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Effect of low and high egg yolk concentrations in the semen extender for goat semen cryopreservation

C.A.S. Bispo[a,b,*], G. Pugliesi[b], P. Galvão[c], M.T. Rodrigues[b], P.G. Ker[b], B. Filgueiras[c], G.R. Carvalho[b]

[a] Federal Institute of Minas Gerais – Campus São João Evangelista, Av. Primeiro de Junho, 1043, Centro, São João Evangelista, CEP: 39705-000, Brazil
[b] Federal University of Viçosa – Animal Science Department, Av. Peter Henry Rolfs, s/n Campus Universitário, CEP: 36570-000, Viçosa, MG, Brazil
[c] Federal University of Viçosa – Veterinary Department, Av. Peter Henry Rolfs, s/n Campus Universitário, CEP: 36570-000, Viçosa, MG, Brazil

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ABSTRACT

This research aimed to evaluate two concentrations of egg yolk inclusion rates (20 and 2.5%) in the semen extender of goat semen cryopreserved during two seasons of the year. The study was conducted during a light-induced breeding season (Experiment 1), and during the natural breeding season (Experiment 2), in the southern hemisphere. Four ejaculates from each buck ($n = 2$) were collected in each experiment. After collection, semen was divided, with each sample being diluted in the semen extender – according to the treatments (T1 – 20% egg yolk or T2 – 2.5% egg yolk, using a glucose–EDTA extender). For T1 treatment in Experiment 2, the semen was also washed before the semen cryopreservation process. The semen samples were frozen, and after thawing evaluated for seminal characteristics i.e. sperm motility, vigor, morphology and membrane integrity. The fertilising capacity of the frozen-thawed semen was evaluated following a single artificial insemination 12 h after the onset of estrus in 50 (Experiment 1) and 60 does (Experiment 2). In Experiments 1 and 2, the mean values for sperm motility and membrane integrity of the frozen-thawed semen did not differ between the T1 and T2 treatments. However, the mean sperm vigor and morphological normal sperm were greater ($P < 0.05$) in T2 than T1 treatment. The fertility rates recorded did not differ between T1 and T2 treatments in Experiment 1, however, it was greater ($P < 0.05$) in the T2 than in the T1 treatment, in Experiment 2. According to obtained results, it can be recommended to use a glucose–EDTA extender with a low egg yolk concentration (2.5%) inclusion, for superior fertility results in goats.

1. Introduction

Artificial insemination (AI) in goats has generally not yet reached the user frequency, compared to the use in bovine – which is a widespread applied technique, to optimally utilize semen proven through progeny testing (Leboeuf et al., 2000). As in other domestic animals, the freezing process of semen reduces the viability of the caprine sperm, with a pregnancy rate after AI with frozen-thawed semen, ranging between 7% and 79% (Leboeuf et al., 2003; Nordstoga et al., 2011).

Egg yolk is routinely used in extenders for the cryopreservation of mammal semen – in order to protect the sperm against thermal shock. It is believed that its action is due to the presence of the low-density lipoproteins (LDL), which adhere to the cellular membrane during the cryopreservation process, thereby preserving the sperm membrane (Moussa et al., 2002). The LDL adherence on the surface of the sperm plasma membrane restores the loss of phospholipids and apparently induces a temporary change

* Corresponding author at: Federal Institute of Minas Gerais – Campus São João Evangelista, Av. Primeiro de Junho, 1043, Centro, São João Evangelista, CEP: 39705-000, Brazil. Tel.: +55 31 3412 2900.
E-mail address: charles.bispo@ifmg.edu.br (C.A.S. Bispo).

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in its composition – consequently preventing rupture of the plasma membrane (Farstard, 1996).

Despite the benefits that were observed during the use of egg yolk in semen extenders, researchers have suggested an alternative inclusion for goat semen. Roy (1957) stated that semen extenders containing egg yolk in their composition are not recommended for use in goat semen. This being due to the fact that buck seminal plasma contains an enzyme secreted by the bulbo-urethral glands, which in the presence of egg yolk, by hydrolysis, leads to the formation of lysophosphatidylcholines – which are toxic to sperm (Leboeuf et al., 2000). In the search of making the process of cellular conservation more practical and less harmful to sperm, Evans and Maxwell (1987) thus proposed the use of an extender with a low egg yolk concentration (2.5%). Washing of goat semen to remove the seminal plasma before dilution and freezing was another alternative when using extenders containing egg yolk. However, washing of the semen is generally time consuming, can affect the sperm viability and promotes sperm loss (Gacitua and Arav, 2005).

