doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Veterinaria México OA
Publicación Digital de la Facultad de Medicina Veterinaria y Zootecnia https://veterinariamexico.fmvz.unam.mx/

Effect of two animal protein-free extenders on cryopreservation of Blackbelly and Pelibuey ram semen

Carlos Adelso Castellanos Zacarías[1]
0000-0001-7148-811X
Julio Porfirio Ramón Ugalde[1]
0000-0002-9312-8438
Henry Jesús Loeza Concha[2]
0000-0001-7686-5113
Roberto Zamora Bustillos[1]
0000-0002-4502-1492
Luis Leonardo Pinzón López[1]
0000-0002-8405-3948
Guadalupe Itzel Rodríguez Gutiérrez[1]
0000-0002-9283-1519
Álvaro Efrén Domínguez Rebolledo[3]*
0000-0002-1444-3844

[1] Tecnológico Nacional de México. Campus Conkal, Yucatán, México.

[2] Colegio de Postgraduados Campus Campeche. Champotón, Campeche, México.

[3] Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). Campo Experimental Mocochá, Yucatán, México.

***Corresponding author:**
Email address:
dominguez.alvaro@inifap.gob.mx

Abstract

The study evaluated the effect of two animal protein-free extenders on ram semen cryopreservation of two tropical hair breeds. The ejaculates were collected from 8 rams (4 Blackbelly and 4 Pelibuey), which were mixed (pooled) by breed, diluted and cryopreserved in three different extenders: Animal protein [Tris egg yolk (Triladyl®)], and animal protein-free extenders AndroMed® (lecithin soy bean) and OPTIXcell® (liposome). Sperm analyses of total (TM) and progressive (PM) motility, viability, mitochondrial activity, acrosome integrity, and plasma membrane integrity (PMI) were carried out at 0 and 6 h after semen thawing. OPTIXcell® and Triladyl® extenders showed similar results between them and differed with AndroMed® in TM, PM, viability, and PMI ($P<0.0017$). In the Blackbelly breed, the TM was higher ($P<0.0159$) in OPTIXcell® than in AndroMed®. In the Pelibuey breed, the OPTIXcell® and Triladyl® showed similar results between them and differed with AndroMed® in TM, PM, viability, mitochondrial activity, and PMI ($P<0.0140$). However, Triladyl® showed a higher percentage of sperm with intact acrosome than AndroMed® ($P<0.0392$). In both breeds, spermatic parameters decreased progressively over the incubation time similarly in all three extenders. In conclusion, OPTIXcell® and Triladyl® proved to be the best extenders to cryopreserve Blackbelly and Pelibuey ram semen. However, OPTIXcell® is an animal protein-free extender that decreases the risk of bacterial contamination, whereas Triladyl® is composed of animal protein (egg yolk), which may impact the fertilizing capacity of sperm.

Keywords: Semen; Extender; Ram; Protein-fre; Cryopreservation.

Submitted: 2022-07-29
Accepted: 2023-03-03
Published: 2023-06-26

Additional information and declarations can be found on page 11

Cite this as:

Castellanos Zacarías CA, Ramón Ugalde JP, Loeza Concha HJ, Zamora Bustillos R, Pinzón López LL, Rodríguez Gutiérrez GI, Domínguez Rebolledo ÁE. Effect of two animal protein-free extenders on cryopreservation of Pelibuey and Blackbelly ram semen. Veterinaria México OA. 2023;(10). doi: 10.22201/fmvz.24486760e.2023.1022.

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Study contribution

The most used component in mammalian semen cryopreservation extenders is egg yolk. Egg yolk acts as a complex biological compound that contains proteins, vitamins, phospholipids, glucose, and antioxidants potentially beneficial for sperm. However, due to its animal origin, the use of the egg yolk in the extender represents a risk of bacterial contamination and transmission of exotic diseases. This study evaluates the effect of three different extenders: animal protein [Tris egg yolk (Triladyl®)] and animal protein-free extenders (OPTIXcell® and Triladyl®) on ram semen cryopreservation of two tropical hair breeds. The result demonstrated that OPTIXcell® and Triladyl® proved to be the best extenders to cryopreserve Blackbelly and Pelibuey ram semen. However, OPTIXcell® is an animal protein-free extender that decreases the risk of bacterial contamination, whereas Triladyl® is composed of animal protein (egg yolk), which may impact the fertilizing capacity of sperm.

Introduction

The germplasm banks allow the storage of biological materials such as tissues, sperm, oocytes, and embryos, among others, of livestock or wild species of high genetic value, threatened or in danger of extinction, to be used in the future through assisted reproduction techniques.[1] One of the tools to achieve the exchange of genetic material between subpopulations that are geographically or biologically isolated is through artificial insemination by using cryopreserved semen. However, a factor that influences this technique is the process of cryopreservation and thawing of semen, which affects the fertilizing capacity of sperm,[2] decreasing fertility and prolificacy. Semen extenders contain nutrients and cryoprotective ingredients that are added to semen to preserve the viability and fertilizing capacity of the sperm during and after cryopreservation.[3] Among the most used components in mammalian semen extenders is egg yolk.[4] Egg yolk acts as a complex biological compound that contains proteins, vitamins, phospholipids, glucose and antioxidants that are potentially beneficial for sperm.[5] However, due to its animal origin, the use of the egg yolk in the extender represents a risk of bacterial contamination[6,7] and transmission of exotic diseases such as avian influenza.[8] In addition, it has endotoxins capable of damaging viability[9] and steroid hormones that reduce sperm motility.[10] For this reason, the World Organization for Animal Health (WOAH), recommended a sanitary code for terrestrial animals in 2003. They stated that all product of animal origin that is used in the processing of semen must be free of any biological risk.[11,12] Likewise, the egg yolk contains granular material that resembles the size and shape of sperm, which makes sperm analysis difficult using computer-assisted semen analysis system[13,14] and it can also interfere with microscopic observations or biochemical assays.[15] Considering these disadvantages, it was proposed the use of extenders free of animal protein such as soy lecithin that acts in a similar way to the lecithins of the egg yolk[16] and liposomes, which are chemically defined molecules that can transfer lipids and cholesterol to the plasma membrane of the sperm, through phosphatidylcholine by changing the transition of the lipid phase of the sperm, making them less sensitive to cooling,[17] helping to counteract the damage caused to cells that have been subjected to thermal shocks.[18]

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Therefore, this study aimed to evaluate the effects of two animal protein-free extenders on the cryopreservation of Blackbelly and Pelibuey ram semen.

Materials and methods

Ethical statement

The animal care procedures and research protocols followed the Committee of Ethics on Animal Handling guidelines of the Official Mexican Standard (NOM-027-ZOO-1995), Zoosanitary Process of Domestic Animal Semen.

