RAM SEMEN CRYOPRESERVATION USING EGG YOLK OR EGG YOLK-FREE EXTENDERS: PRELIMINARY RESULTS*

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Kinematic parameters of thawed ram semen frozen under field conditions were analysed with the use of two commercial (egg yolk vs egg yolk free) semen extenders in different year-seasonal periods. The semen was collected from Suffolk (n = 2) and Charollais (n = 1) rams kept in private breeding farm on 3 test days (pre-mating, at mating, post mating) during year 2016. Two commercial semen extenders (egg yolk-based BullXcell® or egg yolk-free AndroMed®) were used for semen processing. Processed semen was frozen in 0.25 ml plastic cryostraws using the custom-made portable freezing box for ram semen cryopreservation under field conditions. Thawed semen characteristics were evaluated using computer-assisted semen analysis (CASA) system immediately after thawing and after 2 h of heat incubation (±38°C). Significantly higher total motility (+12.3%), straight line velocity (+5.6 μm s^{-1}), and average-path velocity (+6.9 μm s^{-1}) were detected for the semen processed and cryopreserved using egg yolk-based semen extender. Year-seasonal variation and introducing the ram to service had shown to have a significant effect on the cryopreserved ram spermatozoa. These preliminary results confirmed the feasibility of ram semen processing and cryopreservation under field conditions.

spermatozoa, AndroMed®, BullXcell®, freezing box, CASA

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INTRODUCTION

The process of ram semen production is complex and it is strongly influenced by age and breed of ram or by year-seasonal variations, resulting in a great variability of semen quality (Karagiannidis et al., 2000; Kaya et al., 2002; Stadnik et al., 2014). The method of ram semen cryopreservation is established (Salamon, Maxwell, 1995). However, it is known that freezing and thawing procedure decrease drastically the viability and the motility of ram spermatozoa (Fukui et al., 2010). That is the main limitation for using post-thawed semen via the method of cervical insemination (Byrne et al., 2000). To diminish the negative impact of freezing-thawing procedure, ram semen processing and cryopreservation is usually performed under conditions of commercial insemination centres (Jimenez-Rabadan et al., 2016) or under specialized research centres (Rather et al., 2016; Banday et al., 2017). In these centres an expansive high-tech instrumentation (including programmable freezers) is used to ensure optimal regimes of semen processing and cryopreservation. Although crucial for the purpose of large-scale commercial insemination or for advanced frontier research, such expensive high-tech instrumentation is either non-accessible or non-affordable for sheep breeders who own small flocks of animals. Thus, the freezing of ram sperm under field conditions using inexpensive (or even custom-built) freezing instrumentation must be considered. Such instrumentation could be easily available for small-scale sheep breeders. Additionally, this simple instrumentation might help to disseminate the genetically valuable traits in the sheep population more effectively. In spite of the fact that several companies currently offer a relatively inexpensive freezing equipment (such as polystyrene

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freezing boxes of different design), the methodology of ram sperm freezing under field conditions, in general, is poorly established and, therefore, should be improved.

The semen dilutions, a necessary part of semen processing, maintain optimal pH in medium, provide energy for sperm cells, assure the microbial free environment, and protect spermatozoa from coldshock. As well, components of semen diluents protect spermatozoa from cryodamage caused by the freezing-thawing procedure. Egg yolk, a frequently used cryoprotectant in mammalian semen diluents, is highly effective for the maintenance of sperm fertility in different species (Sansone et al., 2000; Garde et al., 2003; Ali et al., 2013). However, Bousseau et al. (1998) demonstrated egg yolk as a major risk of bacterial contamination of semen doses. Moreover, the egg yolk might seriously diminish the quality of frozen-thawed spermatozoa by inappropriate agglutination in spermatozoa, and thus induce the process of spermatozoa premature capacitation. Additionally, the composition of natural egg yolk is extremely variable, making the standardization of egg yolk-based diluents difficult (Masoudi et al., 2016). Commercial semen diluents with egg yolk substitutes, being recently available for freezing bull semen (Gil et al., 2003), were successfully tested to improve cryopreserved ram semen quality characteristics while eliminating the above mentioned egg yolk component disadvantages (Gonzalez et al., 2010; Loaiza-Echeverri et al., 2015).

