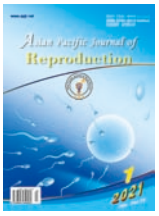

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Dietary selenium supplementation, clarified egg yolk extender and slow cooling improve cryopreserved sperm characteristics of Saanen buck

Kambulu Lukusa[1], Abubeker Hassen[1], Khoboso C. Lehloenya[2]✉

[1]*Department of Animal and Wildlife Sciences, University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa*

[2]*Department of Agriculture, University of Zululand, Private Bag X1001, KwaDlangezwa, 3886, South Africa*

ABSTRACT

Objective: To evaluate the effect of cooling rates, selenium supplementation, and three semen extenders on cooled and frozen-thawed Saanen buck sperm.

Methods: Twenty Saanen bucks were divided into two groups: the selenium supplemented group and the control group. Ejaculates were collected once weekly by artificial vagina. The first experiment examined the effects of cooling rates and selenium supplementation on semen characteristics of Saanen bucks. Pooled semen was diluted with triladyl extender and split into two aliquots for slow and fast cooling. The second experiment explored the effect of selenium supplementation and semen extenders on post-cryopreserved sperm quality. Ejaculates from each group were divided into three aliquots and diluted with three extenders (*i.e.* clarified egg-yolk, whole egg yolk, Tris without egg-yolk extenders). All samples were cooled for 2 h at 4 ℃ and frozen at −196 ℃ for 24 h. The sperm characteristics such as sperm motility, acrosome integrity, normal morphology, and viability were evaluated by using a phase-contrast microscope.

Results: In the first experiment, all sperm characteristics were significantly increased in selenium supplemented samples when slow cooling was used (P<0.05). In the second experiment, in cooled semen, sperm motility and viability were significantly increased in both clarified egg yolk and Tris without egg yolk extenders in selenium supplemented samples as compared with whole egg yolk extender, respectively (P<0.05). After freezing-thawing, all sperm parameters of selenium supplemented samples in clarified egg yolk extender were significantly greater than those in Tris without egg yolk extender (P<0.05). However, normal morphology and acrosome integrity of selenium supplemented samples in whole egg yolk extender were similar to those of selenium supplemented samples in clarified egg yolk extender.

Conclusions: The characteristics of cooled and post-cryopreserved sperm are greater when clarified egg yolk extender is used in semen from selenium supplemented bucks.

KEYWORDS: Extenders; Sperm characteristics; Cryopreservation; Selenium; Cooling rates

1. Introduction

Sperm cryopreservation is an important tool for genetic improvement programs in many species, including goats. In recent years, many studies have been conducted to optimize sperm cryopreservation protocols in goats. Irrespective of the protocol, viability of spermatozoa deteriorates at low temperatures during storage due to oxidative stress and oxidative oxygen species (ROS) that can lead to harmful effects on sperm[1,2].

Antioxidants can prevent or reduce oxidative process by scavenging released free radicals. There are several antioxidants in semen that are known to improve sperm quality such as vitamin E[3-5] and vitamin C[6], as well as selenium (Se) and zinc which are components of antioxidant systems. These antioxidants may be insufficient in seminal plasma to protect spermatozoa against oxidative stress and ROS during the freezing-thawing process[7]. So, oral Se supplementation to animals has been proposed as a potential way to reduce the oxidative damage due to its fast response[8,9]. There are several studies about the effect of oral Se supplementation on reproductive performance in sheep and goats[10,11]. Consequently, it was hypothesized that oral Se supplementation could reduce sperm damage in cooled and frozen-thawed Saanen buck semen.