Place of study

This work was conducted within the autumn (September to October, 2019) at the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), by its acronym in Spanish), located in the Mocochá experimental field at kilometer 25 of the old highway Mérida-Motul, located at 21 ° 05 ' 18 " north latitude and 89 ° 27 ' 12 " west longitude, at an altitude of 9 m above sea level. The predominant climate in the region is warm sub-humid (Awo) with a total rainfall of 900 mm and an average annual temperature of 26.5 °C.[19]

Animals

A total of 8 rams (4 Blackbelly and 4 Pelibuey), with an age of 2 ± 0.5 years and with an average live weight of 43 kg ± 2.95 were used in this study.

Semen collection

A total of 64 ejaculates (8 ejaculates / ram) were obtained per artificial vagina. Ejaculates meeting the following criteria were evaluated: volume > 0.5 mL, motility > 70 %, and sperm concentration > 3000 × 10^6 sperm/mL.

Sperm concentration

It was determined with the Bürker® chamber after a dilution of 1:200 (995 µL of distilled water + 5 µL of semen).[20]

Dilution

The ejaculates collected were mixed (pooled), split and diluted to a final concentration of 400 × 10^6 sperm/mL; with the following extenders: AndroMed® (Minitube, Tiefenbach, Germany) based on soy lecithin; OPTIXcell® (IMV Technologies, L'Aîgle, France) based on liposomes and Triladyl® (Minitube, Tiefenbach, Germany) complemented with 20 % of egg yolk. Subsequently, the diluted samples were packed in 0.25 mL straws (Minitube, Tiefenbach, Germany) for freezing.

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Freezing of semen

At the end of the packaging, the straws were stored for 4 h at 5 °C. Subsequently, they will be placed at 4 cm from the surface of the liquid nitrogen (LN2), for 10 minutes, and then immersed in LN2 and stored in cryogenic tanks until evaluation.

Thawing of semen

The thawing procedure was performed by immersing the straws in a water bath at 37 °C for 30 seconds. Subsequently, the samples were evaluated at 0 and 6 h of incubation at 37 °C.

Experimental design

A completely randomized design was used with a factorial arrangement 2 × 3 × 2 with 2 breeds, 3 extenders and 2 times series with 8 repetitions per extender.

Post-thaw seminal evaluation

Individual and progressive motility

Individual motility was performed by placing 5 µL sample diluted to ~30 × 10^6/mL of spermatozoa on a Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel), preheated to 37 °C and analyzed using a phase contrast microscope with a 10x objective (UOP, Proiser I + D, Valencia, Spain) the percentage of individual motility on a scale of 0 -- 100 %. And for progressive motility, it was performed by diluting 5 µL of thawed semen in 50 µL extender and it was evaluated on a scale of 0-5, with 5 being the highest degree of motility.

Sperm viability

Sperm viability was evaluated according to the technique described by Nagy et al.,[21] with SYBR-14/PI fluorochrome (Live/Dead® kit L-7011, Invitrogen™), adding 1 µL of SYBR-14 (10 µM) and 1 µL IP (12 µM) in 100 µL of sperm sample diluted with saline solution (PBS) and incubated in the dark for 10 minutes at 37 °C. Subsequently, 5 µL of the stained sample was placed on a slide preheated to 37 °C and counted at least 200 sperm with an epifluorescence microscope (LW Scientific i40-ADN, Georgia, US), using the 20x objective and at a wavelength of 488 nm. Those that emitted green fluorescence were considered living cells, while dead cells emitted red fluorescence.

Integrity of the acrosome

The integrity of the acrosome was evaluated using the technique described by Mendoza et al.,[22] with the fluorochrome FITC-PSA (100µg/mL, L0770, Sigma-Aldrich™), adding 2 µL of the fluorochrome in 100 µL of sperm sample diluted in PBS and incubated in the dark for 30 minutes at 37 °C. Subsequently, 5 µL of the sample was placed on a slide and were counted 200 sperm with a fluorescence microscope, using the 20x objective and a wavelength of 488 nm. The cells that emitted green fluorescence in the acrosome part were considered damaged.

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Mitochondrial activity

Mitochondrial activity was analyzed according to the technique described by Celeghini et al.,[23] using the JC-1 fluorochrome (153 µM, Molecular Probes® T-3168, Invitrogen™), mixing 1 µL of the fluorochrome in 100 µL of sperm sample diluted, for 10 minutes at 37 °C. Subsequently, 5 µL of the stained sample was placed on a slide preheated to 37 °C and were counted 200 sperm with a fluorescence microscope, using the 20x objective and with a wavelength of 488 nm. All cells that emitted orange fluorescence in the intermediate part of the flagellum were considered to have mitochondrial activity, while the cells without mitochondrial activity emitted green fluorescence.

The hypo-osmotic swelling test (Host)

The test to determine the plasma membrane integrity of the sperm tail was performed by diluting 5 µL of sperm sample in 50 µL of a hypoosmotic solution composed of 7.35 g/L of sodium citrate and 13.51 g/L of fructose,[24] and incubated for 1 h at 37 °C. Subsequently, 5 µL of the sperm sample was placed on a slide and were counted 200 sperm with a phase contrast microscope, using the 20x objective. If the plasma membrane was intact, the sperm had a helical twist of the tail (Positive Host); if in contrast, the membrane was damaged, the spermatozoa did not present a rolled tail (Negative Host).

Data analysis

The distribution of the variables was determined using the Shapiro-Wilk normality test. The variables expressed as percentages (individual motility, viability, acrosomes, mitochondria and PMI) were transformed to arcsine√(variable)/100 and the progressive motility parameter was transformed to √(variable) before its analysis. Subsequently, it was analyzed with a general linear model with procedure (ANOVA) and to determine the statistical differences between extenders, the Tukey test was used at $P \leq 0.05$. The results were subjected to a statistical analysis system with the Statistical Analysis System software, SAS.[25]

Results

The values of the sperm parameters were similar for the OPTIXcell® and Triladyl® extenders, being higher ($P < 0.0017$) than AndroMed® in TM, PM, viability, and PMI (Table 1). In the Blackbelly ram breed, TM was higher ($P < 0.0159$) in OPTIXcell® than in AndroMed® (table 2), while other sperm parameters did not show a significant difference ($P > 0.0935$) between extenders. On the other hand, in the Pelibuey ram breed, OPTIXcell® and Triladyl® were similar and superior ($P < 0.0140$) to AndroMed®; in TM, PM, viability, mitochondrial activity and PMI. In the acrosome integrity, Triladyl® was higher ($P < 0.0392$) than AndroMed®.

From 0 to 6 h, the sperm parameters progressively decreased ($P < 0.0085$) over incubation time similarly in the three extenders and in both breeds (Tables 3 and 4).