Martin et al. (1979), working on the cryopreservation of equine semen, supplemented the glucose–EDTA extender with an emulsifying substance, Orvus-es-paste, which increases the availability and protective ability of phospholipids. Thawed-frozen semen including Orvus-es-paste exhibited superior sperm motility when compared the same extender without this emulsifying agent – serving as an important protector of the acrosome membrane during the freezing process.

The objective of this study was to evaluate, through in vitro and in vivo fertility tests, which egg yolk concentration (High: 20% or Low: 2.5%) present in a glucose–EDTA extender exhibits better preservation of frozen goat semen during two different breeding seasons.

2. Material and methods

2.1. Location and experimental period

Experiments were conducted at Goat Reproduction Laboratory, located in Federal University of Viçosa, Viçosa, Minas Gerais, Brazil (latitude S 20°45′20″). The experiments were performed during the breeding season (from November to December – Experiment 1), induced by light supplementation and the natural breeding season (from March to June – Experiment 2).

2.2. Animals

In both experiments, 2 fertile bucks (Alpino and Saanen breeds) were selected according to their reproductive history and andrological examination (CBRA, 1998). The bucks received feed composed of corn silage, a protein concentrate, and mineral mixture that met the nutritional requirements for the categories of stud, lactating females and dry females. The bucks were kept in individual stalls (3 m × 8 m) and were maintained in a healthy and good body condition throughout each experiment.

2.3. Semen handling

Four semen samples were taken from each buck in Experiments 1 and 2, respectively. The semen samples were collected three times per week, with the aid of an artificial vagina, according to the method of Memon and Ott (1986). After collection, semen was divided into two equal parts and each sample diluted in the ratio of 1:1 (semen: extender), according to the proposed treatments (T1 or T2). Using a Neubauer chamber, the sperm concentration of each semen sample was determined and the extender volume required for a final concentration of 200×10^6 sperm/mL, added. For the T1 treatment, the extender used was the glucose–EDTA extender as proposed by Martin et al. (1979). For T2 treatment, the glucose–EDTA extender was modified in terms of the egg yolk inclusion. The initial 20 mL of yolk/100 mL (20%) in the extender was thus changed to 2.5 mL (2.5%), while the rest of extender volume (17.5 mL) was made up of a sodium citrate solution.

In Experiment 1 semen was frozen in full i.e. it did not go through the washing process for seminal plasma removal, and the only variable between the treatments was the level of egg yolk inclusion. In Experiment 2, the T2 treatment was similar to Experiment 1. However, for the T1 treatment, the washing of semen samples was performed due to the non-freezing (very poor quality after thawing) of the semen samples recorded in the T1 treatment. Thus Experiment 2 had two variables in the T1 treatment (egg yolk inclusion and semen washing). For the washing process, the semen was diluted with a sodium Ringer lactate solution, and subjected to the centrifugation process for removal of the seminal plasma. The semen centrifugation was performed for 10 min, at 600G (Dell'Aqua Junior and Papa, 2001).

The semen was packaged in 0.5 mL straws, with a total inseminating dose of 100×10^6 sperm. After filling, the semen straws were cooled using the cooling technique of Bispo (2005), and the freezing process of Fürst et al. (2005). The semen was thawed in a water bath at 37 °C for a period of 30 s. After freezing and before insemination, the semen samples were assessed in vitro.

The physical sperm parameters of motility (percentage sperm with progressive movement); vigor (status of sperm motility), scored on a scale of 0 (no movement) to 5 (fast progressive movement); morphological aspects (Pintado and Perez-Llano, 1992); and spermatic membrane integrity (supravital staining) were microscopically evaluated for the fresh and frozen-thawed semen samples. The structural integrity of the plasma membrane of the sperm heads via the supravital test was assessed, classifying them as alive (non-stained sperm) or dead (light pink stain or reddish color) (Smith and Murray, 1997).