The aim of the present study was to evaluate the main kinematic parameters of post-thawed ram spermatozoa, collected at 3 different test days (pre-mating, mating or post-mating), processed with the use of two commercial bull semen diluents (BullXcell® or AndroMed®) initially developed for freezing bull semen and frozen using the portable freezing set adjusted for semen cryopreservation under field conditions.

MATERIAL AND METHODS

Semen collection

The semen was collected from three clinically healthy Suffolk (n = 2) and Charollais (n = 1) rams (18–60 months old) into artificial vagina with all necessary precautions to prevent spermatozoa coldshock. All selected rams were used for breeding during breeding season (each ram had to serve a group of approximately 20 ewes), and they were bred under the same management system of one flock. The flock was located at private sheep breeder (Central Bohemian Region, 285 m a.s.l., average annual rainfall 500 mm, average annual temperature 8.5°C). The semen was collected in 2016 at three test days according to mating the sheep in the flock: 1st collection performed at pre-mating season (22nd September; average daily temperature 14.6°C, average humidity 68.1%); 2nd collection performed at mating season (21st October; average daily temperature 11.1°C, average humidity 73.2%); 3rd collection performed at post-mating season (25th November; average daily temperature 9.6°C, average humidity 84.0%).

Table 1. Composition of BullXcell® and AndroMed® semen diluents

BullXcell®	AndroMed®
Bidistilled water	bidistilled water
TRIS	phospholipids
Glycerol	glycerol
Citric acid	citric acid
Sugars	sugars (fructose)
Buffers	buffers
Antibiotics	antibiotics
+ Fresh egg yolk (1 : 4)	

Semen cryopreservation

Collected semen was evaluated for its volume; subjective spermatozoa concentration and progressive motility were evaluated under light basic microscope before further semen processing. Semen with low progressive motility characteristic was discarded from the processing. In this study, all the freezable semen samples obtained on the same collection day during 30-minute intervals were mixed and defined as semen sample. Each semen sample was subsequently divided into two balanced parts. The first part was diluted with fresh egg yolk-based BullXcell® (IMV Technologies, L'Aigle, France) and the second part was diluted with egg yolk-free phospholipid semen diluent AndroMed® (Minitübe GmbH, Tiefenbach, Germany) prepared accordingly to instruction manual (Table 1). Diluted semen samples were filled into 0.25 ml French straws (IMV Technologies) and equilibrated for 2 h at 4°C. After equilibration, the straws were frozen in liquid nitrogen vapours using a polystyrene freezing box (adapted from Animal Reproduction Systems, Inc., Chino, USA; modified) with a special design enabling precise setting of the distance between the cryostraws and the surface of liquid nitrogen (1st phase, distance 15 cm, 4 min; 2nd phase, distance 9.5 cm, 5 min; 3rd phase, distance 5 cm, 6 min; 4th phase, distance 1.5 cm, 8 min). This freezing rate reflects the modified 4-phase freezing curve (Gil et al., 2000) (Fig. 1). Frozen straws were stored in liquid nitrogen (–196°C) before thawing.

Semen thawing and evaluation of semen characteristics

Straws were thawed in a water bath (38.5°C, 30 s). Thawed semen from each straw was mixed with preheated 500 µl physiological solution with appropriate composition, pH and temperature and incubated in

dry heater (Thermo-block Falc®, Treviglio, Italy) at 38°C. Post-thaw spermatozoa motility was evaluated using the CASA system (SCA® Production v. 5.3.; MICROPTIC S.L., Barcelona, Spain) with a phase contrast microscope Eclipse E200 (Nikon, Tokyo, Japan) at 200–300× magnification. At least five fields of view per each straw were evaluated (Tuncer et al., 2011). The following kinematic parameters were measured: total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μm s^{-1}), straight-line velocity (VSL; μm s^{-1}), velocity path average (VAP; μm s^{-1}), linearity (LIN; %), straightness (STR; %), and wobble (WOB; %). Semen samples were evaluated immediately after thawing (time 0) and after 2 h of incubation at 38°C (time 2). The spermatozoa persistence was calculated as the difference between time 2 and time 0.