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Table 1. Effect of extender on frozen-thawed ram semen quality

Parameters (%)	Extenders			Significance level
	AndroMed®	OPTIXcell®	Triladyl®	
Total motility	33.66 ± 3.09 [b]	43.48 ± 3.43 [a]	41.06 ± 2.88 [a]	**
Progressive motility	2.69 ± 0.17 [b]	3.13 ± 0.15 [a]	3.22 ± 0.15 a	**
Viability	35.87 ± 2.76 [b]	43.31 ± 2.26 [a]	41.63 ± 2.04 [a]	**
Acrosome integrity	39.72 ± 2.97	41.16 ± 2.25	44.16 ± 2.40	ns
Mitochondrial activity	43.13 ± 2.74	44.56 ± 2.16	45.22 ± 1.69	ns
PMI	29.41 ± 1.34 [b]	32.47 ± 1.05 [a]	32.69 ± 1.27 [a]	**

Semen quality = (Mean ± SEM).
PMI: Plasma membrane integrity. ([a, b]) Different literals in the same row show significant differences between extenders. Level of significance between extenders ($P < 0.01$ **), ($P > 0.05$ ns).

Table 2. Effect of extender on frozen-thawed semen quality of Blackbelly and Pelibuey ram

Breed	Parameters (%)	Extenders			Significance level
		AndroMed®	OPTIXcell®	Triladyl®	
Blackbelly	Total motility	36.25 ± 4.82 [b]	45.62 ± 2.50 [a]	40.62 ± 3.09 [ab]	**
	Progressive motility	2.56 ± 0.24	2.63 ± 0.22	2.69 ± 0.20	ns
	Viability	38.50 ± 3.16	44.63 ± 2.49	41.31 ± 2.43	ns
	AcrosomeiIntegrity	37.75 ± 3.70	40.13 ± 3.31	42.75 ± 3.06	ns
	Mitochondrial activity	52.25 ± 3.69	42.88 ± 3.73	45.31 ± 2.49	ns
	PMI	30.94 ± 2.27	32.31 ± 1.77	31.81 ± 1.98	ns
Pelibuey	Total motility	31.06 ± 3.92 [b]	41.25 ± 3.83 [a]	41.50 ± 3.19 [a]	**
	Progressive motility	2.81 ± 0.25 [b]	3.63 ± 0.13 [a]	3.31 ± 0.20 [a]	**
	Viability	31.69 ± 2.03 [b]	41.87 ± 2.59 [a]	42.88 ± 2.01 [a]	**
	Acrosome integrity	37.88 ± 3.10 [b]	42.63 ± 2.91 [ab]	47.81 ± 2.85 [a]	*
	Mitochondrial activity	34.00 ± 2.53 [b]	46.25 ± 2.23 [a]	45.00 ± 2.39 [a]	**
	PMI	26.94 ± 1.47 [b]	32.13 ± 1.41 [a]	32.38 ± 2.08 [a]	**

Semen quality = (Mean ± SEM).
PMI: Plasma membrane integrity. ([a, b]) Different literals in the same row showed significant differences between extenders. The level of significance between extenders by breed ($P < 0.05$ *), ($P < 0.01$ **), and ($P > 0.05$ ns)

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Table 3. Effect of extender on frozen-thawed semen quality

Breed	Sperm parameters (%)	Time (hours)	Extenders		
			Andromed®	Optixcell®	Triladyl®
Pelibuey	Total motility	0	41.88 ± 5.17 ^{A}	50.63 ± 5.13 ^{A}	50.63 ± 2.40 ^{A}
	Progressive motility		3.50 ± 0.27 ^{A}	3.88 ± 0.12 ^{A}	4.00 ± 0.00 ^{A}
	Viability		35.75 ± 2.93 $^{b, A}$	47.63 ± 3.90 $^{ab, A}$	46.75 ± 2.99 $^{a, A}$
	Acrosome integrity		41.63 ± 3.87 $^{b, A}$	48.38 ± 3.22 $^{ab, A}$	54.88 ± 2.94 $^{a, A}$
	Mitochondrial activity		39.63 ± 3.51 ^{A}	48.50 ± 2.99 ^{A}	49.75 ± 2.89 ^{A}
	PMI		31.13 ± 1.78 ^{A}	35.50 ± 1.35 ^{A}	35.75 ± 3.01 ^{A}
	Total motility	6	20.25 ± 2.37 $^{b, B}$	31.88 ± 3.40 $^{ab, B}$	33.12 ± 3.75 $^{a, B}$
	Progressive motility		2.13 ± 0.23 $^{b, B}$	3.38 ± 0.18 $^{a, B}$	2.63 ± 0.18 $^{a, B}$
	Viability		27.63 ± 2.10 $^{b, A}$	36.13 ± 2.05 $^{a, B}$	39.00 ± 2.03 $^{a, B}$
	Acrosome integrity		34.13 ± 4.71 ^{B}	36.88 ± 4.07 ^{B}	40.75 ± 3.45 ^{B}
	Mitochondrial activity		28.38 ± 2.45 $^{b, B}$	44.00 ± 3.29 $^{a, B}$	40.25 ± 3.12 $^{a, B}$
	PMI		22.75 ± 1.05 $^{b, B}$	28.00 ± 1.35 $^{ab, B}$	27.75 ± 1.83 $^{a, B}$
Significance level			**	**	**

The Pelibuey ram´semen was stored at 37 °C for 6 hours.
PMI: Plasma membrane integrity. $^{(a, b)}$ Different literals in the same row show significant differences between extenders per hour. $^{(A, B)}$ Different literals in the same column show significant differences between sperm parameters over time. The level of significance between groups over time ($P < 0.01$ **).

Table 4. Effect of extender on frozen-thawed semen quality

Breed	Sperm parameters (%)	Time (hours)	Extenders		
			Andromed®	Optixcell®	Triladyl®
Blackbelly	Total motility	0	50.63 ± 4.47 ^{A}	49.37 ± 1.75 ^{A}	45.62 ± 2.20 ^{A}
	Progressive motility		3.13 ± 0.12 ^{A}	3.00 ± 0.27 ^{A}	3.13 ± 0.12 ^{A}
	Viability		50.50 ± 2.92 ^{A}	52.50 ± 2.50 ^{A}	48.62 ± 2.42 ^{A}
	Acrosome integrity		53.12 ± 4.79 ^{A}	48.63 ± 3.56 ^{A}	49.38 ± 3.97 ^{A}
	Mitochondrial activity		60.50 ± 4.02 ^{A}	50.88 ± 4.57 ^{A}	51.75 ± 2.50 ^{A}
	PMI		36.25 ± 2.74 ^{A}	36.38 ± 1.58 ^{A}	35.75 ± 2.43 ^{A}
	Total motility	6	21.87 ± 4.52 ^{B}	27.87 ± 1.32 ^{B}	24.50 ± 2.74 ^{B}
	Progressive motility		2.00 ± 2.38 ^{B}	2.25 ± 0.31 ^{B}	2.25 ± 0.31 ^{B}
	Viability		31.62 ± 3.42 ^{B}	38.12 ± 2.71 ^{B}	32.12 ± 2.29 ^{B}
	Acrosome integrity		30.00 ± 4.70 ^{B}	32.00 ± 3.69 ^{B}	31.62 ± 3.45 ^{B}
	Mitochondrial activity		44.00 ± 4.78 ^{B}	34.88 ± 4.52 ^{B}	39.12 ± 2.90 ^{B}
	PMI		25.87 ± 2.56 ^{B}	28.62 ± 2.48 ^{B}	28.25 ± 2.55 ^{B}
Significance level			**	**	**

The Blackbelly ram´semen was stored at 37 °C for 6 hours (Mean ± SEM).
PMI: Plasma membrane integrity. $^{(A, B)}$ Different literals in the same column show significant differences between sperm parameters over time. The level of significance between groups over time ($P < 0.01$ **).