2.4. Artificial insemination and pregnancy diagnosis

In Experiment 1, conducted during a light-induced breeding season, 50 mature does of the Saanen and Alpine breeds, weighing 50.8 ± 9.8 kg (range: 37–52 kg) and in good body condition (BCS: 2.8 ± 0.4) were used. 24 does were inseminated with semen from the T1 treatment (20% egg yolk) and 26 does inseminated with semen from the T2 treatment (2.5% egg yolk). In Experiment 2, conducted during the natural breeding season, 60 mature does of the Saanen and Alpine breeds, weighing 50.0 ± 10.2 kg (range: 35–53 kg) and in good body condition (BCS: 2.8 ± 0.3) were used. Here 30 does were inseminated with semen from T1 treatment (20% egg yolk + washing) and 30 inseminated with semen from the T2 treatment (2.5% egg yolk).

Inseminations were performed using the transcervical technique (Mies Filho, 1987), 24 h after estrous detection. In this procedure the universal applicator for goats (Minitub®) was used together with a coating sheath (Minitub®), with a rounded tip. A vasectomized buck was introduced to the does every 12 h in order to detect the onset of estrus (characterized by the acceptance of mounting by the buck). This teasing occurred until the does no longer exhibited estrus. Before each AI the appearance of the cervical mucus was visualized via the aid of a vaginal speculum, and quantified: 1-crystalline; 2-striated; and 3-caseous. After AI, the site of semen deposition was recorded: 1-superficial cervical; 2-cervical; and 3-intrauterine.

Diagnosis of pregnancy was performed 30 days post AI, by transrectal ultrasonography with the aid of an ultrasound machine, equipped with a linear transducer of 5.0 MHz (ALOKA SSD 500; Aloka American, Wallingford, CT). The number of fetuses was also recorded.

2.5. Statistical analysis

The data which followed a normal distribution, according to the Shapiro–Wilk test, were compared using the one way ANOVA with a subsequent Student–Newman Keuls test (SNK) at the 5% significance level – to compare the means between treatments. Data that were not normally distributed were transformed to log or rank. Qualitative non-parametric data (pregnancy rate) were tested using the chi-square test (χ^2). The program SAEG 9.0 (UFV, 2007) was used to perform the analyses.

Table 1
Mean (±SD) values for physical and qualitative characteristics recorded in frozen buck semen during two breeding seasons.

Treatment	Experiment 1			Experiment 2		
	Motility (%)	Vigor (1–5)	Supravital (%)	Motility (%)	Vigor (1–5)	Supravital (%)
T1	58.7 ± 5.1[a]	2.7 ± 0.3[a]	58.7 ± 7.5[a]	54.3 ± 6.2[a]	2.6 ± 0.2[a]	56.3 ± 8.1[a]
T2	61.2 ± 5.1[a]	3.1 ± 0.2[b]	61.7 ± 6.1[a]	60.6 ± 4.1[a]	3.1 ± 0.2[b]	57.7 ± 6.8[a]
Total	60.0 ± 5.1	2.9 ± 0.3	60.2 ± 6.8	57.5 ± 6.0	2.8 ± 0.3	57.0 ± 7.3

[ab]Means with different superscripts in the same column are significantly different ($P < 0.05$).

3. Results

3.1. Fresh semen

For fresh semen, the mean values for sperm motility (%), vigor (scale: 0–5), concentration ($\times 10^6$ sperm/mL) and seminal volume (mL) of the ejaculate were: 87.5 ± 2.6, 3.8 ± 0.3, 2.5 ± 0.7 and 1.3 ± 0.4, respectively for Experiment 1, and 90.0 ± 3.7, 3.8 ± 0.4, 2.2 ± 1.1 and 1.2 ± 0.1 respectively for Experiment 2. No differences were recorded for the fresh semen parameters in Experiments 1 and 2. Similarly no difference was recorded between bucks in both experimental studies. The values for semen volume, sperm motility and vigor obtained during the two seasons were above to the minimum standard required for fresh goat semen – as suggested for the specie (CBRA, 1998). The mean values recorded for membrane plasma integrity by supravital staining were 87.6 ± 3.7% and 76.2 ± 6.2% in Experiments 1 and 2, respectively.