To cryopreserve semen, dilution with a protective extender is important to maintain fertilizing capacity of sperm during *in vitro* storage at low temperatures. The most widely used extenders for freezing goat semen are Tris-egg yolk and skim milk extenders and

✉To whom correspondance may be addressed. E-mail: khoboso.lehloenya@gmail.com

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their components are shown to be the primary cause of damage in semen. Egg yolk (lysolecithin) and a protein identified as SBUⅢ interact with the seminal plasma lipase, a content of the bulbourethral secretion, and this interaction is known to be harmful to the sperm[12]. In addition, whole egg yolk has been reported to interfere with microscopic observations or biochemical assays, as it contains granular material of the same size and shape as spermatozoa and also reduces respiration and motility of sperm cells[13]. By centrifugation, egg yolk can be separated into its two main fractions (plasma and granules)[14]. Therefore, it is essential to remove large particles in whole egg yolk by centrifugation, and only plasma (clarified) is used to obtain better post-cryopreserved sperm quality. This clarified egg yolk extender has been successfully used for stallion and bull semen cryopreservation[15,13]. Therefore, it can be assumed that the use of clarified egg yolk extender will preserve better the quality of buck sperm before and after cryopreservation.

Cooling of semen prior to sperm cryopreservation is an important step to minimize damages during the freezing process. The cooling period is necessary to decrease the effect of temperature changes and to allow equilibration of the spermatozoa with the diluents before freezing. However, cooling is a highly stressful process, which leads to irreparable damages to the spermatozoa membrane that result in either cell death or premature capacitation-like changes[16]. These detrimental effects can be reduced by optimizing cooling rates before freezing. The optimal cooling rates have been established in bull, boar, stallion, and ram[17]. But for goat sperm, temperatures over which goat semen must be cooled are quite diverse for frozen sperm, ranging from -0.3 ℃/min to 0.55 ℃/min[18,19]. These cooling rates lead to different results, making it difficult to establish optimal cooling rates.

Consequently, it is can be hypothesized that appropriate cooling rate for cooled and frozen semen from bucks supplemented with Se and extended with clarified egg yolk medium may improve sperm quality. Therefore, the present study aims to evaluate the effect of different types of extenders and cooling rates on cooled and post-cryopreserved sperm of Se-supplemented Saanen bucks.

2. Materials and methods

2.1. Study area

The study was conducted at the University of Pretoria, Hatfield Experimental Farm in Pretoria (latitude 25°45' South, longitude 28°16' East). The experimental farm is situated in the Highveld region of South Africa, at an altitude of 1 327 m above mean sea level[20]. The present study was carried out for 3 months during autumn (1st of February to 30th of April, 2017).

2.2. Animals

A total of 20 Saanen bucks aged 18-19 months and weighing (55.13±2.75) kg were used. The animals were raised only on locally available milled lucerne containing 207 g/kg dry matter of crude protein and 1.9 Mcal metabolize energy (ME/kg dry matter). The animals had no access to fresh growing forages or other feed for four months before the start of the experiment. Fresh water was provided *ad libitum* during the experimental period. Lucerne hay was milled and tested for Se concentration prior to supplementation and no Se traces were detected by using the spectrophotometer (Perkin-Elmer 2380 Atomic Absorption Spectrophotometer; Varian, Australia)[11]. The treated animals received Se (ACECHEM, South Africa) at the dose rate of 0.34 mg/kg body weight, administered orally at 10-day intervals for 3 months[11]. The control animals did not receive Se supplementation.

2.3. Semen collection and evaluation

A total of 240 semen samples (12 ejaculates from each buck) were collected by using artificial vagina according to previous studies[11,21]. Ejaculates were routinely collected once weekly throughout the experiment during three-month period. The bucks were trained for more than six weeks before successful semen collection using a doe on heat. All semen samples were evaluated macroscopically for ejaculate volume and pH, and microscopically for sperm mass motility, progressive motility, concentration, normal morphology, acrosome integrity, and viability under a phase-contrast microscope (OLYMPUS, CX21FS1; Tokyo, Japan) as described previously[11].