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Discussion

In vivo tests were excluded from this study. In this sense, different studies are related to post-thawing semen quality where this type of test is not included, such as in the ram,[26, 27] collared peccary,[28] horses,[29] goat,[30] among others. Moreover, *in vitro* fertility does not depend solely on the fertilizing capacity of the sperm, but also on the capacity of the oocyte to be fertilized.[31, 32] On the other hand, the *in vivo* test depends largely on the fertility of the female, which is affected by a wide range of factors, such as farm, year, season, artificial insemination technique, and technician,[33] heat stress,[34–36] diet,[37] health status, as well as parity, lambing interval, body condition score, genetic traits, full functionality of reproductive organs, herd management, female prolificacy, nutritional management before and after artificial insemination, the type of estrus (natural or hormonally manipulated), and the site of deposition of the semen and climate factors.[38, 39]

On the other hand, the sperm parameters evaluated in this study were selected due to their predictive capacity in fertility. For instance, several studies have indicated that sperm viability (live/dead) is related to sheep fertility[40] and bull fertility[41–44] after artificial insemination. Christensen et al.[45] and Kumaresan et al.[46] reported that the most precise estimation of a bull's nonreturn rate at 56 days was achieved through the assessment of sperm viability in neat semen and post-thaw sperm viability. Likewise, it has been shown a that a reduced sperm viability is correlated with impaired male fertility.[47] The assessment of sperm motility *in vitro*, it is essential to understand factors which alter motility.[48]

Poor sperm motility has been associated with low rates of fertilization success in many species.[49, 50–52] Mass motility, in particular, is useful in ram semen motility assessment due to its correlation to *in vivo* sperm fertilizing capacity.[53] In addition, it has been shown that sperm motility is a convincing indicator of fertility in boar[54] and human.[55] Mitochondrial activity is related to sperm motility and has been associated to the ability of spermatozoa to fertilize oocytes.[56–58] The hypoosmotic swelling test (HOST) has proved to be a good tool for evaluating the plasma membrane integrity of the sperm tail of various domestic animals.

This method contributes to conventional sperm quality tests in predicting the success of *in vitro* fertilization.[59] Oliveira et al.[60] reported that the sire that presented numerically lower pregnancy after AI also presented a lower percentage of sperm with a swelling tail. The presence of an acrosome (the acrosomal status) and the ability of the spermatozoa to release the acrosomal content (the acrosomal responsiveness) and expose zona pellucida binding sites in response to female factors correlates with the outcomes of *in vitro* fertilization.[61, 62] Kumaresan et al.[46] reported that acrosome status is highest correlated with bull's nonreturn rate at 56 days.

The results of this study showed that the effect of extender on sperm parameters upon thawing are similar to those reported by Stewart et al.[63] in semen samples from white-tailed deer and in bulls of the Holstein-Friesian breed,[64] where the TM and PM of the spermatozoa were higher with the OPTIXcell® extender upon thawing. Likewise, the viability was also similar to the OPTIXcell® extender in frozen-thawed semen samples of buffalo,[18] Holstein bull and Red Swiss bull,[65] Korean bull,[66] and dromedary camel.[67] However, the results obtained from viable spermatozoa differed from those reported by Ondřej et al.[68] Frozen-thawed seminal samples from the bull were higher with AndroMed® extender than with OPTIXcell®. In the PMI, the results are like those reported by Ansari et al.[18] in

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

bull semen samples frozen with the OPTIXcell® extender, while in the dromedary camel semen, the Triladyl® extender showed a higher percentage of sperm with the membrane integrated upon thawing. The other parameters did not show differences between extenders, being like that reported in frozen-thawed samples of buffalo,[18] in Ghezel [69] and Dorper ram,[70] in bull[65] and rhinoceros.[71]

In the Blackbelly breed, the results are like those obtained in buffalo semen samples,[17] in Balinese bull,[72] in Korean bull,[66] and in *Bos indicus* bull,[73] which reported a higher TM in frozen spermatozoa with OPTIXcell® extender. However, Gomes-Alves et al.[74] reported that in Brown bear, TM was higher in seminal samples frozen-thawed with the Tris extender complemented with egg yolk than the AndroMed® extender. The sperm parameters PM, viability, acrosome integrity, mitochondrial activity and PMI are similar to those reported by Fleisch et al.[75] in frozen-thawed bull semen samples. In the Pelibuey breed, the results are similar to those reported in semen from the Holstein bull and the Swiss red bull,[66] where TM, PM, mitochondrial activity, and PMI had higher percentages with the OPTIXcell® extender. However, in acrosome integrity differs from that reported by Singh et al.[17] in Bos indicus bull, no differences were found with the extenders AndroMed®, OPTIXcell®, and Triladyl®. In contrast to these results, Souza et al.[76] showed that Tris extender improves acrosome integrity more than the Optixcell® extender in frozen/thawed semen sample of Santa Inês ram. These differences between results may be associated with the species and breed of the animal, as well as between individuals within a species.[77] This may be attributed, in part, to the lipid composition[78, 79] and the polyunsaturated fatty acids (PUFAs) content present in the sperm membranes,[80] which has an important role in the functionality of the sperm cell, by providing fluidity and permeability, which are related to an increase in cryoresistance.

The sperm parameters evaluated at 0 h and at 6 h of incubation are similar to those found in previous studies with thawed semen samples. TM, PM, acrosome integrity, mitochondrial activity and PMI decreased progressively over time similarly with the extenders AndroMed®, OPTIXcell® and Tris complemented with egg yolk in buffalo semen samples; 0 to 2 hours.[81] On one hand, they are similar to those reported by Wojtusik et al.[71] in samples of thawed rhinoceros semen; from 0 to 1 h and from a Balinese bull; from 0 to 4 hours[73] where TM, PM, viability, acrosome integrity, and PMI decreased progressively over time similarly with extenders OPTIXcell® and Tris complemented with egg yolk. On the other hand, Amal et al.[72] mention that the response time of the spermatozoa in incubation will depend on the metabolism of the spermatozoa, as it may depend on the variation of breed and/or species. However, Talini et al.[82] quote that the incubation time after thawing does not provide consistent parameters to determine semen quality.

