VSL/VAP (straightness); VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, VAP/VCL (wobble).

TABLE 3. EFFECT OF THE FREEZING MEDIUM ON *IN VIVO* RESULTS AFTER ARTIFICIAL INSEMINATION WITH THAWED RAM SPERM

Medium	Pregnancy rate (%)	Prolificacy (No. of lambs/ewe)	Average daily weight gain of lambs (kg/d)
Control medium (n=97)	72.2±0.4	1.8±0.8	0.34±0.1
CRYO3-based medium (n=98)	67.3±0.5	1.6±0.6	0.35±0.1
p	0.51	0.65	0.55

Results are presented as the means ± standard deviation.

remains difficult, however, as reflected by the low fertility rates after AI observed in this species.² This study was performed to investigate the effect of a chemically defined sperm cryopreservation medium on *in vitro* parameters and *in vivo* fertility in ovine species.

For this study, we showed that all *in vitro* parameters (by flow cytometry, HOST, and CASA), except for LIN and STR, yielded better results for the control medium compared with the CRYO3-based medium. After laparoscopic inseminations on 195 ewes, however, no significant difference was found between media for the pregnancy rate, prolificacy, and average DWG of lambs.

In this study, egg yolk and milk appeared to protect spermatozoa better than the tested medium with regard to *in vitro* results. Membrane integrity, acrosome integrity, hMMP, and progressive motility of sperm cryopreserved in control medium were three times higher compared with the CRYO3-based medium. Indeed, egg yolk and milk are used in cryopreservation media as a source of lipoproteins, which are known to protect sperm cells from cold shock mainly by maintaining membrane phospholipid integrity.²⁹ Numerous authors have suggested that LDL could be responsible for the resistance of sperm during cold shock and for the improvement of motility.^{30–32}

Soy lecithin, a vegetal phospholipid, is known to replace egg yolk³³ and has been studied in many experiments involving ram semen cryopreservation, as reported earlier.^{15,33,34} Indeed, semen quality (progressive motility, functional membrane integrity, acrosome integrity, and mitochondrial activity) was comparable or even better (25%, 56%, 85%, 56%, respectively) when cryopreserved with soy lecithin than in presence of egg yolk (21%, 50%, 76%, ~54%, respectively).³³

In our study, we tested a synthetic product composed of HA. Several studies used HA as an antioxidant supplement in sperm freezing media,^{35–37} or as a cryoprotectant.³⁸ HA is known to compose the extracellular matrix and to be present in several tissue and fluids.³⁹ More specifically, it appears that HA could regulate sperm metabolism and could be involved in the sperm capacitation process.⁴⁰ HA supplementation after thawing human sperm has been shown to be beneficial.⁴¹ However, the addition of HA in the freezing medium does not improve sperm quality in human⁴¹ or ram,³⁶ as observed in our study.

Our *in vitro* results (especially the sperm motility) are different from some previous studies^{42,43} but consistent with others.^{33,34,44–46} These discrepancies could be explained by the low freezing rate method (20 cm above liquid nitrogen). Furthermore, the significant difference observed for *in vitro*

results between our two media can also, in part, be explained by the differences in the composition of antibiotics. Indeed, Salvetti et al.⁴⁷ showed that antibiotics in rabbit sperm extenders can change the temperature of crystallization and the quantity of ice formed during the freezing step. Therefore, even if HA from CRYO3 does not sufficiently mimic the surfactant properties of phospholipids in vegetal or animal-derived product we should be careful since composition of media in terms of antibiotics are different.

Despite the obvious differences observed between control and CRYO3-based media, some parameters such as LIN and STR were not statistically different for the control and CRYO3-based media. The nonsignificant differences in LIN and STR could explain the *in vivo* results. Indeed, Hirano et al.⁴⁸ reported that VSL and ALH were correlated with *in vitro* fertilization results for human sperm. Similarly, Lavara et al.⁴⁹ found a negative correlation for LIN and kindling rate with rabbit sperm, whereas Larsen et al.⁵⁰ found a significant effect of STR on the fertility rate with human sperm. A positive correlation between BCF and fertility has also been reported in human and ovine sperm.^{50,51}

In this study, however, no relationship between CASA parameters and *in vivo* results was found. Similarly, Holden et al.⁵² did not observe a correlation between *in vitro* sperm parameters (viability or total motility and pregnancy rates) when nonsorted bull sperm was evaluated. It appears that motility or viability parameters are considered as compensable traits of semen for fertility, as they depend on the number rather than the rate of spermatozoa affected.^{43,44,52,53}

