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The effect of heterologous seminal plasma from ram, buck or camel on the freezability of ram semen

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ABSTRACT: Here, we compared the effects of ram, buck and dromedary camel seminal plasma mixed with TRIS-egg yolk glycerol extender on the freezing preservation of ram semen. Awassi ram semen samples underwent primary evaluation and were then pooled and diluted with the following diluents: TRIS-egg yolk glycerol mixed with (1) whole ram semen as a control (T); (2) ram sperm after seminal plasma removal (W); or (3) ram, (4) buck or (5) camel seminal plasma (R, B and C, respectively). The diluted semen was frozen using liquid nitrogen vapor. Various sperm parameters were evaluated in the frozen semen. Total motility before and after freezing was significantly higher in R, B and C diluents than in T and W diluents. Progressive motility after freezing was significantly higher in R, B, C and T diluents than in W diluent. Vitality after freezing was significantly higher in B than in W diluent. DNA fragmentation before and after freezing was significantly lower in R, B, C and T diluents than in W diluent. Plasma membrane integrity before and after freezing was significantly higher in R, B and C diluents than in W diluent. Sperm abnormalities before freezing were significantly lower in R, B and C diluents than in W diluent. Malondialdehyde concentration was significantly higher in T and W diluents than other diluents. Reduced glutathione concentration was significantly higher in B diluent than other diluents. Moreover, reduced glutathione concentration was significantly higher in C, R and W diluents than in T diluent. Thus, the addition of ram, buck or camel seminal plasma to TRIS-egg yolk glycerol extender improved the quality of frozen ram semen, while seminal plasma removal adversely affected it. Ram, buck and camel seminal plasma had similar effects, with no significant differences between them on the evaluated parameters of frozen ram semen.

Keywords: sperm; extender; cryopreservation; sheep; goat; post-thawing; TRIS

Artificial insemination (AI) is a commonly used assisted reproductive technology that can achieve rapid genetic improvement with high efficiency and low costs. Furthermore, AI is used to prevent disease spread between animals, especially venereal diseases. The use of different semen extenders to improve semen extenders for the protection of sperm during freezing and thawing processes has

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been the focus of reproductive biology research in the last two decades (Gil et al. 2003; Fukui et al. 2008; Forouzanfar et al. 2010). The quality of sperm is reduced considerably after thawing because of sperm exposure to osmotic stress and cold shock during the freezing and thawing process (Salamon and Maxwell 2000). Most of these types of damage can be minimised by using suitable extenders (Gil et al. 2003; Barbas and Mascarenhas 2009). The use of frozen semen for AI in different animal species, which usually includes removal of or highly diluted seminal plasma (SP), leads to lower fertility rates than those after natural mating (Tummaruk et al. 2000). Therefore, SP components are crucial in sperm function and fertilisation.

SP activates and augments of the motility of spermatozoa, acts as a buffer to ensure optimum osmotic pressure and as a nutrient medium, prevents premature sperm activation during its physiological journey, protects its plasma membrane (PM) and inhibits its capacitation (Villemure et al. 2003). SP protects sperm from phagocytosis and damage in inflammatory conditions (Alghamdi et al. 2004) and regulates sperm transportation and elimination (Troedsson et al. 2005). SP plays an important role in the induction of ovulation in camelids (Ratto et al. 2005) and facilitates sperm-ovum interactions and fertility (Souza et al. 2008). Furthermore, SP activates the expression of embryotrophic cytokines and helps in preparing the genital tract for the developing embryo, mainly by promoting immune alterations essential for accommodation of pregnancy (Robertson 2005).

SP consists of ions, energy substrates, organic compounds, nitrogenous components and reducing substances, etc. The pH and chemical composition of SP vary among animal species and are greatly affected by the presence, size, secretory output and storage capacity of different male genital tract organs, especially the accessory sex glands (Killian et al. 1993; Aurich et al. 1996). The addition of SP or its components into post-thawed semen increases sperm motility and oxygen uptake (Simpson et al. 1987), recovers some sperm surface proteins (Dominguez et al. 2008), reverses cryodamage to ram sperm PM (Rebolledo 2007) and increases overall sperm quality parameters (Maxwell et al. 2007).

The addition of ovine, bovine or equine SP to ram semen before freezing or after thawing has beneficial effects on ram sperm (Garcia-Lopez et al. 1996; Gunay et al. 2006; Martins et al. 2013; Rodrigues et al. 2013). The homology between SP proteins (SPPs) of different animal species indicates that SPPs might play similar biological roles in different animal species (Menard et al. 2003; Bergeron et al. 2005; Jobim et al. 2005). Therefore, because of the higher SP content in camel and buck semen ejaculates compared to ram semen ejaculates, it appears promising to test the addition of buck or camel SP to ram semen diluent. We hypothesised that addition of seminal plasma of ram, buck or camel to a cryopreservation diluent could improve the post-thaw quality of ram semen. To our knowledge, the influence of buck or camel SP addition to ram semen diluent has not been studied yet. Therefore, the present study was aimed at evaluating the effect of the addition of ram, buck or camel SP to TRIS-egg yolk glycerol extender on the quality of frozen ram semen. We also evaluated the effect of the removal of ram SP before dilution with TRIS-egg yolk glycerol extender on the quality of frozen ram semen.

MATERIAL AND METHODS

Animals. This study was conducted on ten Awassi rams, seven Aardi bucks and seven dromedary camels. All animals were mature, with proven fertility and had passed a breeding soundness evaluation test, including semen evaluation using a computer-assisted sperm analysis device called the Sperm Class Analyzer® (SCA®, version 5.4.0.0; Microptic SL, Barcelona, Spain). All animals were healthy, free from disease and had a mean body condition score of three. The animals were housed in yards under a roof in an open-sided barn at the Experimental Farm, Department of Animal Production, King Saud University, Riyadh, Saudi Arabia (latitude, 24°48'N; longitude, 46°31'E). The animals were fed a commercial pellet diet (14.5% crude protein; 2.78 Mcal metabolisable energy/kg dry matter) that met daily energy and protein requirements.