It was observed that the OPTIXcell® extender made from liposomes presented the highest percentage in most of the evaluated sperm variables, compared to the AndroMed® extender made from soy lecithin and the Triladyl® extender complemented with animal protein (chicken egg yolk). The difference between extender (animal protein-free) may be due to the percentage of soy lecithin added to the extender; since it has been proven that high concentrations may be toxic and lower concentrations may be insufficient to protect the plasma membrane from sperm during its cryopreservation.[83] On the other hand, Salmin et al.[84] mention that the enzyme phospholipase A, that found in the seminal plasma of ram semen can tolerate

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 2023

the levels of soy lecithin, so it does not catalyze the levels of soy lecithin into fatty acids and lysolecithins, causing coagulation in the extender and toxicity in the sperm. Likewise, Ansari et al.[18] mentioned that liposomes act by adhering to the sperm, exchanging cholesterol and phospholipids, replacing the lipoproteins in seminal plasma. One or more biological layers of concentric lipids, which help encapsulate molecules[85] capable of rehydrating sperm,[86, 87] counteract the deterioration of phospholipids and the loss of cholesterol[88] caused by thermal shocks in freezing-thawing,[18] especially in ruminants,[64] helping improve the percentage of sperm fertility.

Conclusions

In conclusion, the extenders OPTIXcell® and Triladyl® are the ones that best cryopreserve Blackbelly and Pelibuey ovine semen. However, OPTIXcell® extender, being free of animal protein in its preparation, reduces the risk of bacterial contamination; unlike Triladyl® extender, which, as it is composed of animal protein (egg yolk), can affect the fertilizing capacity of the sperm.

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

Data availability

All relevant data are within the manuscript and its supporting information files.

Acknowledgments

The authors thank Dr. José Luis Ros Santaella for his comments and suggestions of this manuscript.

Funding statement

This work was supported by Fiscal Project Number. 1610133865 of INIFAP.

Conflicts of interest

The authors have no conflict of interest to declare in regard to this publication.

Author contributions

Conceptualization: CA Castellanos-Zacarías, AE Domínguez-Rebolledo,
Data curation: CA Castellanos-Zacarías, AE Domínguez-Rebolledo, HJ Loeza-Concha.
Formal analysis: CA Castellanos-Zacarías, AE Domínguez-Rebolledo, JP Ramón-Ugalde, HJ Loeza-Concha.
Funding acquisition: AE Domínguez-Rebolledo
Investigation: CA Castellanos-Zacarías, AE Domínguez-Rebolledo, JP Ramón-Ugalde
Methodology: CA Castellanos-Zacarías, AE Domínguez-Rebolledo, JP Ramón-Ugalde.
Project administration: AE Domínguez-Rebolledo, JP Ramón-Ugalde.
Resources: AE Domínguez-Rebolledo.
Software: CA Castellanos-Zacarías, AE Domínguez-Rebolledo.
Supervision: AE Domínguez-Rebolledo, JP Ramón-Ugalde, R Zamora-Bustillos.
Validation: AE Domínguez-Rebolledo, JP Ramón-Ugalde, R Zamora-Bustillos, LL Pinzón-López.
Visualization: AE Domínguez-Rebolledo, JP Ramón-Ugalde, R Zamora-Bustillos, LL Pinzón-López, GI Rodríguez-Gutiérrez.
Writing-original draft: CA Castellanos-Zacarías.
Writing-review and editing: AE Domínguez-Rebolledo, JP Ramón-Ugalde, R Zamora-Bustillos, LL Pinzón-López, GI Rodríguez-Gutiérrez, HJ Loeza-Concha.

References

1. Comizzoli P, Mermillod P, Mauget R. Reproductive biotechnologies for endangered mammalian species. Reproduction Nutrition Development. 2000;40(5):493–504. doi: 10.1051/rnd:2000113.
2. Roca J, Rodríguez M, Gil M, Carvajal G, García E, Cuello C, et al. Survival and *in vitro* fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. Journal of Andrology. 2005;26:15–24. PMID: 15611562.
3. Viveiros A, Lock E, Woelders H, Komen J. Influence of cooling rates and plunging temperatures in an interrupted slow-freezing procedure for semen of the African catfish, *Clarias gariepinus*. Cryobiology. 2002;43(3):276–287. doi: 10.1006/cryo.2001.2362.
4. Crespilho AM, Sá Filho MF, Dell'Aqua JA, Nichi M, Monteiro GA, Avanzi BR, et al. Comparison of *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. Livestock Science. 2012;149(1–2):1–6. doi: 10.1016/j.livsci.2012.05.011.

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

5. Huopalahti R, López-Fandiño R, Anton M, Schade R. Use of egg compounds for cryopreservation of spermatozoa. In: Bioactive Egg Compounds. Berlin, Heidelber: Springer-Verlag; 2007. 259–262 p.
6. Aires A, Hinsch K, Mueller F, Mueller-Schloesser F, Bogner K, Mueller-Schloesser S, et al. *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. Theriogenology. 2003;60(2):269–279. doi: 10.1016/s0093-691x(02)01369-9.
7. Amirat L, Anton M, Tainturier D, Chatagnon G, Battut I, Courtens JL. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reproduction. 2005;129(4):535–543. doi: 10.1530/rep.1.00011.
8. Yildiz C, Bozkurt Y, Yavas I. An evaluation of soybean lecithin as an alternative to avian egg yolk in the cryopreservation of fish sperm. Cryobiology. 2013;67(1):91–94. doi: 10.1016/j.cryobiol.2013.05.008.
9. Vidal AH, Batista AM, da Silva ECB, Gomes WA, Pelinca MA, Silva SV, et al. Soybean lecithin-based extender as an alternative for goat sperm cryopreservation. Small Ruminant Research. 2013;109(1):47–51. doi: 10.1016/j.smallrumres.2012.07.022.
10. El-Sisy GA, El-Nattat WS, El-Sheshtawy RI, El-Maaty AM. Substitution of egg yolk with different concentrations of soybean lecithin in tris-based extender during bulls semen preservability. Asian Pacific Journal Reproduction. 2016;5(6):514–518. doi: 10.1016/j.apjr.2016.10.011.
11. Marco-Jiménez F, Puchades S, Mocé E, Viudes-de-Cartro MP, Vicente JS, Rodriguez M. Use of powdered egg yolk vs fresh egg yolk for the cryopreservation of ovine semen. Reproduction in Domestic Animals. 2004;39(6):438–441. doi: 10.1111/j.1439-0531.2004.00537.x.
12. Layek SS, Mohanty TK, Kumaresan A, Parks JE. Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. Animal Reproduction Science. 2016;172:1–9. doi: 10.1016/j.anireprosci.2016.04.013.
13. Ansari MS, Rakha BA, Andrabi SM, Akhter S. Usefulness of powdered and fresh egg yolk for cryopreservation of Zebu bull spermatozoa. Reproductive Biology. 2010;10(3):235–240. doi: 10.1016/s1642-431x(12)60043-6.
14. Singh AK, Singh VK, Narwade BM, Mohanty TK, Atreja SK. Comparative quality assessment of buffalo (*Bubalus bubalis*) semen chilled (5 °C) in egg yolk and soya milk based extenders. Reprodution in Domestic Animals. 2012;47(4):596–600. doi: 10.1111/j.1439-0531.2011.01928.x.
15. Pillet E, Duchamp G, Batellier F, Beaumal V, Anton M, Desherces S, et al. Egg yolk plasma can replace egg yolk in stallion freezing extenders. Theriogenology. 2011;75(1): 105–114. doi: 10.1016/j.theriogenology.2010.07.015.
16. Belala R, Delay J, Amirat L, Ropers MH, Guillou JL, Anton M, et al. The benefits of liposomes for chilling canine sperm for 4 days at 4 °C. Animal Reproduction Science. 2016;168:100–109. doi: 10.1016/j.anireprosci.2016.02.032.
17. Singh AK, Kumar A, Honparkhe M, Kaur S, Kaur H, Ghuman S, et al. Comparison of in vitro and in vivo fertilizing potential of buffalo bull semen frozen in egg yolk, soya bean lecithin and liposome based extenders. Reproduction in Domestic Animals. 2018;53(1):195–202. doi: 10.1111/rda.13092.