Statistical analysis

Statistical analyses were conducted using SAS software (SAS/STAT® Version 9.3., 2011). The correlation analysis was used to express the relationship between the factors for further statistical analysis in the model. A generalized linear model (GLM) with fixed effects of extender (AndroMed®, $n = 10$; BullXcell®, $n = 10$) and test day of semen collection (1st day of semen collection, $n = 4$; 2nd day of semen collection, $n = 8$; 3rd day of semen collection, $n = 8$) was used to examine the variability of spermatozoa kinematic characteristics immediately after thawing, after 2 h of incubation at 38°C and the spermatozoa persistence. The lower number of insemination doses (IDs) from the 1st semen collection was due to the limited production of freezable semen from rams during a defined time interval (see above). The interaction effect of extender × day of semen collection was also tested during ongoing analysis. However, this factor was non-significant for all the evaluated traits, and therefore excluded. The characteristics of actual spermatozoa motility at time 0 and time 2 are presented in the form of table, while spermatozoa persistence traits are expressed as figures using MS Excel software. The differences between the variables were tested by the Tukey-Kramer method at the significance level $P < 0.05$.

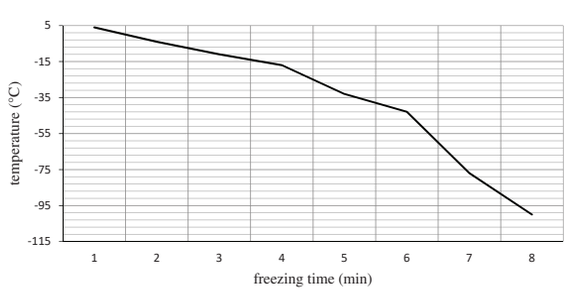

Fig. 1. The 4-phase freezing curve

RESULTS

The influence of extender on post thawed semen characteristics

The influence of extender or the test day of semen collection on the ram spermatozoa motility and selected movement characteristics is presented in Table 2. No significant differences were demonstrated between both extenders immediately after thawing the straws. However, numerically higher results of all the observed traits were detected for egg yolk extender (BullXcell®) compared to phospholipid extender (AndroMed®). The TM and PM characteristics at time 0 rapidly increased from the 1st day to the 2nd day of semen collection (27.3% increase for TM, $P < 0.05$; 12.7% increase for PM, $P < 0.05$). However, both these traits decreased significantly during the mating season on the final level of 35.8% for TM, and 14.7% for PM (post-mating). Additionally, significantly lowest values, concerning all the other spermatozoa characteristics, were demonstrated for the 3rd day of semen collection (post-mating season) compared to the 2nd one (mating season). The differences of VCL, VSL, VAP, LIN, STR, and WOB on the 1st day of semen collection were close to significant with the 3rd day of semen collection; therefore, there was a clear decreased tendency demonstrated also between the 1st day and 3rd day of semen collection. Significantly higher TM (+ 12.3%) was detected for BullXcell® in comparison with AndroMed® after 2 h of incubation after thawing (time 2). Semen diluted in extender with egg yolk addition also reached 5.6 μm s^{-1} higher VSL ($P < 0.05$) and 6.9 μm s^{-1} higher VAP ($P < 0.05$) at the same time. Numerically higher PM (+ 6%), VCL (+ 2.6 μm s^{-1}), LIN (+ 9.1%) and WOB (+ 9.8%) characteristics were noticed for straws frozen in BullXcell® at time 2. The day of semen collection showed very similar results at 2 h of incubation as the results detected immediately after thawing. Significantly the highest TM was thus demonstrated at mating with a 21% difference to the 1st day or with a 16.7% difference to the 3rd day. Significantly lower VCL (– 12.5 μm s^{-1}), VSL (– 5.8 μm s^{-1}), VAP (– 7.9 μm s^{-1}), LIN (– 16.4%), STR (– 24.6%), and WOB (– 21.7%) were estimated at the 3rd day of semen collection (post-mating) in comparison with the results obtained at mating (2nd day of semen collection). Differences between the 1st day and the 3rd day of semen collection showed a clear tendency; however, they were non-significant in the majority, due to the higher standard error at the 1st day of semen collection. As important, egg yolk extender showed a more favourable persistence during 2 h of incubation as presented (Fig. 2). The highest difference was demonstrated for TM when AndroMed® showed more than two-fold times higher decrease for this trait (6.05% decrease in BullXcell® vs 15.36% decrease in AndroMed®).