Despite poor *in vitro* results, laparoscopic insemination could allow a high pregnancy rate with CRYO3-based medium (67% vs. 72% for egg yolk and milk-based medium, $p > 0.05$). The number of spermatozoa per dose and the pregnancy rate associated were similar to other studies using laparoscopic inseminations.^{54–56} Our *in vivo* results were even better compared to other studies using fresh or frozen-thawed ram semen.^{57,58}

In stallions, a freezing medium was not optimal based on *in vitro* analyses but was better after insemination compared with the control medium with egg yolk.⁵⁹ The authors made the assumption that after cryopreservation, it is possible that only a small subpopulation of spermatozoa that is still sufficient for fertilizing the egg was preserved in the medium tested. Consequently, this optimal but small subpopulation of sperm has not been highlighted by the laboratory examination of sperm quality. Indeed, we assume that CRYO3-based medium via its main component (HA) might select the “strongest spermatozoa” and eliminate the less resistant during cryopreservation.

Recently, it was shown that it is therefore important to identify and compare sperm subpopulations using a clustering analysis because of the heterogeneity of a single ejaculate.⁶⁰ Thus, in this study, CRYO3 failed to protect ram spermatozoa while it appeared to be a good substitute to serum-based media in rabbit and bovine embryo freezing.

Conclusion

This preliminary study showed that CRYO3 cannot replace egg yolk and milk in commercially available freezing extenders. Indeed, this product was established to replace serum for somatic and stem cells cryopreservation. However, since laparoscopic insemination has allowed to recover a pregnancy rate

of 67%, CRYO3-based medium remains an option in some instances. The use of CRYO3 may be appropriate to overcome the forthcoming regulatory requirements in the international trade of semen from animals of high genetic value. It could also be considered for the long-term storage of rams resources in sheep cryobanking programs. Adjustments of the freezing procedure must nevertheless be studied to improve the *in vitro* viability of spermatozoa.

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Author Disclosure Statement

The authors have no competing financial interests.

References

- Holt WV. Basic aspects of frozen storage of semen. *Anim Reprod Sci* 2000;62:3–22.
- Salamon S, Maxwell WMC. Storage of ram semen. *Anim Reprod Sci* 2000;62:77–111.
- Gil J, Rodriguez-Iraozqui M, Lundeheim N, Söderquist L, Rodriguez-Martinez H. Fertility of ram semen frozen in Bioexcell[®] and used for cervical artificial insemination. *Theriogenology* 2003;59:1157–1170.
- Phillips PH. Preservation of bull semen. *J Biol Chem* 1939; 130:415.
- Paulenz H, Söderquist L, Pérez-Pé R, Andersen Berg K. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. *Theriogenology* 2002; 57:823–836.
- Vishwanath R, Shannon P. Storage of bovine semen in liquid and frozen state. *Anim Reprod Sci* 2000;62:23–53.
- Bergeron A, Manjunath P. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. *Mol Reprod Dev* 2006;73:1338–1344.
- Therrien A, Manjunath P, Lafleur M. Chemical and physical requirements for lipid extraction by bovine binder of sperm BSP1. *Biochim Biophys Acta* 2013;1828:543–551.
- Bergeron A, Brindle Y, Blondin P, Manjunath P. Milk caseins decrease the binding of the major bovine seminal plasma proteins to sperm and prevent lipid loss from the sperm membrane during sperm storage. *Biol Reprod* 2007; 77:120–126.