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

18. Ansari MS, Rakha BA, Akhter S, Ashiq M. OPTIXcell improves the postthaw quality and fertility of buffalo bull sperm. Theriogenology. 2016;85(3):528–532. doi: 10.1016/j.theriogenology.2015.09.035.
19. INEGI. Anuario estadístico y geográfico de Yucatán. 2017.
20. Kvist U, Björndahl L. Sperm concentration. In: Manual on Basic Semen Analysis. Oxford: Oxford University Press; 2002. pp. 7–9.
21. Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biology Reproduction. 2003;68(5):1828–1835. doi: 10.1095/biolreprod.102.011445.
22. Mendoza C, Carreras A, Moos J, Tesarik J. Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. Journal of Reproduction and Fertility. 1992;95(3):755–763. doi: 10.1530/jrf.0.0950755.
23. Celeghini EC, De Arruda RP, De Andrade AF, Nascimento J, Raphael CF. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reproduction in Domestic Animals. 2007;42(5);479–488. doi: 10.1111/j.1439-0531.2006.00810.x.
24. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Journal of Reproduction and Fertility. 1984;70(1):219–228. doi: 10.1530/jrf.0.0700219.
25. Statistical Analysis System (SAS). Release 9.1 for windows. Cary, NC, USA: SAS Institute Inc; 2003.
26. Carrera-Chávez JM, Jiménez-Aguilar EE, Acosta-Pérez TP, Núñez-Gastélum JA, Quezada-Casasola A, Escárcega-Ávila AM, et al. Effect of *Moringa oleifera* seed extract on antioxidant activity and sperm characteristics in cryopreserved ram semen. Journal of Applied Animal Research. 2020;48(1):114–120. doi: 10.1080/09712119.2020.1741374.
27. Shehab-El-Deen M, Ali M, Al-Sharari M. Effects of extenders supplementation with gum arabic and antioxidants on ram spermatozoa quality after cryopreservation. Animals. 2023;13(1):111. doi: 10.3390/ani13010111.
28. Moreira SSJ, Silva AM, Pereira AG, Santos RP, Dantas MRT, Souza-Júnior JBF, et al. Effect of detergents based on sodium dodecyl sulfate on functional metrics of frozen–thawed collared peccary (*Pecari tajacu*) semen. Animals. 2023;13(3):451. doi: 10.3390/ani13030451.
29. Gutiérrez-Cepeda L, Crespo F, Blazquez JC, Serres C. Optimization of the equine-sperm freeze test in purebred Spanish horses by incorporating colloidal centrifugation. Animals. 2023;13(3):382. doi: 10.3390/ani13030382.
30. Galián S, Peinado B, Almela L, Poto Á, Ruiz S. Post-thaw quality of spermatozoa frozen with three different extenders in the Murciano Granadina goat breed. Animals. 2023;13(2):309. doi: 10.3390/ani13020309.
31. Ectors FJ, Vanderzwalmen P, Van Hoeck J, Nijs M, Verhaegen G, Delvigne A, et al. Relationship of human follicular diameter with oocyte fertilization and development after in-vitro fertilization or intracytoplasmic sperm injection. Human Reproduction. 1997;12(9):2002–2005. doi: 10.1093/humrep/12.9.2002.

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

32. Swain JE, Pool TB. ART failure: oocyte contributions to unsuccessful fertilization, Human Reproduction Update. 2008;14(5):431–446. doi: 10.1093/humupd/dmn025.
33. Anel L, Alvarez M, Martinez-Pastor F, Garcia-Macias V, Anel E, De Paz P. Improvement strategies in ovine artificial insemination. Reproduction in Domestic Animals. 2006;41(s2):30–42. doi: 10.1111/j.1439-0531.2006.00767.x.
34. Dixon AB, Knights M, Winkler JL, Marsh DJ, Pate JL, Wilson ME, et al. Patterns of late embryonic and fetal mortality and association with several factors in sheep. Journal of Animal Science. 2007;85(5):1274–1284. doi: 10.2527/jas.2006-129.
35. Gharibzadeh Z, Riasi A, Ostadhosseini S, Hosseini SM, Hajian M, Nasr-Esfahani MH. Effects of heat shock during the early stage of oocyte maturation on the meiotic progression, subsequent embryonic development and gene expression in ovine. Zygote. 2015;23(4):573–582. doi: 10.1017/S0967199414000203.
36. Abecia JA, Arrébola F, Macías A, Laviña A, González-Casquet O, Benítez F, Palacios C. Temperature and rainfall are related to fertility rate after spring artificial insemination in small ruminants. International Journal of Biometeorology. 2016;60:1603–1609. doi: 10.1007/s00484-016-1150-y.
37. Errandonea N, Fierro S, Viñoles C, Gil J, Banchero G, Olivera-Muzante J. Short term protein supplementation during a long interval prostaglandin-based protocol for timed AI in sheep. Theriogenology. 2018;114(1):34–39. doi: 10.1016/j.theriogenology.2018.03.021.
38. Gibbons AE, Fernandez J, Bruno-Galarraga MM, Spinelli MV, Cueto MI. Technical recommendations for artificial insemination in sheep. Animal Reproduction. 2019;16(4):803–809. doi: 10.21451/1984-3143-AR2018-0129.
39. Priskas S, Termatzidou SA, Gargani S, Arsenos G. Evaluation of factors affecting pregnancy rate after cervical insemination of dairy ewes in Greece. Journal of Veterinary Science and Medicine. 2019;7(2):1–7.
40. Santolaria P, Vicente-Fiel S, Palacín I, Fantova E, Blasco ME, Silvestre MA, et al. Predictive capacity of sperm quality parameters and sperm subpopulations on field fertility after artificial insemination in sheep. Animal Reproduction Science. 2015;163:82–88. doi: 10.1016/j.anireprosci.2015.10.001.
41. Christensen P, Boelling D, Pedersen KM. Korsgaard IR, Jensen J. Relationship between sperm viability as determined by flow cytometry and nonreturn rate of dairy bulls. Journal of Andrology. 2005;26(1):98–106. doi: 10.1002/j.1939-4640.2005.tb02878.x.
42. Januskauskas A, Johannisson A, Rodriguez-Martinez H. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure and field fertility. Theriogenology 2003;60(4):743–758. doi: 10.1016/s0093-691x(03)00050-5.
43. Tartaglione CM, Ritta MN. Prognostic value of spermatological parameters as predictors of *in vitro* fertility of frozen–thawed bull semen. Theriogenology 2004;62(7):1245–1252. doi: 10.1016/j.theriogenology.2004.01.012.
44. Rodríguez-Martinez H. State of art in farm animal sperm evaluation. Reproduction, Fertility and Development. 2007;19(1):91–101. doi: 10.1071/rd06104.
45. Christensen P, Labouriau R, Birck A, Boe-Hansen GB, Pedersen J, Borchersen S. Relationship among seminal quality measures and field fertility of young dairy