3.2. Frozen semen

Regarding frozen semen, the values for sperm motility, vigor and supravital staining are summarized in Table 1. In Experiments 1 and 2, significant differences ($P < 0.05$) were recorded in sperm vigor and major defects in sperm morphology between treatments (Tables 1 and 2). The minor sperm defects recorded were lower ($P < 0.05$) in the T2 treatment, than in the T1 treatment in Experiment 1 and T1 + washing in Experiment 2.

3.3. Frozen semen fertility

The values regarding deposition site, appearance of the mucus, and duration of estrus in does did not differ between the treatments – either in Experiment 1 or Experiment 2. The mean values for deposition site (1–3), appearance of the mucus (1–3), and duration of estrus (h) were 2.9 ± 0.3, 2.0 ± 0.2 and 31.6 ± 9.4 in Experiment 1, and 2.9 ± 0.3, 2.0 ± 0.3 and 31.6 ± 9.3 in Experiment 2, respectively.

The results regarding fertility are set out in Table 3. In Experiment 1 it was observed that the fertility rate recorded was lower ($P < 0.05$) in the T1 treatment, than in the T2 treatment. In Experiment 2, however, no difference in fertility rate was recorded between treatments.

4. Discussion

The mean sperm concentration and sperm integrity following the supravital test were normal, when compared to the standard values for goat semen (Betini et al., 1998; Rovay, 2006; Castilho et al., 2009). The lack of difference in fresh semen parameters, when Experiments 1 and 2 were compared, indicated that season of the year did not have an effect on the seminal parameters in this trial – which is consistent with the results in frozen-thawed semen, recorded by Dorado et al. (2010). The effect of photoperiod on seminal quality has been previously reported in goats (Zarazaga et al., 2009), and according to Mies Filho (1987), has a marked effect at the mid and high latitudes. However, in the tropical zones (low latitude) as in the present study (S 20°45′20″), this was not observed.

In Experiment 1, semen was processed for cryopreservation without seminal plasma being removed (washing), and in Experiment 2 the seminal plasma was taken from semen used in the T1 treatment. This difference was due to the non-freezing of buck semen samples with a high level of egg yolk (T1 treatment) without the washing process. Previous studies (Ritar and Salamon, 1991; Cabrera et al., 2005; Choe et al., 2006) also observed this negative effect on goat semen. La Falci et al. (2002), studying the changes in the production of enzymes in the seminal plasma of goats during the year, reported differences in the production of the egg yolk coagulating enzyme. This enzyme may produce higher quantities of lysophosphatidylcholines, and consequently have an effect on the quality of the frozen-thawed semen – as these substances are toxic to goat sperm (Leboeuf et al., 2000). The benefits obtained from seminal plasma are variable. Some researchers indicate seminal plasma removal to be necessary – maximizing the post-freezing sperm motility and acrosomal integrity of the goat

Table 2
Mean (±SD) major and minor sperm defects recorded in frozen buck semen during two breeding seasons.

Treatment	Experiment 1		Experiment 2	
	Major defects	Minor defects	Major defects	Minor defects
T1	5.1 ± 2.2[a]	2.0 ± 1.1[a]	4.7 ± 1.4[a]	2.2 ± 1.4[a]
T2	2.6 ± 1.4[b]	0.7 ± 0.8[b]	2.5 ± 0.7[b]	3.1 ± 1.8[a]
Total	3.8 ± 2.2	1.3 ± 1.2	3.6 ± 1.6	2.6 ± 1.6

[ab]Means with different superscripts in the same column are significantly different ($P < 0.05$).

Table 3
Fertility rate of goat semen cryopreserved in different seasons by different treatments.

Treatment	Experiment 1		Experiment 2	
	Total animals	Pregnancy rate (%)	Total animals	Pregnancy rate (%)
T1	30	14 (46.7)[a]	24	12 (50.0)[a]
T2	30	21 (70.0)[b]	26	20 (76.0)[a]

[ab]Means with different superscripts in the same column are significantly different ($P < 0.05$).

semen (Ritar and Salamon, 1991; Cabrera et al., 2005; Choe et al., 2006). Others have reported positive results for frozen goat semen, without washing (Ritar and Salamon, 1982; Azeredo et al., 2001). It is also important to note that in a trial conducted by Cavalcante (2003), the serum testosterone levels were 346.7 ng/dL during the natural breeding season, and 288.57 ng/dL outside the breeding season. This difference in testosterone level may be sufficient to stimulate the bulbo-urethral glands, which then secrete more enzymes related to the egg yolk coagulation, compared to during the natural breeding season.