Preparation of diluents. TRIS-egg yolk glycerol diluent was prepared according to an earlier study (Salamon and Maxwell 1995) as follows: 3.07 g Tris(hydroxymethyl)aminomethane (Merck, Darmstadt, Germany), 1.26 g fructose and 1.64 g monohydrous citric acid (Winlab®, Laboratory Chemicals Reagents, Fine Chemicals, UK) were dissolved in 100 ml glass-distilled water. Next, 15% egg yolk, 7% glycerol and 10 mg gentamycin/100 ml diluent (Sigma®, USA) were added.

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SP was obtained during the breeding season from ejaculates collected with an artificial vagina three times a week from ten rams (ram SP; RSP), seven bucks (buck SP; BSP) and seven camels (camel SP; CSP) with a female as a teaser. Each semen sample was evaluated macroscopically and microscopically immediately after collection using the SCA®. Ejaculates of ram, buck or camel that met minimum quality standards were homogeneously pooled within the species according to Swellum et al. (2018a; Swellum et al. 2018b) and then centrifuged twice at room temperature (3000 × *g* for 10 min) until a clear sperm-free supernatant was recovered.

Part of the prepared TRIS-egg yolk glycerol extender was mixed (1 : 4) (v/v) with one of three prepared SPs (RSP, BSP or CSP) in a water bath (30 °C) to obtain the following three extenders: TRIS-egg yolk glycerol with RSP (R), TRIS-egg yolk glycerol with CSP (C) and TRIS-egg yolk glycerol with BSP (B). The other part of the TRIS-egg yolk glycerol diluent was either used to dilute whole ram semen and considered a control without SP addition or removal (T), or, in addition to the 1 : 4 dilution ratio, ram SP was removed to form diluent without SP (W). The extenders were prepared and kept in a refrigerator for 12 h before use.

Semen processing. Ejaculates with a thick consistency, rapid wave motion (3–5 on a 0–5 scale) and > 75% initial motility were accepted and pooled. The accepted pooled ram semen was divided into two parts. The first part was divided into four equal parts and diluted gradually in a water bath using the three different types of diluents (T, R, B or C) in a 1 : 2 dilution ratio. The second part of the accepted pooled ram semen was centrifuged to obtain ram sperm without SP, and the removed SP was replaced by TRIS-egg yolk glycerol diluent and diluted 1 : 2 with TRIS-egg yolk glycerol extender to form the diluent without SP (W).

The diluted semen was cooled gradually to 5 °C within 2 h, loaded into 0.25-ml Minitube straws using a semiautomatic filling and sealing machine (Minitub GmBH, Tiefenbach, Germany) and left for 2 h at 5 °C for glycerol equilibration. After equilibration, the straws were frozen in liquid nitrogen vapor by placing them 8 cm above the surface of the liquid nitrogen for 15 min. Then, the straws were plunged into liquid nitrogen (–196 °C) and stored at this temperature. All the steps of the experiment were carried out seven times (seven replicates).

Semen evaluation parameters. Accepted diluted semen samples (> 70% motility) were re-evaluated on two different occasions. The first evaluation was of cooled semen samples (at least three samples/diluent/replicate) after equilibration and just before freezing (BF). The second evaluation was of post-thaw semen samples (at least three straws/diluent/replicate) after at least 48 h of freezing and storage in liquid nitrogen (AF). The thawing and/or reactivation of semen was done in a water bath at 37 °C for 30 s. The semen samples were evaluated using the SCA® for the parameters described in the following subsections.

Motility and motion kinetic parameters of sperm. The velocity of each sperm cell was evaluated with phase-contrast microscopy using the SCA® motility program to analyse three different velocities (linear, curvilinear, and straight line) and was classified as slow, medium or rapid. Average pathway velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) were measured and expressed in micrometers per second. Linearity (LIN), straightness (STR) and wobble (WOB), all ratios of the velocity parameters (VSL/VCL, VSL/VAP and VAP/VCL, respectively), were used for assessing progressiveness on a relative scale and were expressed in percentage. The amplitude of lateral head displacement (ALH) expressed in micrometers and beat cross frequency (BCF) expressed in Hertz were both used to measure sperm cell oscillation and were based on each specific sperm cell path.

Sperm morphology and morphometric evaluation. Sperm morphology and morphometry were evaluated under high-power microscopy using the SpermBlue protocol and the SCA® morphology program. Briefly, semen samples were smeared on a glass slide and allowed to air-dry. The slides with sperm smears were placed in a staining tray and fixed with SpermBlue fixative (pH 7.6 and osmotic concentration 375 mOsm/kg) for 10 min. Then, the slides were placed in a staining tray containing SpermBlue stain and stained for 15 min (immersion technique). After staining, the excess stain was drained off, and the slides were gently dipped once in distilled water for about 1 s. The slides then were placed at an angle of about 60–80° to remove excess fluid and left to air-dry. Sperm size, sperm shape and pixels were used to differentiate sperm cells from debris and cell abnormalities (Microptic SL). Thirteen morphometric parameters were calculated

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automatically: four for head size: length in micrometers (the main axis along the sperm head), width in micrometers (the smaller axis along the sperm head), area in square micrometers and perimeter in micrometers; four for head shape calculated from the previous parameters: ellipticity (L/W), rugosity (4πA/P2), elongation ((L – W)/(L + W)) and regularity (πLW/4A); and four for the midpiece: width (w, at the intersection of the midpiece with the sperm head, in micrometers), area (a, the area occupied by the entire midpiece, in square micrometers), distance (d, between the main axis of the sperm head and the intersection with the midpiece, in micrometers) and angle (°) (the angle of divergence of the midpiece and the head axis).