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

bulls using low-dose inseminations. Journal of Dairy Science. 2011;9484):1744–1754. doi: 10.3168/jds.2010-3087.

46. Kumaresan A, Johannisson A, Al-Essawe EM, Morrell JM. Sperm viability, reactive oxygen species, and DNA fragmentation index combined can discriminate between above- and below-average fertility bulls. Journal of Dairy Science. 2017;100(7):5824–5836. doi: 10.3168/jds.2016-12484.
47. Samplaski MK, Dimitromanolakis A, Lo KC, Grober ED, Mullen B, Garbens A, Jarvi KA. The relationship between sperm viability and DNA fragmentation rates. Reproductive Biology and Endocrinology. 2015;14:13-42. doi: 10.1186/s12958-015-0035-y.
48. Van de Hoek M, Rickard JP, de Graaf SP. Motility assessment of ram spermatozoa. Biology. 2022;11(12):1715. doi: 10.3390/biology11121715.
49. Kjaestad H, Ropstad E, Berg KA. Evaluation of spermatological parameters used to predict the fertility of frozen bull semen. Acta Veterinaria Scandinavica. 1993;34(3):299–303. doi: 10.1186/BF03548194.
50. Wierzbowski S, Kareta W. An assessment of sperm motility estimation for evaluation in rams. Theriogenology. 1993;40(1):205–209. doi: 10.1016/0093-691X(93)90354-8.
51. Love CC. Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 2011;76(3):547–557. doi: 10.1016/j.theriogenology.2011.03.007.
52. Puglisi R, Pozzi A, Foglio L, Spanò M, Eleuteri P, Grollino MG, et al. The usefulness of combining traditional sperm assessments with *in vitro* heterospermic insemination to identify bulls of low fertility as estimated *in vivo*. Animal Reproduction Science. 2012;132(1-2):17–28. doi: 10.1016/j.anireprosci.2012.04.006.
53. David I, Kohnke P, Lagriffoul G, Praud O, Plouarboué F, Degond P, et al. Mass sperm motility is associated with fertility in sheep. Animal Reproduction Science. 2015;161:75-81. doi: 10.1016/j.anireprosci.2015.08.006.
54. Fernández-López P, Garriga J, Casas I, Yeste M, Bartumeus F. Predicting fertility from sperm motility landscapes. Communications Biology. 2022;5:1027. doi: 10.1038/s42003-022-03954-0.
55. Villani MT, Morini D, Spaggiari G, Falbo AI, Melli B, La Sala GB, et al. Are sperm parameters able to predict the success of assisted reproductive technology? A retrospective analysis of over 22,000 assisted reproductive technology cycles. Andrology. 2022;10(2):310–321. doi: 10.1111/andr.13123.
56. Malić Vončina S, Golob B, Ihan A, Kopitar AN, Kolbezen M, Zorn B, et al. Sperm DNA fragmentation and mitochondrial membrane potential combined are better for predicting natural conception than standard sperm parameters. Fertility and Sterility. 2016;105(3):637-644. doi: 10.1016/j.fertnstert.2015.11.037.
57. Ferreira JJ, Cassina A, Irigoyen P, Ford M, Pietroroia S, Peramsetty N, et al. Increased mitochondrial activity upon CatSper channel activation is required for mouse sperm capacitation. Redox Biology. 2021;48. No. 102176. doi: 10.1016/j.redox.2021.102176.
58. Giaccagli MM, Gómez-Elías MD, Herzfeld JD, Marín-Briggiler CI, Cuasnicú PS, Cohen DJ, et al. Capacitation-induced mitochondrial activity is required for sperm fertilizing ability in mice by modulating hyperactivation. Frontiers in Cell and Developmental Biology. 2021;9. No. 767161. doi: 10.3389/fcell.2021.767161.

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

59. Brito LFC, Barth AD, Bilodeau-Goeseels S, Panich PL,Kastelic JP. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology. 2003;60(8):1539–1551. doi: 10.1016/s0093-691x(03)00174-2.
60. Oliveira LZ, de Arruda RP, de Andrade AFC, Celeghini ECC, dos Santos RM, Beletti ME, et al. Assessment of field fertility and several *in vitro* sperm characteristics following the use of different Angus sires in a timed-AI program with suckled Nelore cows. Livestock Science. 2012;146(1):38–46. doi: 10.1016/j.livsci.2012.02.018.
61. Liu DY, Baker HW. Calcium ionophore-induced acrosome reaction correlates with fertilization rates *in vitro* in patients with teratozoospermic semen. Human Reproduction. 1998;13(4):905–910. doi: 10.1093/humrep/13.4.905.
62. Xu F, Zhu H, Zhu W, Fan L. Human sperm acrosomal status, acrosomal responsiveness, and acrosin are predictive of the outcomes of *in vitro* fertilization: a prospective cohort study. Reproductive Biology. 2018;18(4):344–354. doi: 10.1016/j.repbio.2018.10.007.
63. Stewart JL, Shipley CF, Katich AS, Po E, Ellerbrock RE, Lima FS, et al. Cryopreservation of white-tailed deer (*Odocoileus virginianus*) semen using soybean, liposome and egg yolk-based extenders. Animal Reproduction Science. 2016;171:7–16. doi: 10.1016/j.anireprosci.2016.05.006.
64. Murphy EM, O'Meara C, Eivers B, Lonergan P, Fair S. Comparison of plant and egg yolk-based semen diluents on *in vitro* sperm kinematics and *in vivo* fertility of frozen-thawed bull semen. Animal Reproduction Science. 2018;191:70–75. doi: 10.1016/j.anireprosci.2018.02.010.
65. Lima-Verde IB, Johannisson A, Ntallaris T, Al-Essawe E, Al-Kass Z, Nongbua T, et al. Effect of freezing bull semen in two non-egg yolk extenders on post-thaw sperm quality. Reproduction in Domestic Animals. 2018;53(1):127–136. doi: 10.1111/rda.13080.
66. Kang SS, Lee MS, Kim UH, Lee SD, Yang BC, Yang BS, et al. Effect of Optixcell and Triladyl extenders on frozen-thawed sperm motilities and calving rates following artificial insemination in Hanwoo. Korean Journal Agricultural Science. 2019;46(1);195–204. doi: 10.7744/kjoas.20190009.
67. Abdel-Aziz SA, Saadeldin IM, Ba-Awadh H, Al-Mutary MG, Moumen AF, Alowaimer AN, et al. Efficiency of commercial egg-yolk free and egg-yolk supplemented tris-based extenders for dromedary camel semen cryopreservation. Animals. 2019;9(11):999. doi: 10.3390/ani9110999.
68. Ondřej Š, Jiří Š, Jan B, Pavla MP, Lucie T, Doležalová M, et al. Low density lipoprotein important player in increasing cryoprotective efficiency of soybean lecithin-based bull semen extenders. Animal Reproduction. 2019;16(2):267–276. doi: 10.21451/1984-3143-AR2018-0107.
69. Mehdipour M, Daghigh Kia H, Nazari M, Najafi A. Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. Cryobiology. 2017;78:34–40. doi: 10.1016/j.cryobiol.2017.07.005.
70. Luna-Orozco JR, González-Ramos MA, Calderón-Leyva G, Gaytán-Alemán LR, Arellano-Rodríguez F, Ángel-García O, et al. Comparison of different diluents based on liposomes and egg yolk for ram semen cooling and cryopreserva-