Table 2. Influence of the extender and day of semen collection on spermatozoa motility characteristics immediately after thawing, and after 2 h of incubation (Least Squares Means)

		TM	PM	VCL	VSL	VAP	LIN	STR	WOB
Time 0 h	**Extender**								
	BullXcell®	44.2	17.6	40.5	23.1	30.4	50.5	68.0	66.9
	AndroMed®	41.1	15.4	40.2	20.9	28.4	46.7	66.2	63.6
	Day								
	1	32.4^{A}	11.1^{A}	43.4^{AB}	23.1^{AB}	31.4^{AB}	52.6^{AB}	73.1^{AB}	71.7^{A}
	2	59.7^{B}	23.8^{B}	45.5^{A}	25.6^{A}	33.7^{A}	55.9^{A}	75.5^{A}	73.8^{A}
	3	35.8^{A}	14.7^{A}	32.0^{B}	17.2^{B}	23.0^{B}	37.3^{B}	52.7^{B}	50.4^{B}
Time 2 h	**Extender**								
	BullXcell®	38.1^{A}	14.4	33.5	18.8^{A}	25.0^{A}	45.2	61.0	60.0
	AndroMed®	25.8^{B}	8.4	30.9	13.2^{B}	18.1^{B}	36.1	61.9	50.2
	Day								
	1	23.5^{A}	8.8	33.6^{AB}	18.6^{AB}	23.8^{AB}	45.8^{A}	65.1^{AB}	59.0^{AB}
	2	44.5^{B}	14.9	37.7^{A}	17.6^{A}	24.3^{A}	46.3^{A}	71.9^{A}	64.0^{A}
	3	27.8^{A}	10.4	25.2^{B}	11.8^{B}	16.4^{B}	29.9^{B}	47.3^{B}	42.3^{B}

TM = total spermatozoa motility (%), PM = progressive spermatozoa motility (%), VCL = curvilinear velocity (μm s^{-1}), VSL = straight line velocity (μm s^{-1}), VAP = average-path velocity (μm s^{-1}), LIN = linearity (%), STR = straightness (%), WOB = wobble (%), DAY 1 = 1st collection day performed pre-mating the sheep, DAY 2 = 2nd collection day performed at mating the sheep, DAY 3 = 3rd collection day performed post-mating the sheep

^{A,B}means within columns with different letters differed significantly ($P < 0.05$)

A significantly higher decline of persistence characteristics was demonstrated for straws frozen in AndroMed® extender. Namely, these differences were 3.8% for PM, 3.7 μm s^{-1} for VSL, 5.2 μm s^{-1} for VAP, 5.7% for LIN, and 6.8% for WOB. The differences between days of semen collection were more stable (Fig. 3), despite of significantly the highest decline for TM detected at the 2nd day of semen collection (7.0% compared to the 3rd day) or for PM at the 2nd day of semen collection (6.6% compared to the 1st one or 4.6% compared to the 3rd one).