Plasma membrane integrity. The plasma membrane integrity (PMI) of spermatozoa was assessed using the hyposmotic swelling (HOS) technique. The HOS solution consisted of 0.73 g sodium citrate and 1.35 g fructose (Winlab®) dissolved in 100 ml distilled water (osmotic pressure ~ 190 mOsm/kg). For the assessment of sperm PMI, each semen sample was mixed with HOS solution (1 : 10) and incubated for 40 min at 37 °C. Subsequently, a drop of the mixed sample was placed on a glass slide, covered with a cover-slip and examined under a microscope (400 ×). Swollen spermatozoa with coiled tail were considered to have intact PMs.

Sperm vitality. Sperm vitality was evaluated in frozen semen with aid of fluorescence using the FluoVit protocol and the SCA® vitality program. Briefly, 1 µl of warm (37 °C) trihydrochloride trihydrate stain was added to 10 µl of semen sample and incubated at 37 °C for 5 min. Then, 1 µl of warm (37 °C) propidium iodide stain was added to the mixture followed by incubation at 37 °C for 5 min. Next, 10 µl of stained sample were evaluated using fluorescence. Live sperm cells stained blue, while dead cell stained red.

Sperm DNA fragmentation. The level of DNA fragmentation was evaluated for frozen semen using the Sperm-Halomax® kit (Halotech-DNA SL, Madrid, Spain) and SCA® DNA fragmentation program. Briefly, 25 µl of diluted semen samples were mixed with melted agarose gel at 37 °C. Twenty-five microliters of the mixture were placed onto a supercoated slide on a cold surface (4 °C) and covered with a 22 × 22 mm coverslip. The slides were kept for 5 min at 4 °C in the refrigerator to create a microgel with implanted sperm. Coverslips were carefully removed, and slides were immersed horizontally into the previously prepared acid solution (80 µl HCl in 10 ml distilled water) for 7 min. Next, the slides were transferred to the tray with a lysing solution to remove membranes and proteins and incubated for 25 min. Rinsing with distilled water was followed by dehydration for 2 min in increasing concentrations of ethanol (70%, 90% and 100%). After drying, slides were stained with Wright's stain, rinsed under tap water and allowed to dry at room temperature. The slides were examined by light microscopy at 20 × magnification, and at least 200 sperm per sample were automatically counted. The SCA® software discriminated between sperm with fragmented DNA, which showed a large and spotty halo of chromatin dispersion, and sperm with unfragmented DNA, which showed a small and compact halo of chromatin dispersion.

Malondialdehyde (MDA) and reduced glutathione (GSH) measurements. Lipid peroxide (MDA) was measured in the thawed semen samples according to a colorimetric method (Satoh 1978) using a commercial kit (Biodiagnostic, Dokki, Giza, Egypt) and based on the reaction of thiobarbituric acid (TBA) with MDA in acidic medium at a temperature of 95 °C for 30 min to form thiobarbituric acid-reactive product. The absorbance of the resultant pink product can be measured at a wavelength of 534 nm. GSH levels in the thawed semen samples were measured according to a colorimetric method (Smith et al. 1988) with a commercial kit (Biodiagnostic, Dokki, Giza, Egypt). The method was based on the reduction of 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The level of reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at a wavelength of 405 nm.

Statistical analysis. Comparisons among semen evaluation parameters in different types of diluents were evaluated using two-way ANOVA in SAS (SAS Institute, Cary, USA) to clarify the effect of freezing and the effect of using different diluents on semen evaluation parameters. A difference was considered significant at $P < 0.05$. The data were expressed as means ± standard error.

RESULTS

Rapid progressive motility before freezing was significantly ($P < 0.05$) higher in R diluent than

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Table 1. Effects of adding ram, buck or camel seminal plasma to TRIS-egg yolk glycerol extender on the percentages of sperm in different motility grades, according to the path velocity and straightness of spermatozoa, (WHO 1999) in frozen ram semen

Parameter	Time	T	W	R	B	C
Rapid progressive	BF	14.31 ± 2.37^{c}	18.97 ± 2.44^{bc}	27.64 ± 2.59^{a}	21.18 ± 2.47^{abc}	24.29 ± 1.91^{ab}
	AF	4.5 ± 0.75^{a}	4.76 ± 0.74^{a}	6.62 ± 0.73^{a}	6.49 ± 1.25^{a}	3.87 ± 0.71^{a}
Medium progressive	BF	17.56 ± 2.27^{c}	19.73 ± 2.45^{bc}	25.13 ± 2.44^{ab}	31.63 ± 1.84^{a}	28.4 ± 2.98^{a}
	AF	11.23 ± 2.12^{a}	6.15 ± 0.88^{b}	11.61 ± 1.20^{a}	11 ± 1.10^{a}	14.78 ± 2.33^{a}
Non-progressive	BF	44.25 ± 2.44	32.7 ± 4.66	33.82 ± 4.15	34.72 ± 1.98	35.29 ± 5.24
	AF	29.21 ± 2.44^{bc}	25.69 ± 2.35^{c}	37.35 ± 2.76^{a}	39.11 ± 2.45^{a}	34.61 ± 3.15^{ab}
Immotile	BF	23.89 ± 2.55^{b}	31.59 ± 2.53^{a}	13.41 ± 2.35^{c}	12.44 ± 1.00^{c}	10.86 ± 1.84^{c}
	AF	55.06 ± 1.57^{b}	63.15 ± 2.6^{a}	44.41 ± 3.84^{c}	43.4 ± 2.37^{c}	46.74 ± 2.34^{c}