2.4. Extenders preparation

Different freezing extenders namely the ready-to-use triladyl, clarified egg yolk, whole egg yolk, and Tris without egg yolk extenders were used. Triladyl extender was purchased from Faculty of Veterinary Sciences, University of Pretoria. The clarified egg yolk was prepared as described by Wilber *et al*[22]. The whole egg yolk-based extender consisted of Tris 2.422 g, citric acid monohydrate 1.36 g, glucose 1 g, gentamycin 1 000 μg/mL, kanamycin 1 000 μg/mL, egg-yolk (v/v) 20%, glycerol (v/v) 16%, and distilled H_2O to final volume (mL). The composition of Tris without egg yolk-based extender was comprised of Tris 4.54 g, citric acid monohydrate 2.61 g, glucose 0.82 g, gentamycin 1 000 μg/mL, kanamycin 1 000 μg/mL, distilled H_2O to final volume (mL). The pH was then adjusted to approximately 7.2[23].

2.5. Cooling and freezing procedures

2.5.1. Experiment Ⅰ: Effects of cooling rates and Se supplementation on semen characteristics of Saanen bucks

The twenty animals were divided into two equal groups: the Se-supplemented and control groups. The pooled semen samples for both groups were diluted with no-glycerolated fraction of freezing

extender (fraction A) and further subdivided into two equal aliquots each. One aliquot was cooled by using a slow cooling rate and the other for a fast cooling rate for 24 h at 4 ℃.

Semen was considered for cooling using standard criteria established by Hidalgo *et al*[24]. The ready-to-use triladyl extender (Veterinary Sciences, University of Pretoria) was used as a cooling extender. The pooled semen samples were diluted at a ratio of 1:2 (semen: extender) at 33 ℃, to obtain a sperm concentration of 150×10^9 sperm/mL and mixed gently to ensure homogeneity of the mixture. The extended semen was transferred into two 15 mL conical tubes for either slow or fast cooling and kept for 2 h and 30 min in the refrigerator at 4 ℃, respectively. All cooling processes were performed as described by Memon *et al*[18]. The sperm characteristics such as progressive motility, normal morphology, acrosome integrity, and viability were evaluated microscopically immediately after cooling and at 24 h following storage at 4 ℃.

2.5.2. Experiment Ⅱ: Effects of selenium supplementation and semen extenders on post-cryopreserved sperm characteristics of Saanen bucks by slow cooling

Since the slow cooling rate produced better results in experiment Ⅰ, it was used in experiment Ⅱ to evaluate the effect of Se supplementation and extenders type on cooled and frozen-thawed sperms. A total of 12 pooled semen samples from each group were used in the study. Each sample was divided into three equal aliquots that were diluted with clarified egg yolk, whole egg yolk, and Tris without egg yolk using a 2-step dilution method. In the first step, solution A (without glycerol) was added to semen samples to a ratio of 1:2 (semen: extender) at 33 ℃, to obtain a sperm concentration of 150×10^9 sperm/mL and cooled to 4 ℃ for 2 h. The cooled semen was further diluted to a ratio of 1:1 (semen: extender) with solution B (containing 16% glycerol) to obtain a final sperm concentration of 75×10^9 sperm/mL. After equilibration time at 4 ℃ for 2 h, semen samples were aspirated into 0.25 mL French straws. Thereafter, the straws were sealed with polyvinyl alcohol powder followed by their suspension in liquid nitrogen vapour inside a cooler box container at a height of 4 cm above liquid nitrogen for 10 min. Then, they were subsequently submerged into liquid nitrogen at –196 ℃, where they were stored for 24 h. A minimum of 3 straws from each treatment (clarified egg yolk, whole egg yolk, and Tris without egg yolk) were thawed at 37 ℃ for 30 s in a water bath 24 h after freezing to evaluate post-cryopreserved semen characteristics.

2.6. Statistical analysis

Statistical analysis was performed by the General Linear Model procedures using statistical software SPSS (2015) (IBM SPSS Statistics for Windows, Version 23.0. NY, USA). Normality distribution of data was verified before its analysis using the Shapiro–Wilk test as a parametric test assumption. All data on sperm characteristics passed the normality test were used in further analysis. In experiment Ⅰ, a completely randomized block design in a 2×2 factorial arrangement for Se treatment (the Se-supplemented and control groups) and cooling rates (slow and fast) was used. In experiment Ⅱ, a 2×3 factorial arrangement for Se treatment (the Se-supplemented and control groups) and extenders (clarified egg yolk, whole egg yolk, and Tris without egg yolk) was used. The fixed effects were the Se supplemention, cooling rate, extenders, and their interaction. The results were expressed as mean±standard deviation (mean±SD). The analysis of variance using repeated measures was used to test differences between the treatments for each variable. Mean±SD were separated by using Duncan's multiple range tests. A probability of $P<0.05$ was considered to be statistically significant.