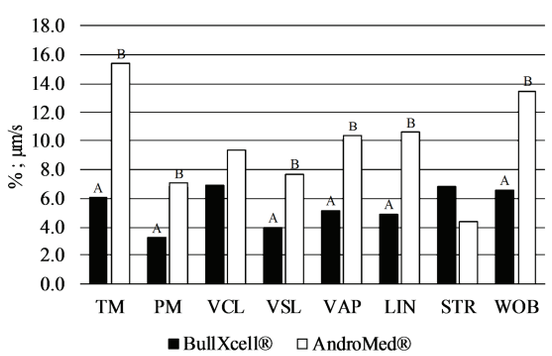

Fig. 2. Influence of the extender on spermatozoa persistence characteristics during 2 h of incubation (the decrease of kinematic characteristics)

TM = total spermatozoa motility (%), PM = progressive spermatozoa motility (%), VCL = curvilinear velocity (μm s–1), VSL = straight line velocity (μm s–1), VAP = average-path velocity (μm s–1), LIN = linearity (%), STR = straightness (%), WOB = wobble (%)
^{A,B}different letters within particular characteristics indicate significant differences at P < 0.05 level of significance

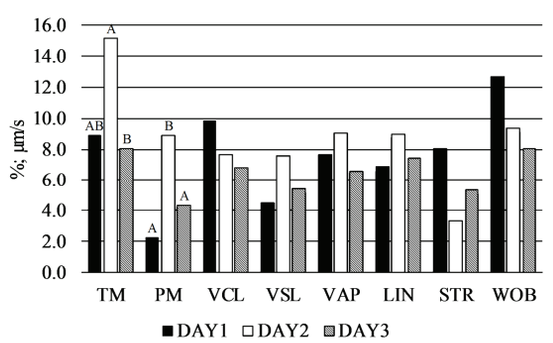

Fig. 3. Influence of the day of semen collection on spermatozoa persistence characteristics during 2 h of incubation (the decrease of kinematic characteristics)

TM = total spermatozoa motility (%), PM = progressive spermatozoa motility (%), VCL = curvilinear velocity (μm s–1), VSL = straight line velocity (μm s–1), VAP = average-path velocity (μm s–1), LIN = linearity (%), STR = straightness (%), WOB = wobble (%), DAY 1 = 1st collection day performed pre-mating the sheep, DAY 2 = 2nd collection day performed at mating the sheep, DAY 3 = 3rd collection day performed post-mating the sheep
^{A,B}different letters within particular characteristics indicate significant differences at P < 0.05 level of significance

DISCUSSION

Since as early as 1930s it has been known that a sudden fall in temperature from 40°C to 0°C (coldshock) affects negatively the spermatozoa viability and motility, that the sperm cell plasma membrane is the primary site of coldshock damage, and that the hen egg yolk is a potent spermatozoa coldshock protector (Blackshaw, 1954). Early researchers have reported that egg yolk aids the sperm cell in resisting coldshock. Later, it was discovered that egg yolk is also the non-permeable cryoprotective agent (the same as permeable glycerol), preventing the spermatozoa cryodamage following the freezing-thawing (Pace, Graham, 1974). This is due to inhibition of ice crystals growth outside the cells (Gharajelar et al., 2016), and due to egg yolk Low Density Lipoproteins (LDL) which are able to adhere to the cell membrane and replace the damaged phospholipids (Rehman et al., 2013; El-Sisy et al., 2016; Simonik et al., 2016). Next spermatozoa protection mechanism for egg yolk LDL was reported by Manjunath et al. (2002) who demonstrated that egg yolk LDL are able to diminish the detrimental activity of semen plasma proteins (collectively called BSP proteins) in bull and possibly in ram through rapid and stable charge-based complex formation between LDL and BSP. Moreover, egg yolk lecithin is an energy source for spermatozoa in the absence of oxidisable soluble carbohydrate (van Tran et al., 2017); e.g. incubation of ram semen in the absence of any exogenous substrate resulted in spermatozoa oxidizing the long chain fatty acid resides from phosphatidylcholine (which is the principal phospholipid of lecithin). As a result, egg yolk was used for decades as the 'gold standard' component in ram sperm cryopreservation protocols due to its effective coldshock and cryoprotective ability.