AF = after freezing, B = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional buck seminal plasma, BF = before freezing, C = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional camel seminal plasma, R = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional ram seminal plasma, T = whole ram semen diluted with TRIS-egg yolk glycerol extender, W = ram semen without seminal plasma diluted with TRIS-egg yolk glycerol extender

^{a,b,c}Mean ± standard error with different superscript letters within the same row differ at $P < 0.05$ (diluent effect)

in T and W diluents. All diluents showed similar rapid progressive motility after freezing. Medium progressive motility before freezing was significantly ($P < 0.05$) higher in B and C diluents than in T and W diluents. Medium progressive motility after freezing was significantly ($P < 0.05$) higher

Table 2. Effects of adding ram, buck or camel seminal plasma to TRIS-egg yolk glycerol extender on the motion kinetic parameters of spermatozoa in ram frozen semen

Parameter	Time	T	W	R	B	C
VCL (μm/s)	BF	66.88 ± 0.80^{*c}	59.00 ± 0.70^{*d}	77.48 ± 0.70^{*b}	79.46 ± 1.00^{*a}	67.94 ± 0.70^{*c}
	AF	44.56 ± 1.81^{b}	41.57 ± 0.82^{c}	51.73 ± 1.20^{a}	52.70 ± 0.70^{a}	44.16 ± 1.91^{b}
VSL (μm/s)	BF	40.90 ± 0.70^{*d}	34.58 ± 0.60^{*e}	45.7 ± 0.63^{*b}	51.18 ± 0.90^{*a}	43.34*± 0.62^{c}
	AF	28.06 ± 1.50^{a}	24.20 ± 1.00^{b}	27.32 ± 0.72^{a}	28.48 ± 0.50^{a}	28.76 ± 1.61^{a}
VAP (μm/s)	BF	52.94 ± 0.80^{*d}	46.11 ± 0.60^{*e}	65.27 ± 0.91^{*a}	60.71 ± 0.63^{*b}	54.89 ± 0.7^{*c}
	AF	36.04 ± 1.71^{bc}	33.67 ± 0.8^{c}	35.20 ± 1.20^{bc}	39.49 ± 0.60^{a}	36.98 ± 1.9^{b}
ALH (μm)	BF	2.8 ± 0.03^{*d}	2.72 ± 0.02^{*e}	3.35 ± 0.03^{*a}	3.09 ± 0.031^{*b}	2.89 ± 0.03^{*c}
	AF	1.87 ± 0.05^{d}	1.92 ± 0.02^{d}	2.79 ± 0.06^{a}	2.68 ± 0.03^{b}	2.05 ± 0.06^{c}
BCF (Hz)	BF	3.63 ± 0.04^{*c}	3.56 ± 0.04^{*d}	4.06 ± 0.05^{*a}	4.06 ± 0.03^{*a}	3.87 ± 0.04^{*b}
	AF	3.05 ± 0.05^{c}	2.8 ± 0.10^{d}	3.20 ± 0.08^{b}	3.47 ± 0.04^{a}	3.21 ± 0.11^{b}
WOB (%)	BF	0.735 ± 0.003^{b}	0.711 ± 0.003^{c}	0.73 ± 0.004^{b}	0.758 ± 0.004^{a}	0.749 ± 0.003^{a}
	AF	0.74 ± 0.01	0.72 ± 0.005	0.60 ± 0.01	0.70 ± 0.004	0.73 ± 0.01
LIN (%)	BF	0.57 ± 0.006	0.53 ± 0.004	0.54 ± 0.005	0.50 ± 0.004	0.57 ± 0.004
	AF	0.57 ± 0.01	0.54 ± 0.006	0.40 ± 0.01	0.48 ± 0.005	0.54 ± 0.01
STR (%)	BF	0.71 ± 0.006	0.67 ± 0.004	0.69 ± 0.006	0.65 ± 0.004	0.70 ± 0.004
	AF	0.70 ± 0.01	0.68 ± 0.006	0.60 ± 0.01	0.64 ± 0.005	0.66 ± 0.01

AF = after freezing, B = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional buck seminal plasma, BF = before freezing, C = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional camel seminal plasma, R = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional ram seminal plasma, T = whole ram semen diluted with TRIS-egg yolk glycerol extender, W = ram semen without seminal plasma diluted with TRIS-egg yolk glycerol extender

^{a,b,c}Mean ± standard error with different superscript letters within the same row differ at $P < 0.05$ (diluent effect)

*Mean ± standard error with (*) within the same column and evaluation parameter differ at $P < 0.05$ (time effect)

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in R, B, C and T diluents than in W diluent. Non-progressive motility was significantly ($P < 0.05$) higher after freezing in R, B, and C diluents than in W diluent. The percentage of immotile sperm before and after freezing was significantly ($P < 0.05$) lower in R, B and C diluents than in T and W diluents. In addition, the percentage of immotile sperm before and after freezing was significantly ($P < 0.05$) lower in T than in W diluent (Table 1).