2.7. Ethics statement

All animal care and procedures used were performed in accordance with Animal Ethics Committee of the University of Pretoria (Project No: EC079-14).

3. Results

3.1. Experiment Ⅰ: Effects of cooling rates and Se supplementation on semen characteristics of Saanen bucks

The sperm progressive motility, acrosome integrity, normal morphology, and viability of sperm cooled using slow cooling rate in Se supplemented samples were significantly increased as compared with sperm cooled with both slow and fast cooling in the control group ($P<0.05$). The interaction between Se supplementation treatment and cooling rates were significant as compared with the control group ($P<0.05$). All sperm characteristics were significantly increased in Se-supplemented samples when slow cooling was used ($P<0.05$) (Table1).

3.2. Experiment Ⅱ: Effects of Se supplementation and semen extenders on post-cryopreserved sperm characteristics of Saanen buck by slow cooling

In cooled semen, sperm motility and viability were significantly increased in both clarified egg yolk and Tris without egg yolk extenders in Se-supplemented samples as compared with whole egg yolk extender, respectively ($P<0.05$) (Table 2). After freezing-thawing, all sperm parameters of Se-supplemented samples in clarified egg yolk extender were significantly greater than those in the Tris without egg yolk extender ($P<0.05$). However, normal morphology and acrosome integrity of Se-supplemented samples in whole egg yolk extender were similar to those of Se-supplemented samples in clarified egg yolk extender (Table 3).

Table 1. Interaction of selenium treatment and cooling rates on overall semen characteristics of Saanen bucks (%) .

Parameters	Se treatment group		Control group	
	Slow cooling	Fast cooling	Slow cooling	Fast cooling
Sperm motility	74.20±0.98^{a}	69.51±1.02^{b}	67.70±2.01^{bc}	62.50±1.60^{d}
Acrosome integrity	70.32±1.70^{a}	68.71±1.15^{ab}	66.81±1.79^{b}	63.60±1.15^{c}
Normal morphology	76.21±1.60^{a}	70.00±0.50^{b}	71.40±0.48^{b}	64.20±0.44^{c}
Viability	75.15±1.80^{a}	69.10±0.80^{bc}	67.70±0.26^{b}	51.20±0.64^{c}

Data are expressed as mean±SD. Different superscripts in a line differ significantly at $P<0.05$. Se: selenium.

Table 2. Effects of selenium supplementation on sperm of Saanen bucks in cooled semen stored in different extenders by slow cooling (%).

Parameters	Clarified egg yolk extender		Whole egg yolk extender		Tris without egg yolk extender	
	Se treatment group	Control group	Se treatment group	Control group	Se treatment group	Control group
Sperm motility	72.11±2.01^{a}	67.15±6.03^{b}	66.52±3.14^{b}	64.74±3.02^{bc}	70.50±2.03^{a}	67.05±4.04^{b}
Acrosome integrity	74.13±3.22^{a}	70.10±2.01^{b}	69.55±4.05^{b}	66.70±5.15^{c}	70.30±6.11^{b}	68.81±5.11^{bc}
Normal morphology	74.11±3.06^{a}	73.15±3.64^{a}	67.20±6.82^{b}	64.50±4.03^{bc}	73.21±7.02^{a}	66.10±6.04^{b}
Viability	73.10±5.50^{a}	70.00±4.04^{b}	70.15±2.21^{b}	62.00±2.11^{c}	72.65±2.03^{a}	71.01±3.03^{b}

Data are expressed as mean±SD. Different superscripts in a line differ significantly at $P<0.05$. Se: selenium.