However, there has been a movement recently to eliminate egg yolk as it might seriously diminish the quality of frozen-thawed spermatozoa due to its several flaws. As Cabrera et al. (2005) and Akhter et al. (2012) have reviewed, the use of egg yolk associated with sanitary risks may contribute to lower fertility of cryopreserved semen directly through deteriorating the semen quality by producing harmful metabolites and toxins or indirectly through local infection leading to abortion; egg yolk in semen diluent can reduce the respiration and motility of the spermatozoa. Structurally, the hen egg yolk is a complex system consisting of non-soluble protein aggregates (granules) in suspension in a clear yellow fluid (plasma). Granules, a minor part of yolk dry matter, are mainly composed of High Density Lipoproteins (HDL), while plasma, corresponding to about 78% of yolk dry matter, is made up of LDL (Anton, 2013). HDL in egg yolk induce the efflux of cholesterol from the sperm plasma membrane resulting in the change of fluidity that increases the sensitivity to coldshock; HDL of egg yolk induce premature sperm capacitation and subsequent acrosome reaction. HDL can be easily removed out from the egg yolk by a mild centrifugation (10 000 *g* per 30 min) (Anton, 2013). The method of HDL removal ('clarification of egg yolk') was established for semen processing in bull (Pace, Graham, 1974), red deer (Fernandez-Santos et al., 2006), gazelle (Holt et al., 1996) and stallion (Pillet et al., 2011). Egg yolk might increase an inappropriate agglutination in spermatozoa (Masoudi et al., 2016). It is not possible to prepare egg yolk-based semen diluents consistent with any interlaboratory quality standards because of the individual quality differences inherent in egg yolk due to the numbers of days after egg laying and the storage period (Fukui et al., 2008). Furthermore, the greater viscosity and the presence of particulate debris in semen diluents due to egg yolk globules interferes in the microscopic assessment of the sample and could be the cause of reduced fertility, as reviewed by de Paz et al. (2010). Additionally, diluents containing animal proteins could have had adverse effects on biochemical studies of polypeptides derived from frozen-thawed spermatozoa (Hinsch et al., 1997). The soybean lecithin-based semen diluent AndroMed® (Minitübe GmbH) has been developed initially for bovine spermatozoa cryopreservation. This commercially available bovine semen diluent contains a high content of egg yolk-like phospholipids (mainly phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol), as reviewed by Fukui et al. (2008), de Paz et al. (2010), and Simonik et al. (2016). In general, soybean lecithin consists not only of a mixture of phospholipids but also of triglycerides, fatty acids, pigments, sterols, sterol glycosides and esters, tocopherols, and carbohydrates (Scholfield, 1981). Soybean lecithin has recently been recognized as a chemically-defined pathogen-free alternative of egg yolk for successful low temperature storage of ram semen (Fukui et al. 2008; de Paz et al., 2010; Khalifa et al., 2013; Snoeck et al., 2017). Despite reported success in the application of commercial soybean lecithin-based semen diluents for ram spermatozoa cryopreservation (in terms of *in vitro* and *in vivo* sperm function assays), some workers reported a marked decrease in the rate of *in vitro* blastocyst formation, a reduction in the pregnancy rate and embryonic losses after ram spermatozoa processing in commercial soybean lecithin-based semen diluents, as reviewed by Khalifa et al. (2013). Hegedusova et al. (2012) compared commercial diluents free of products of animal origin and diluents containing products of animal origin for short-term and long-term ram semen preservation; processed semen was stored under the temperature of 16–18°C and spermatozoa motility was assessed at 24, 48, 72 and 96 h. Best motility was observed in semen processed in diluents, containing products of animal origin (egg yolk). Interestingly, the superiority of one soybean