VCL, VSL, VAP, ALH and BCF significantly ($P < 0.05$) decreased after freezing in the five types of diluents. VCL before freezing was the highest in B diluent, followed by R diluent, and it was the lowest in W diluent ($P < 0.05$). VCL after freezing was significantly higher in R and B diluents than in other diluents ($P < 0.05$), and it was significantly higher in T and C diluents than in W diluent ($P < 0.05$). VSL before freezing was the highest in B diluent, followed by R diluent, and it was significantly ($P < 0.05$) higher in C diluent than in W and T diluents. VSL before freezing, as well as after freezing, was the lowest in W diluent ($P < 0.05$). VAP before freezing was the highest in R diluent, followed by B, C, T and W diluents in decreasing order ($P <$

Table 3. Effects of adding ram, buck, or camel seminal plasma to TRIS-egg yolk glycerol extender on the morphometry of ram frozen sperm

Parameter	Time	T	W	R	B	C
Head length (μm)	BF	9.71 ± 0.10^{a}	9.53 ± 0.15^{*ab}	9.25 ± 0.16^{bc}	9.16 ± 0.06^{c}	9.6 ± 0.10^{a}
	AF	9.89 ± 0.10^{ab}	10.02 ± 0.15^{a}	9.49 ± 0.16^{c}	9.75 ± 0.06^{*abc}	9.67 ± 0.10^{bc}
Head width (μm)	BF	5.33 ± 0.08	5.30 ± 0.04	5.21 ± 0.05	5.81 ± 0.07	5.53 ± 0.05
	AF	5.46 ± 0.10^{a}	5.36 ± 0.70^{a}	5.18 ± 0.05^{b}	5.40 ± 0.06^{a}	5.45 ± 0.06^{a}
Head area (μm^2)	BF	42.69 ± 1.20^{a}	41.4 ± 0.70^{*ab}	39.84 ± 0.70^{b}	40.04 ± 0.70^{b*}	42.15 ± 0.90^{a}
	AF	44.21 ± 0.80^{a}	43.87 ± 0.80^{a}	40.88 ± 1.10^{b}	42.81 ± 0.70^{a}	43.3 ± 0.80^{a}
Head peri-meter (μm)	BF	26.42 ± 0.50	26.2 ± 0.30	25.25 ± 0.30	25.75 ± 0.20	26.5 ± 0.30
	AF	26.82 ± 0.30^{a}	26.99 ± 0.40^{a}	25.55 ± 0.40^{b}	26.21 ± 0.30^{ab}	26.34 ± 0.30^{ab}
Acrosome (%)	BF	58.55 ± 0.50^{a}	57.69 ± 0.40^{a}	57.31 ± 0.30^{a}	53.82 ± 0.40^{b*}	59.28 ± 0.40^{a}
	AF	57.89 ± 0.30^{a}	58.49 ± 0.60^{a}	58.55 ± 0.40^{a}	57.42 ± 0.50^{a}	57.8 ± 0.40^{a}
Elongation	BF	0.29 ± 0.005^{bc}	0.28 ± 0.006^{*bc}	0.28 ± 0.005^{c}	0.33 ± 0.008^{ab}	0.34 ± 0.05^{a}
	AF	0.3 ± 0.007	0.3 ± 0.008	0.31 ± 0.004	0.28 ± 0.007	0.28 ± 0.004
Ellipticity	BF	1.81 ± 0.03	1.8 ± 0.02*	1.77 ± 0.02	1.75 ± 0.05*	1.77 ± 0.02
	AF	1.8 ± 0.03	1.88 ± 0.03	1.88 ± 0.02	1.81 ± 0.03	1.78 ± 0.02
Regularity	BF	0.96 ± 0.002	0.96 ± 0.001	0.96 ± 0.001	0.96 ± 0.001	0.95 ± 0.001
	AF	0.96 ± 0.001	0.96 ± 0.001	0.95 ± 0.002	0.95 ± 0.001	0.96 ± 0.001
Rugosity	BF	0.77 ± 0.008	0.76 ± 0.006	0.79 ± 0.007	0.89 ± 0.009	0.78 ± 0.006
	AF	0.77 ± 0.007^{ab}	0.76 ± 0.008^{b}	0.77 ± 0.006^{ab}	0.79 ± 0.008^{a}	0.78 ± 0.004^{ab}
Midpiece width (μm)	BF	1.47 ± 0.13	1.77 ± 0.13	1.64 ± 0.21	1.66 ± 0.15	1.79 ± 0.11
	AF	1.6 ± 0.08^{ab}	1.78 ± 0.19^{a}	1.41 ± 0.08^{b}	1.55 ± 0.06^{ab}	1.79 ± 0.13^{a}
Midpiece area (μm^2)	BF	9.37 ± 0.93	10.76 ± 0.72	9.73 ± 1.30	9.07 ± 1.30	9.84 ± 1.18
	AF	9.5 ± 1.00	8.93 ± 0.90	9.64 ± 1.00	9.26 ± 0.62	8.97 ± 0.93
Distance (μm)	BF	0.4 ± 0.03^{b}	0.5 ± 0.05^{a}	0.4 ± 0.08^{b}	0.4 ± 0.04^{*ab}	0.4 ± 0.04^{*b}
	AF	0.4 ± 0.04	0.5 ± 0.06	0.31 ± 0.04	0.33 ± 0.03	0.54 ± 0.94
Angle (°)	BF	2.4 ± 0.30	1.9 ± 0.20	2.7 ± 0.40	2.5 ± 0.31	2.3 ± 0.23
	AF	2.7 ± 0.30	2.9 ± 0.30	2.2 ± 0.30	2.7 ± 0.22	2.2 ± 0.21

AF = after freezing, B = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional buck seminal plasma, BF = before freezing, C = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional camel seminal plasma, R = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional ram seminal plasma, T = whole ram semen diluted with TRIS-egg yolk glycerol extender, W = ram semen without seminal plasma diluted with TRIS-egg yolk glycerol extender

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*Mean ± standard error with (*) within the same column and evaluation parameter differ at $P < 0.05$ (time effect)

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0.05). VAP after freezing was also the highest in B diluent ($P < 0.05$), and it was significantly higher in C diluent than in W diluent ($P < 0.05$). ALH before freezing was also the highest in R diluent, followed by the other diluents in the following order: B > C > T > W ($P < 0.05$). Meanwhile, ALH after freezing showed the following decreasing order: R > B > C W = T ($P < 0.05$). BCF before freezing was significantly ($P < 0.05$) higher in R and B diluents than in the other diluents, followed by C, T and W diluents, of which the latter had the lowest value. For BCF after freezing, the decreasing order of the diluents was B > R = C > T > W ($P < 0.05$). For WOB before freezing, the trend of the diluents was as follows: B = C > T = R > W ($P < 0.05$) (Table 2).