Despite the sperm motility not differing between the treatments, the low inclusion rate of egg yolk without the semen washing process (Experiment 1), showed better results in terms of sperm vigor and sperm morphology (Tables 1 and 2). A similar result was observed in Experiment 2, when the low inclusion rate of egg yolk (T2) was compared with the high inclusion rate of egg yolk, coupled with semen washing (T1). The results indicated semen extenders with a low inclusion rate of egg yolk (2.5%) to preserve the capacity of movement better, and to reduce the morphologic injuries to the goat sperm during the cryopreservation process. This could be due to the less depletory effect originating from the hydrolysis of egg yolk compounds (Pellicer-Rubio et al., 1997). The sperm morphology defects remained low in both treatments, and all the frozen-thawed goat semen was acceptable for use in the AI procedures in goats (CBRA, 1998).

The deposition site of semen, aspect of mucus and the time of AI play an important role in the fertility rate obtained (Siqueira et al., 2009). Regarding the deposition site, the mean values recorded in this trial (2.8–2.9 – on a scale of 1–3), indicated that most inseminations were performed in the uterus. The mean values for the mucous appearance recorded remained close to 2.0 (on a scale of 1–3). The high rate of passage through the cervix and the aspect of mucus recorded may be related to the AI procedures being performed 24 h after the onset of estrus. Characterizing that most AI's were performed at a time near to the end of estrus – possibly coinciding with the time of ovulation. The best time to perform AI is said to be when the mucus is striated and abundant – which corresponds to the middle third to the end of estrus (Siqueira et al., 2009). The approximate time of the AI procedure and ovulation in the present study could have positively accounted to the fertility rate obtained.

The better results of parameters observed in vitro (sperm vigor and morphology) following T2 treatment (low egg yolk level) in Experiment 1, are in agreement with the results affecting fertility – showing a higher pregnancy rate when using the low level of egg yolk inclusion in the semen extender. In the present study, the fertility rate for the T1 treatment with or without washing process were similar to that reported in others studies (Ritar et al., 1990; Salvador et al., 2005; Dorado et al., 2007) using semen extenders with 20% egg yolk. The increase in fertility rate for low egg yolk inclusion indicated in Experiment 1 was not observed in certain other studies (Ferrari et al., 1998; Barbas et al., 2006), when semen extenders containing low egg yolk were also used. The differences in these studies may be due to the difference in egg yolk inclusion rate, breed, age of animals, season and others factors that can influence the fertility rate and consequently challenge the comparison between the studies.

The pregnancy rates obtained in Experiment 2 did not differ between the T1 and T2 treatments – possibly due to the number of animals used. Another observation was that Experiment 2 was conducted during the natural breeding season of the goat, which may affect the semen plasma composition and its interaction with goat semen during the cooling and freezing processes. However, the results in Experiment 1 indicated that a low concentration of egg yolk promoted better preservation of goat semen following the cryopreservation process, outside the natural breeding season. This effect could have occurred due to less formation of the lysophosphatidylcholines, as low levels of egg yolk have less substrate for the action of the bulbo-urethral 60-kilodaltonglycoprotein, with triglyceride lipase activity (Pellicer-Rubio et al., 1997). Thus, the low level of egg yolk probably reduced the toxic effect towards the sperm cell during semen cryopreservation. Further studies regarding the measurement of lysophosphatidylcholines after the semen cryopreservation technique using low and high levels of egg yolk on semen extenders are however still required.

5. Conclusion

The use of a low concentration (2.5%) egg yolk in the glucose–EDTA extender improved the preservation of frozen goat semen outside the natural breeding season, compared to a high concentration (20%) of egg yolk. The low level of egg yolk in semen extender can be used in the cryopreservation of goat semen, regardless of the season.

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