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

tion. Iranian Journal of Veterinary Research. 2019;20(2):126–130. PMCID: PMC6716279.

71. Wojtusik J, Stoops MA, Roth TL. Animal protein-free OptiXcell and shortened equilibration periods can replace egg yolk-based extender and slow cooling for rhinoceros semen cryopreservation. Cryobiology. 2019;89:21–25. doi: 10.1016/j.cryobiol.2019.06.003.
72. Amal AS, Arifiantini RI, Setiadi MA, Said S. Characteristics of the post-thawed Balinese bull semen extended in three different extenders and equilibration times. Journal of the Indonesian Tropical Animal Agriculture. 2019:44(2);135-145. doi: 10.14710/jitaa.44.2.135-145.
73. Singh A, Bhakat M, Mohanty TK, Mondal S, Yadav SK, Kumar P, et al. Effect of tris-egg yolk, soya milk and liposome based extenders on sahiwal (*Bos indicus*) sperm quality during pre and post cryopreservation stages. CryoLetters. 2019;40(2):94–102. PMID: 31017609.
74. Gomes-Alves S, Alvarez M, Nicolas M, Lopez-Urueña E, Martínez-Rodríguez C, Borragan S, et al. Use of commercial extenders and alternatives to prevent sperm agglutination for cryopreservation of brown bear semen. Theriogenology. 2014;82(3):469–474. doi: 10.1016/j.theriogenology.2014.05.015.
75. Fleisch A, Malama E, Witschi U, Leiding C, Siuda M, Janett F, et al. Effects of an extension of the equilibration period up to 96 hours on the characteristics of cryopreserved bull semen. Theriogenology. 2017:89;255–262. doi: 10.1016/j.theriogenology.2016.10.018.
76. Souza C, Brandao F, Santos J, Alfradique V, Brair V, Prellwitz L, et al. 38 Ram sperm longevity after cryopreservation in extender containing L-carnitine. Reproduction, Fertility and Development. 2020;32(2):145. doi: 10.1071/RDv32n2Ab38.
77. Loomis PR, Graham JK. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Animal Reproduction Science. 2008;105(1–2):119–128. doi: 10.1016/j.anireprosci.2007.11.010.
78. Arav A, Pearl M, Zeron Y. Does membrane lipid profile explain chilling sensitivity and membrane lipid phase transition of spermatozoa and oocytes? CryoLetters. 2000;21(3):179–186. PMID: 12148049.
79. Holt WV. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology. 2000;53(1):47–58. doi: 10.1016/S0093-691X(99)00239-3.
80. Waterhouse KE, Hofmo PO, Tverdal A, Miller RR. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction. 2006;131(5):887–894. doi: 10.1530/rep.1.01049.
81. Kumar P, Saini M, Kumar D, Balhara AK, Yadav SP, Singh P, et al. Liposome-based semen extender is suitable alternative to egg yolk-based extender for cryopreservation of buffalo (*Bubalus bubalis*) semen. Animal Reproduction Science. 2015;159:38–45. doi: 10.1016/j.anireprosci.2015.05.010.
82. Talini R, Kozicki LE, Gaievski FR, Polo G, Lima LG, Santiago J, et al. Bovine semen thermoresistance tests and their correlation with pregnancy rates after fixed-time artificial insemination. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2019;71(06):1917–1925. doi: 10.1590/1678-4162-10994.
83. Naijian HR, Kohram H, Shahneh AZ, Sharafi M. Effects of various concentrations of BSA on microscopic and oxidative parameters of Mahabadi goat semen fol-

doi: http://dx.doi.org/10.22201/fmvz.24486760e.2023.1022
Vol. 10 | 2023

lowing the freeze–thaw process. Small Ruminant Research. 2013:113(2);371–375. doi: 10.1016/j.smallrumres.2013.03.015.

84. Salmin S, Ismaya I, Kustopo K, Beliarti E. The effect of semen washing and soybean lecithin level on motility and viability of ram spermatozoa stored at 5 °C. Journal of the Indonesian Tropical Animal Agriculture. 2012;37(4):244–249. doi: 10.14710/jitaa.37.4.244-249.
85. Asadpour R, Jafari R, Tayefi-Nasrabadi H. The effect of antioxidant supplementation in semen extenders on semen quality and lipid peroxidation of chilled bull spermatozoa. Iranian Journal of Veterinary Research. 2012;13(3):246–249. doi: 10.22099/IJVR.2012.365.
86. Pillet E, Labbe C, Batellier F, Duchamp G, Beaumal V, Anton M, et al. Liposomes as an alternative to egg yolk in stallion freezing extender. Theriogenology. 2012;77(2):268–279. doi: 10.1016/j.theriogenology.2011.08.001.
87. Belala R, Briand-Amirat L, Vinciguerra L, Tainturier D, Kaidi R, Thorin C, et al. Effect of equilibration time on the motility and functional integrity of canine spermatozoa frozen in three different extenders. Research in Veterinary Science. 2016;106:66–73. doi: 10.1016/j.rvsc.2016.03.010.
88. Sieme H, Oldenhof H, Wolkers WF. Sperm membrane behaviour during cooling and cryopreservation. Reproduction in Domestic Animals. 2015;50(3):20–26. doi: 10.1111/rda.12594.