The effects of ram, buck or camel seminal plasma addition to TRIS-egg yolk glycerol extender on the morphometry of ram frozen sperm are presented in Table 3. After freezing, sperm head size (length, width, area and perimeter) was significantly higher in T and W diluents than R diluent.

The total motility, progressive motility, vitality and PMI significantly ($P < 0.05$) decreased after freezing in different diluents. The total motility before and after freezing was significantly ($P < 0.05$) higher in R, B and C diluents than in T and W diluents. Furthermore, the total motility after freezing was significantly ($P \leq 0.05$) higher in T diluent than in W diluent. The progressive motility before freezing was significantly ($P < 0.05$) higher in R, B, and C diluents than in T and W diluents. The progressive motility after freezing was significantly ($P < 0.05$) higher in R, B, C and T diluents than in W diluent. PMI before and after freezing was significantly ($P < 0.05$) higher in R, B, and C diluents than in W diluent. The vitality before freezing was significantly ($P < 0.05$) higher in R, B and C diluents than in W diluent. The vitality after freezing was significantly higher in B than in W diluent ($P < 0.05$). The DNA fragmentation before and after freezing was significantly ($P < 0.05$) lower in R, B, C and T diluents than in W diluent. Sperm abnormalities before freezing were significantly ($P < 0.05$) lower in R, B and C diluents than in W diluent (Table 4).

MDA concentration was significantly higher in T and W diluents than other diluents. Additionally, MDA concentration was significantly lower in B diluent than in T and W diluents. GSH concentration was significantly higher in B diluent than in other diluents. Moreover, GSH concentration was

Table 4. Effects of adding ram, buck or camel seminal plasma to TRIS-egg yolk glycerol extender on the percentages of motility grades (WHO 2010), vitality, plasma membrane integrity, DNA fragmentation and abnormalities of spermatozoa in ram frozen semen

Parameter	Time	T	W	R	B	C
Total motility	BF	76.11 ± 2.55^{*b}	71.4 ± 4.56^{*b}	86.59 ± 2.35^{*a}	87.53 ± 1.01^{*a}	87.97 ± 2.26^{*a}
	AF	44.94 ± 1.57^{b}	36.6 ± 2.41^{c}	55.58 ± 3.84^{a}	56.6 ± 2.37^{a}	53.26 ± 2.34^{a}
Progressive motility	BF	31.87 ± 1.17^{*b}	38.7 ± 1.18^{*b}	52.76 ± 5.03^{*a}	52.81 ± 2.14^{*a}	52.69 ± 4.68^{*a}
	AF	15.73 ± 1.54^{a}	10.91 ± 1.61^{b}	18.24 ± 1.83^{a}	17.49 ± 1.15^{a}	18.65 ± 2.55^{a}
Plasma membrane integrity	BF	79.00 ± 2.08^{*ab}	75.00 ± 5.77^{*b}	87.00 ± 4.04^{*a}	86.67 ± 1.33^{*a}	88.00 ± 2.31^{*a}
	AF	46.00 ± 5.51^{ab}	38.00 ± 5.69^{b}	57.00 ± 7.81^{a}	60.00 ± 2.31^{a}	53.00 ± 4.04^{a}
Vitality	BF	80.00 ± 1.73^{*ab}	72.33 ± 2.85^{*b}	89.33 ± 3.48^{*a}	88.33 ± 4.63^{*a}	87.67 ± 3.18^{*a}
	AF	45.07 ± 4.56^{ab}	39.33 ± 2.33^{b}	56.5 ± 6.25^{ab}	57.37 ± 6.66^{a}	54.33 ± 3.48^{ab}
DNA fragmentation	BF	2.34 ± 0.42^{b}	6.05 ± 2.11^{a}	1.23 ± 0.14^{b}	3.20 ± 0.77^{b}	1.89 ± 0.46^{b}
	AF	5.21 ± 0.77^{*b}	12.51 ± 1.23^{*a}	6.95 ± 1.00^{*b}	6.04 ± 1.52^{*b}	5.45 ± 0.28^{*b}
Sperm abnormalities	BF	12.10 ± 1.31^{ab}	13.89 ± 1.14^{b}	9.11 ± 1.13^{a}	9.99 ± 1.95^{a}	10.87 ± 1.09^{a}
	AF	25.22 ± 3.82*	28.11 ± 3.92*	23.88 ± 2.73*	22.33 ± 2.96*	25.77 ± 3.99*

AF = after freezing, B = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional buck seminal plasma, BF = before freezing, C = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional camel seminal plasma, R = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional ram seminal plasma, T = whole ram semen diluted with TRIS-egg yolk glycerol extender, W = ram semen without seminal plasma diluted with TRIS-egg yolk glycerol extender

^{a,b,c}Mean ± standard error with different superscript letters within the same row differ at $P < 0.05$ (diluent effect)

*Mean ± standard error with (*) within the same column and evaluation parameter differ at $P < 0.05$ (time effect)

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Table 5. Effects of adding ram, buck or camel seminal plasma to TRIS-egg yolk glycerol extender on malondialdehyde (MDA) and reduced glutathione (GSH) concentrations in ram frozen semen (mean ± SD)