- Lusignan M-F, Bergeron A, Lafleur M, Manjunath P. The major proteins of bovine seminal plasma interact with caseins and whey proteins of milk extender. *Biol Reprod* 2011;85:457–464.
- Bousseau S, Brillard JP, Marquant-Le Guienne B, Guérin B, Camus A, Lechat M. Comparison of bacteriological qualities of various egg yolk sources and the *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology* 1998;50:699–706.
- Aires VA, Hinsch K-D, Mueller-Schloesser F, Bogner K, Mueller-Schloesser S, Hinsch E. *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology* 2003;60:269–279.
- Shannon P, Curson B. Kinetics of the aromatic l-amino acid oxidase from dead bovine spermatozoa and the effect of catalase on fertility of diluted bovine semen stored at 5°C and ambient temperatures. *J Reprod Fertil* 1982;64:463–467.
- Gil J, Lundeheim N, Söderquist L, Rodríguez-Martínez H. Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. *Theriogenology* 2003;59:1241–1255.
- Sharafi M, Zhandi M, Akbari Sharif A. Supplementation of soybean lecithin-based semen extender by antioxidants: Complementary flow cytometric study on post-thawed ram spermatozoa. *Cell Tissue Bank* 2015;16:261–269.
- Nishijima K, Kitajima S, Koshimoto C, et al. Motility and fertility of rabbit sperm cryopreserved using soybean lecithin as an alternative to egg yolk. *Theriogenology* 2015;84:1172–1175.
- Ansari MS, Rakha BA, Akhter S, Ashiq M. OPTIXcell improves the postthaw quality and fertility of buffalo bull sperm. *Theriogenology* 2016;85:528–532.
- Lasic DD. On the thermodynamic stability of liposomes. *J Colloid Interface Sci* 1990;140:302–304.
- Colas G. Effect of initial freezing temperature addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. *J Soc Reprod Fertil* 1975;42:277–285.
- Jurga M, Forraz N, Basford C, et al. Neurogenic properties and a clinical relevance of multipotent stem cells derived from cord blood samples stored in the biobanks. *Stem Cells Dev* 2012;21:923–936.
- Sarnowska A, Jablonska A, Jurga M, et al. Encapsulation of mesenchymal stem cells by bioscaffolds protects cell survival and attenuates neuroinflammatory reaction in injured brain tissue after transplantation. *Cell Transplant* 2013;22:67–82.
- Mueller AA, Forraz N, Gueven S, et al. Osteoblastic differentiation of Wharton jelly biopsy specimens and their mesenchymal stromal cells after serum-free culture. *Plast Reconstr Surg* 2014;134:59e–69e.
- Ducret M, Fabre H, Farges J-C, et al. Production of human dental pulp cells with a medicinal manufacturing approach. *J Endod* 2015;41:1492–1499.
- Bruyère P, Baudot A, Joly T, et al. A chemically defined medium for rabbit embryo cryopreservation. *PLoS One* 2013;8:e71547.
- Bruyère P, Baudot A, Guyader-Joly C, Guérin P, Louis G, Buff S. Improved cryopreservation of *in vitro*-produced bovine embryos using a chemically defined freezing medium. *Theriogenology* 2012;78:1294–1302.
- Jeyendran RS, Van der Ven HHV, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 1984;70:219–228.
- R Development Core Team. R: A language and environment for statistical computing. Vienna; 2011. www.R-project.org (last accessed July 12, 2017).
- Layek SS, Mohanty TK, Kumaresan A, Parks JE. Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. *Anim Reprod Sci* 2016;172:1–9.
- Moussa M, Martinet V, Trimeche A, Tainturier D, Anton M. Low density lipoproteins extracted from hen egg yolk by an easy method: Cryoprotective effect on frozen-thawed bull semen. *Theriogenology* 2002;57:1695–1706.
- Evans RJ, Bauer DH, Bandemer SL, Vaghefi SB, Flegal CJ. Structure of egg yolk very low density lipoprotein. Polydispersity of the very low density lipoprotein and the role of