Table 3. Effects of selenium supplementation on sperm of Saanen bucks in frozen-thawed semen stored in different extenders by slow cooling (%).

Parameters	Clarified egg yolk extender		Whole egg yolk extender		Tris without egg yolk extender	
	Se treatment group	Control group	Se treatment group	Control group	Se treatment group	Control group
Sperm motility	51.02±4.12^{a}	46.03±3.71^{b}	45.41±9.61^{bc}	45.00±8.46^{bc}	38.00±7.47^{d}	37.50±9.27^{d}
Acrosome integrity	56.25±4.73^{a}	53.81±6.11^{b}	55.06±3.04^{a}	44.60±7.02^{c}	33.15±8.85^{d}	35.30±4.23^{d}
Normal morphology	64.03±7.04^{a}	60.01±9.03^{b}	63.22±7.12^{a}	54.55±9.78^{c}	40.51±5.84^{d}	39.75±5.41^{d}
Viability	60.45±3.01^{a}	58.35±7.18^{ab}	52.20±8.29^{c}	50.65±9.69^{cd}	49.35±7.44^{cd}	39.75±9.24^{e}

Data are expressed as mean±SD. Different superscripts in a line differ significantly at $P<0.05$. Se: selenium.

4. Discussion

4.1. Experiment Ⅰ: Effects of cooling rates and Se supplementation on semen characteristics of Saanen bucks

The improved characteristics of Se-supplemented Saanen buck sperm treated with slow cooling rate demonstrated clearly that Se had a significant protective effect against lipid peroxidation on slowly cooled sperm parameters. Lukusa and Lehoenya[11] confirmed the protective and beneficial effects of Se on semen quality and suggested that better quality semen can be obtained by supplementing bucks with Se.

Se might have enhanced the protective effects during slow cooling rate by reducing the production of ROS and maintaining better sperm quality during cooling[25]. However, it can be suggested that in order to start the freezing process with acceptable sperm quality, supplementing the animals with Se to boost their antioxidant status is necessary. The biological function of Se is accomplished through the selenoproteins, such as glutathione peroxidise (GSH-Px) in which Se is a structural component[26]. The enzyme has been reported to increase in blood plasma of Se-supplemented bucks[11]. This enzyme plays a major role in the protection of sperm membrane against oxidative damage[27]. Therefore, spermatozoa may be more vulnerable to oxidative stress if the Se content in selenoproteins is low and likely decreases the possibility of fertilization[28].

The increase in sperm motility in slowly cooled semen indicated that stabilization of sperm cells with a slow cooling rate coupled with Se supplementation could enable the sperm to cope with detrimental effects of physical, osmotic, and cold stresses during cooling. This may be attributed to the combined role of Se and slow cooling in the protection of sperm membrane integrity and lowering enzyme leakages during cooling process[18]. In addition, Se may also act in the maintenance of mitochondrial structural integrity, leading to an increase in adenosine triphosphate of the spermatozoon, thereby causing an increase in sperm motility[29].

The higher percentages of morphologically normal sperm in slowly cooled semen from Se-supplemented bucks in the current study may be attributed to a higher level of GSH-Px activity. Kehr *et al*[30] documented that higher GSH-Px activity plays an indispensable role in chromatin structure protection of the sperm in the epididymis, thereby leading to increased population of morphologically normal sperms. Similar results were observed by Rezaeian *et al*[31] who indicated that the addition of 5 mg/mL of Se to human sperm before freeze-thawing procedures caused an increase in spermatozoa with normal morphology.

In the present study, an improvement was observed in viability of spermatozoa with slow cooling in Se-supplemented bucks, which indicates that slow cooling rate in combination with Se have significant protective effects on sperm viability, and there is a strong interaction between cooling rate and Se supplementation. Similar results were reported by Memon *et al*[18] with slow cooling in Boer goat semen. The current results confirmed that a slow cooling rate is required to reduce damage to Saanen buck sperm cells during the freezing process. The temperature of 4 ℃ must be attained within 2 h for desirable viability after thawing. On the other hand, the beneficial effects of Se can be attributed to the fact that Se is a very

efficient antioxidant and a scavenger of oxygen free radicals that are toxic to metabolic activity and cellular viability of cryopreserved spermatozoa.