Parameter	T	W	R	B	C
MDA (nmol/ml	3.44 ± 0.08[a]	3.26 ± 0.09[a]	2.31 ± 0.08[b]	1.47 ± 0.10[c]	2.43 ± 0.09[b]
GSH (mmol/l)	140.20 ± 4.84[c]	159.38 ± 6.23[b]	161.50 ± 5.85[b]	219.78 ± 6.53[a]	161.05 ± 4.23[b]

AF = after freezing, B = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional buck seminal plasma, BF = before freezing, C = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional camel seminal plasma, R = whole ram semen diluted with TRIS-egg yolk extender glycerol with additional ram seminal plasma, T = whole ram semen diluted with TRIS-egg yolk glycerol extender, W = ram semen without seminal plasma diluted with TRIS-egg yolk glycerol extender

[a,b,c]Mean ± standard error with different superscript letters within the same row differ at $P < 0.05$ (diluent effect)

significantly higher in C, R and W diluents than in T diluent (Table 5).

DISCUSSION

The frozen storage of ram spermatozoa markedly affects sperm viability because of the damaging effects of cold shock. These detrimental effects can be avoided or at least reduced by supplementing the diluents with some additives and by gradual cooling of diluted semen. We examined the effects of SP addition to semen diluent on spermatozoa motility, viability and abnormalities during freezing.

The overall results showed that ram, buck and camel SP are beneficial in ram semen freezing, as indicated by the maintenance of high levels of sperm motility, vitality and integrity of sperm DNA and PM, and reduced percentage of abnormal sperm. In addition, SP removal had adverse effects on sperm evaluation parameters after freezing. These improvements might be explained by the beneficial roles of SP. Briefly, SP has an important role in maintaining sperm motility and viability in many animal species (Maxwell and Watson 1996) and in increasing the resistance of sperm to cold shock injury (Barrios et al. 2005) owing to components that protect the sperm cell membrane (Maxwell and Watson 1996; Ollero et al. 1997).

SP contains components that are critical for the metabolic regulation of sperms (Barrios et al. 2000). Fructose in SP has a vital role in sperm energy production and metabolism (Mortimer and Maxwell 1999). Moreover, SPPs cover and protect sperm cells (Simpson et al. 1987; Ashworth et al. 1994; Mortimer and Maxwell 1999), and they are associated with the ability to repair the sperm membrane after cryoinjury (Barrios et al. 2000; Perez-Pe et al. 2001). SP lipids play important roles in the structure of the sperm cell membrane, sperm capacitation, sperm metabolism and oocyte fertilisation (Hafez and Hafez 1993). Therefore, a decrease in SP lipid content is associated with a reduction in sperm motility (Kelso et al. 1997; Taha et al. 2000), which was observed in the W diluent in the present study.

Our results correspond with the findings of many researchers who used SP to decrease the amount of damage that occurs during the cryopreservation process. Sperm motility, PM stability and fertility have been found to improve in frozen-thawed ram semen resuspended in artificial SP or ram SP compared to those resuspended in PBS (Mortimer and Maxwell 1999). Furthermore, adding SP during cooling, freezing and thawing increases motility in ram sperm and reverses cold-shock damage on the ram sperm membrane (Jobim et al. 2005). SP has an important role in preserving spermatozoa motility in bull and ram semen, increasing ram sperm viability and improving the cold-shock resistance of boar sperm (Jobim et al. 2005). The addition of bull SP to the diluent has a supportive effect on sperm motility, morphological integrity and on the acrosomes of thawed semen. Moreover, the addition of bull SP to ram semen extender can improve its fertility (Gunay et al. 2006).

The addition of bovine SP to ram semen before freezing (Gunay et al. 2006) and after thawing (Kareta et al. 1972; Garcia-Lopez et al. 1996) has beneficial effects on ram sperm viability. Meanwhile, the addition of ovine or equine whole SP to post-thawing incubation medium affects the viability of thawed ram sperm in a time-dependent manner. The positive effect of equine SP on the DNA integrity of ram sperm can be explained by the presence of SPPs or other components (Rodrigues

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et al. 2013). The homology between bovine and ovine SPPs (Bergeron et al. 2005; Jobim et al. 2005) and between bovine and equine SPPs (Menard et al. 2003) indicates that SPPs play similar biological roles in different animal species.

The addition of whole SP or its components to post-thawed semen increases the motility of spermatozoa and their oxygen uptake (Simpson et al. 1987), helps to recover some surface proteins (Dominguez et al. 2008), reverses damage to ram sperm PM resulting from cryopreservation (Rebolledo 2007) and increases overall sperm quality parameters (Maxwell et al. 2007). The addition of SPPs to the diluent before cooling prevents sperm membrane damage caused by cold shock by maintaining the activities and distribution of antioxidant enzymes on the sperm surface (Marti et al. 2008; Sharma et al. 2016) and inhibiting protein tyrosine phosphorylation (Perez-Pe et al. 2002), which ensures a viable sperm population (Perez-Pe et al. 2001). Meanwhile, certain harmful effects of SP on motility, viability and post-thaw survival rate of sperms have also been reported (Iwamoto et al. 1993; Kawano et al. 2004).

We detected no significant differences among the three types of SP (ram, buck or camel) for most sperm evaluation parameters after freezing. These results might be explained by the presence of some component in SP that can compensate for the shortage of other components. For example, the presence of glucose in camel SP compensates for the shortage of fructose.