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- lipovitellenin in the structure. *Arch Biochem Biophys* 1973;154:493–500.
31. Pace MM, Graham EF. Components in egg yolk which protect bovine spermatozoa during freezing. *J Anim Sci* 1974;39:1144–1149.
 32. Quinn PJ, Chow PYW, White IG. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. *J Reprod Fertil* 1980;60:403–407.
 33. Emamverdi M, Zhandi M, Zare Shahneh A, Sharafi M, Akbari-Sharif A. Optimization of ram semen cryopreservation using a chemically defined soybean lecithin-based extender. *Reprod Domest Anim* 2013;48:899–904.
 34. Najafi A, Najafi M, Zanganeh Z, Sharafi M, Martinez-Pastor F, Adeldust H. Cryopreservation of ram semen in extenders containing soybean lecithin as cryoprotectant and hyaluronic acid as antioxidant. *Reprod Domest Anim* 2014;49:934–940.
 35. Peña FJ, Johannisson A, Wallgren M, Rodriguez-Martinez H. Effect of hyaluronan supplementation on boar sperm motility and membrane lipid architecture status after cryopreservation. *Theriogenology* 2004;61:63–70.
 36. Bucak MN, Ateşşahin A, Varışlı Ö, Yüce A, Tekin N, Akçay A. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: Microscopic and oxidative stress parameters after freeze–thawing process. *Theriogenology* 2007;67:1060–1067.
 37. Bucak MN, Sariözkan S, Tuncer PB, Ulutaş PA, Akçadağ Hİ. Effect of antioxidants on microscopic semen parameters, lipid peroxidation and antioxidant activities in Angora goat semen following cryopreservation. *Small Rumin Res* 2009;81:90–95.
 38. Qian L, Yu S, Zhou Y. Protective effect of hyaluronic acid on cryopreserved boar sperm. *Int J Biol Macromol* 2016;87:287–289.
 39. Rodriguez-Martinez H, Tienthai P, Atikuzzaman M, Vicente-Carrillo A, Rubér M, Alvarez-Rodriguez M. The ubiquitous hyaluronan: Functionally implicated in the oviduct? *Theriogenology* 2016;86:182–186.
 40. Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodriguez-Martinez H. Capacitation status and in vitro fertility of boar spermatozoa: Effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. *Int J Androl* 2002;25:84–93.
 41. Sbracia M, Grasso J, Sayme N, Stronk J, Huszar G. Hyaluronic acid substantially increases the retention of motility in cryopreserved/thawed human spermatozoa. *Hum Reprod* 1997;12:1949–1954.
 42. Gil J, Söderquist L, Rodriguez-Martinez H. Influence of centrifugation and different extenders on post-thaw sperm quality of ram semen. *Theriogenology* 2000;54:93–108.
 43. Gil J, Rodriguez-Irazaqui M, Söderquist L, Rodriguez-Martinez H. Influence of centrifugation or low extension rates prefreezing on the fertility of ram semen after cervical insemination. *Theriogenology* 2002;57:1781–1792.
 44. Martins KR, Gheller SSM, Ferreira CER, et al. Post-thawing quality of ram spermatozoa is impaired by inclusion of boar seminal plasma in the freezing extender. *Small Rumin Res* 2016;144:229–233.
 45. Fang Y, Zhong R, Zhang X, Zhang J, Zhou D. Boar seminal plasma inhibits cryo-capacitation of frozen-thawed ram sperm and improves fertility following intracervical insemination. *Theriogenology* 2018;105:84–89.
 46. Masoudi R, Sharafi M, Zare Shahneh A, et al. Effect of dietary fish oil supplementation on ram semen freeze ability and fertility using soybean lecithin- and egg yolk-based extenders. *Theriogenology* 2016;86:1583–1588.
 47. Salvetti P, Joly T, Baudot A. Effect of antibiotics on thermodynamic properties of freezing media in rabbit species: A first calorimetric approach. *Cryobiology* 2006;53:268–275.
 48. Hirano Y, Shibahara H, Obara H, et al. Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates in vitro. *J Assist Reprod Genet* 2001;18:215–220.
 49. Lavara R, Mocé E, Lavara F, Viudes de Castro MP, Vicente JS. Do parameters of seminal quality correlate with the results of on-farm inseminations in rabbits? *Theriogenology* 2005;64:1130–1141.
 50. Larsen L, Scheike T, Jensen TK, et al. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. *Hum Reprod* 2000;15:1562–1567.
 51. Del Olmo E, Bisbal A, Maroto-Morales A, et al. Fertility of cryopreserved ovine semen is determined by sperm velocity. *Anim Reprod Sci* 2013;138:102–109.
 52. Holden SA, Fernandez-Fuertes B, Murphy C, et al. Relationship between in vitro sperm functional assessments, seminal plasma composition, and field fertility after AI with either non-sorted or sex-sorted bull semen. *Theriogenology* 2017;87:221–228.
 53. Saacke RG, Dalton JC, Nadir S, Nebel RL, Bame JH. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. *Anim Reprod Sci* 2000;60–61:663–677.
 54. King ME, McKelvey WAC, Dingwall WS, et al. Lambing rates and litter sizes following intrauterine or cervical insemination of frozen/thawed semen with or without oxytocin administration. *Theriogenology* 2004;62:1236–1244.
 55. Fair S, Hanrahan JP, O'Meara CM, et al. Differences between Belclare and Suffolk ewes in fertilization rate, embryo quality and accessory sperm number after cervical or laparoscopic artificial insemination. *Theriogenology* 2005;63:1995–2005.
 56. Khalifa T, Lymberopoulos A, Theodosiadou E. Association of soybean-based extenders with field fertility of stored ram (*Ovis aries*) semen: A randomized double-blind parallel group design. *Theriogenology* 2013;79:517–527.
 57. Richardson L, Hanrahan JP, Donovan A, et al. Effect of site of deposition on the fertility of sheep inseminated with frozen-thawed semen. *Anim Reprod Sci* 2012;131:160–164.
 58. Casali R, Pinczak A, Cuadro F, Guillen-Muñoz JM, Mezzalira A, Menchaca A. Semen deposition by cervical, transcervical and intrauterine route for fixed-time artificial insemination (FTAI) in the ewe. *Theriogenology* 2017;103:30–35.
 59. Pillet E. Development of a new extender for cryopreservation of stallion semen and mechanisms of cryoprotection involved. [PhD thesis]. France: Agrocampus Ouest; 2009. <http://www.theses.fr/2009NSARI054>
 60. Ledesma A, Zalazar L, Fernández-Alegre E, Hozbor F, Cesari A, Martínez-Pastor F. Seminal plasma proteins modify the distribution of sperm subpopulations in cryopreserved semen of rams with lesser fertility. *Anim Reprod Sci.* 2017;184:44–50.

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