4.2. Experiment Ⅱ: Effects of Se supplementation and semen extenders on post-cryopreserved sperm characteristics of Saanen bucks by slow cooling

Our results showed clearly that supplementing Se as a component of antioxidant system increased percentages of cooled sperm parameters in both clarified egg yolk and Tris without egg yolk extenders. The present results are in agreement with the findings of earlier researchers who reported that Se supplementation led to significant increases in sperm viability as well as motility before and after freezing[25]. El-Sheshtawy *et al*[32] also stated that sperm motility and semen characteristics were improved with clarified egg yolk extender as compared with whole egg yolk in bull cooled-stored semen. Protective effect of Se supplementation on cooled sperm motility, acrosome integrity, normal morphology, and also viability in both clarified egg yolk and Tris without egg yolk extenders observed in the current study may be explained by the increase of GSH-Px in Se-supplemented bucks as reported by Lukusa and Lehloenya[11]. These findings suggest that Se supplementation could increase antioxidative status of seminal plasma and spermatozoa to reduce excessive production of ROS during cooling process.

In the present study, all post-cryopreserved sperm characteristics were higher on Se-supplemented samples preserved in clarified egg yolk extender. These results support the report of Fernández-Santos *et al*[33] who indicated that centrifuged egg yolk provided higher protection than whole egg yolk during the freeze-thawing of Iberian red deer epididymal spermatozoa. Similarly, El-Sheshtawy *et al*[32] revealed that bull sperm motility and semen characteristics were improved with clarified egg yolk extender supplemented with strawberry juice as an antioxidant as compared with whole egg yolk. Buck semen frozen using clarified egg yolk extender supplemented with antioxidants such as Se can produce acceptable post-cryopreserved sperm quality that can be used in artificial insemination programs. The removal of some detrimental components from egg yolk by centrifugation in clarified egg yolk also played a greater role in the cryoprotective effect of Se on post-cryopreserved sperm quality during the freeze-thawing process. Therefore, antioxidants such as Se may be necessary to protect sperm against ROS in both clarified egg yolk and whole egg yolk extenders for cryopreservation of Saanen buck semen.

The results of the present study will help to improve the breeding performance of the Saanen goat in particular and other goat breeds in general. However, the measurement of post-thawed sperm parameters using subjective method is not sufficient to determine the effect of dietary Se supplementation, extenders, and cooling rates on post-thawed semen quality. Measurement of sperm velocity and kinematic parameters by computer-assisted sperm analysis as well as other parameters such as DNA fragmentation index, mitochondrial membrane potential, and *in-vitro* fertility or pregnancy rates could be assessed to understand the level of effectiveness of Se supplementation, extenders, and cooling rates on post-cryopreserved sperm quality.

In conclusion, the slow cooling rate used in semen from Se-supplemented bucks preserves sperm by maintaining an acceptable percentage of sperm progressive motility, acrosome integrity, normal morphology, and viability. In addition, cooled and post-cryopreserved sperm characteristics are greater when clarified egg yolk extender is used in semen from Se-supplemented bucks, which suggests that before starting freezing process, supplementing animals with Se to boost their antioxidant status and then using clarified egg yolk extender with slow cooling rate may be beneficial to yield acceptable post-cryopreserved sperm quality.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Authors′ contributions

Khoboso C. Lehloenya is a corresponding author of this manuscript; he has contributed substantially to conception and design, revising it critically for important intellectual content; and final approval of the version to be published. Kambulu Lukusa is the co-author of this manuscript; he contributed substantially in samples collection, data analysis and interpretation as well as drafting of the manuscript. Abubeker Hassen is a co-supervisor and co-author of this manuscript; he has contributed with his constructive criticism of ideas and approaches related to this research work.

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