Given the role of sugar in energy production in sperm, the sugar concentration of SP is positively correlated with fertility (Garner et al. 2001). Although seminal glands are absent in camelids which might cause a reduction in fructose concentration (El-Manna et al. 1986), glucose is the main sugar in camel SP and is converted into fructose. Ram sperms express the sorbitol dehydrogenase enzyme, which converts sorbitol to fructose, and fructose is used as a metabolic substrate (Setchell and Brooks 1988). In addition, a strong positive correlation between Mg and apoptosis-free viable ram sperms has been reported (Juyena and Stelletta 2012).

The ability to freeze ram semen is positively correlated with the total protein in SP (Barrios et al. 2000; Chaudhari et al. 2015). SPPs are secreted mostly from the cauda epididymis (Moura et al. 2010) or accessory sex glands (Moura et al. 2007) and/or are shed from the sperm membrane (Thimon et al. 2005). The presence of some types of SPPs compensates for the absence of other types. There are significant homologies between the Fn-2 proteins identified in ruminants, for example, between ram RSVP14, ram RSP15, bovine PDC-109 and goat GSP-14/15 (Cardozo et al. 2006).

The improvement in post-thaw semen quality with the use of R and B diluents may be explained by an increase the concentration of spermadhesins secreted from the seminal vesicles of rams, bulls and bucks (Einspanier et al. 1994; Teixeira et al. 2002; Bergeron et al. 2005; Teixeira et al. 2006; Melo et al. 2008). Spermadhesins are multifunctional, with ligand-binding affinity for some oligosaccharides, serine protease inhibitors, sulfated polysaccharides, phospholipids and glycosaminoglycans (Topfer-Petersen et al. 1998). This ligand-binding affinity suggests that these proteins are involved in sperm-egg interactions and sperm capacitation. Moreover, spermadhesins regulate mitochondrial function, limit lipid peroxidation, have an energy-preserving effect, can maintain sperm viability and help in the elimination of waste sperm that fail to fertilise (Schoneck et al. 1996; Teixeira et al. 2002).

Ashworth et al. (1994) described valuable SPPs that can overcome the effects of dilution on ram spermatozoa. The molecular sizes of SPPs are similar between rams and bucks (Villemure et al. 2003; Bergeron et al. 2005; Jobim et al. 2005). They also share heparin- and phosphorylcholine-binding properties. The exact functions of these SPPs in small ruminants have not yet been determined (Barrios et al. 2005). RSP-14 might have a role in stabilising sperm membrane phospholipid and the cytoskeleton. In addition, RSP-14 and RSP-20 have protective effects which include the repair of membranes of sperms subjected to cold shock (Perez-Pe et al. 2001).

The improvement in post-thaw semen quality with the R diluent may also be explained by an increase in the concentration of calsemin and membrane vesicles. Calsemin is a heat-stable, low-molecular weight acidic protein fraction detected in ram SP (Bradley and Forrester 1982). It acts as a Ca^{2+}-dependent regulator of the Ca^{2+}-dependent Mg^{2+}-ATPase enzyme and 39,59-cyclic nucleotide phosphodiesterase enzyme. Calsemin acts like calmodulin and stimulates flagellar beat activity (Bradley and Forrester 1982). Membrane vesicles or vesicular fractions are secreted in whole ram

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ejaculate from the testis and epididymis and have a beneficial effect on sperm progressive motility (El-Hajj Ghaoui et al. 2004; El-Hajj Ghaoui et al. 2007). The attachment of membrane vesicles to the sperm surface delays capacitation and the acrosome reaction (Arienti et al. 1997).

In the present study, MDA concentration was significantly higher in T and W diluents than other diluents. GSH concentration was significantly higher in B diluent than other diluents. Moreover, GSH concentration was significantly higher in C and R diluents than in T diluent. These observations explain the protective effects of the seminal plasma of buck, camel and ram including high vitality, high intact plasma membrane and low DNA fragmentation, as the main reason for abnormal sperm function is lipid peroxidation (LPO) and antioxidant imbalance caused by oxidative stress (Aitken et al. 1996; Aitken et al. 2006). Therefore, reactive oxygen species (ROS) have a vital role in the functioning of spermatozoa and their fertilising ability after cryopreservation (Bilodeau et al. 2000; Chatterjee and Gagnon 2001; Neild et al. 2005; Guthrie and Welch 2007; Thuwanut et al. 2009). Oxidative stress is associated with an increase in the oxidation rate of sperm cellular components and massive production of ROS (Alvarez and Storey 1982; Aitken et al. 1996). Low levels of oxidative stress may have useful effects on sperm cells (de Lamirande and Gagnon 1993), while high-intensity oxidative stress can cause cell death via damage to nucleic acids, proteins, lipids and carbohydrates (Park et al. 2003; Agarwal et al. 2005; Peris et al. 2007). ROS production was increased during the freeze-thaw process of semen (Chatterjee and Gagnon 2001; Park et al. 2003). The main sites of ROS formation are mitochondria (Brouwers and Gadella 2003) and sperm cell membranes (Agarwal et al. 2005), which are particularly vulnerable to damage from sudden temperature changes. Damage to cellular membranes during freezing and subsequent thawing is caused by lipid peroxidation products, leading to reduced membrane integrity (Aitken 1995; Neild et al. 2005). Moreover, analysis of cryopreserved semen in many mammalian species showed increased ROS production during the freeze-thaw process (Bilodeau et al. 2000; Chatterjee and Gagnon 2001).

In conclusion, the addition of ram, buck or camel SP to TRIS-egg yolk glycerol extender improved the quality of frozen ram semen. In addition, the removal of SP was found to adversely affect the quality of frozen ram semen. The effects of ram, buck and camel SP on the parameters evaluated in frozen semen did not differ significantly. Based on the greater SP content in camel and buck ejaculates compared to ram ejaculates, it appears advisable to add buck or camel SP to ram semen diluent.

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