lecithin-based bull semen diluent (BioXcell®) over the other soybean lecithin-based bull semen diluent (AndroMed®) was detected and ascribed to the improvement of semen fertility of young rather than mature rams (Khalifa et al., 2013). This might be due to non-identical biochemical composition between two commercial soybean lecithin-based semen diluents, both developed for bull. Workers who wish to adapt commercial soybean lecithin-based bull semen diluents for ram spermatozoa processing have to be aware of this fact. In this connection, the results of our study are in line with all previous results confirming the beneficial effect of egg yolk during ram spermatozoa cryopreservation. This is despite the fact that in our study we used the whole egg yolk, containing HDL granules. It is known that HDL might cause cholesterol efflux from the sperm plasma membrane and by this might increase the sensitivity of processed spermatozoa to coldshock (Akhter et al., 2012). The level of IDs processing could be regulated by appropriate time of semen collection. Year-seasonal variation of semen characteristics was demonstrated by Karagiannidis et al. (2000) or Stadnik et al. (2014). Also the ram ejaculation frequency influenced significantly the semen characteristics (Kaya et al., 2002). This is clearly indicated by the present results, when ram semen characteristics increased up to mating season and then rapidly decreased during mating the sheep (and this is despite of fulfilling the mating ratio 1 ram per 30–50 ewes) (Ridler et al., 2012). The quality of IDs is usually demonstrated not only by the absolute values of sperm kinematic characteristics, but also by the dynamics of the values degradation after prolonged incubation (Dolezalova et al., 2017). In our study, slower degradation of spermatozoa kinematic characteristics was demonstrated for the egg yolk extender, supporting the above-mentioned qualities of the egg yolk component.

In this study, we demonstrated kinematic characteristics of thawed ram spermatozoa which were either lower (Pradiee et al., 2016; Saieed et al., 2016) or higher (Jimenez-Rabadan et al., 2016; Souza et al., 2017) than those obtained in commercial insemination centres or specialized research centres. Importantly, several workers trying to cryopreserve the ram semen in field conditions showed very similar results (Masoudi et al., 2016). This confirms the possibility of obtaining ram sperm IDs of an appropriate quality even when standardized laboratory conditions and expensive freezing equipment is omitted. Ledesma et al. (2016) showed extraordinarily greater kinematic characteristics while investigating the effect of seminal plasma proteins on Assaf rams. These results were more than two- or three-fold higher than our results and overreached also many of the other studies. Many studies have been concerned with extender additives (cryoprotectants), demonstrating their positive effect on spermatozoa kinematic characteristics. Therefore, additives such as soybean lecithin (Masoudi et al., 2016), skim-milk (Salmon et al., 2017), glycerol (Najafi et al., 2017), LDL (Loaiza-Echeverri et al., 2015), cyclodextrin, cholesterol, vitamin E (Benhenia et al., 2016) or antioxidants (Rather et al., 2016) should be investigated in more detail as promising aids for improving the quality of ram sperm IDs after ram semen collection, processing and cryopreservation under field conditions.

CONCLUSION

In this study, an attempt was given to improve the methodology of ram sperm freezing under field conditions with the use of inexpensive custom-built freezing instrumentation. Two commercial (egg yolk or egg yolk-free) semen extenders in different year-seasonal periods were used to confirm the feasibility of ram semen cryopreservation under field conditions. The preliminary results of the present study clearly indicated a beneficial effect of egg yolk component in ram spermatozoa cryopreservation. This effect, having a tendency immediately after thawing the straws, was more significant after 2 h of incubation and in evaluating the persistence of particular spermatozoa kinematic characteristics. Year-seasonal variation and introducing a ram to service showed to have a significant effect on cryopreserved ram spermatozoa. In conclusion, the effective cryopreservation of ram semen can be achieved under field conditions without the needs of expensive accessories such as programmable freezers.

Further research is needed to optimize ram semen processing and cryopreservation under field conditions. The improved methodology of semen cryopreservation under field conditions might help to disseminate genetically valuable traits in the sheep population more effectively.

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