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Effects of frozen diluents on storage of ram sperm

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ABSTRACT

Semen collection evaluation and addition of preservatives to increase storage period of sperm are essential for successful artificial insemination. This study was conducted on 4rams (2 Ghezel Merinos and 2 Merinos Moghani) to evaluate the effect of two diluents in Khalatposhan research station. Average age of rams was 3 years. Rams were trained to serve the artificial insemination and semen samples were collected weekly. It was started from October 2011 to June 2012. After collection semen samples were mixed with diluents. They were stored in liquid nitrogen. After diluting and storage semen was assessed after 0, 1, 2 and 3 day for pH, viability and progressive motility. Frozen straws were thawed individually at 37 °C for 20 s in a water bath for evaluation. Effect of diluent and storage day on pH, viability and progressive motility of sperm were significant ($P< 0.01$). With the increasing storage day pH, viability and progressive motility of sperm decreased. The success of these diluent has been attributed the tris which may act as a buffer against changes in pH and tonicity. Fructose and citric acid are energy source. Egg- yolks protect the cell membrane during cooling. Glycerol protects the spermatozoa against membrane damage during freezing. This study showed that diluents containing of 7% glycerol and 20% egg-yolk had better sperm protection ability than extender containing 5% glycerol and 5% egg-yolk according to sperm motility, pH and sperm viability.

Key words: Artificial insemination, Diluent, Ram, Semen.

INTRODUCTION

The sheep industry has not been able to utilize many of the assisted reproductive technologies (ART) in general and AI in particular, as other livestock industries, due to inefficiencies in collecting, freezing and inseminating frozen ram semen. Furthermore, some studies routinely collect and freeze ram semen, and there is still a need to optimize cryopreservation and breeding protocols for ram semen (Blackburn, 2004). Cryopreservation is an extensively used technique for the long-term storage of semen, but it causes partial irreversible damage to the sperm cells (Amann and Pickett, 1987; Purdy, 2006). This has been attributed to sperm cold shock, oxidative stress, sperm membrane modification, cryoprotectant toxicity, intracellular

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ice crystal formation, and fluctuations in osmotic pressure (Watson andMartin, 1975; Watson, 1995; Isachenko, 2003). Such sperm injuries can then ultimately lead to a reduction in the sperm motility and poor fertility following AI (Salamon and Maxwell, 2000; Matsuoka et al., 2006; Rodriguez-Martinezan and Barth, 2007). Diluents for freezing ram semen should have similar properties to diluents for fresh use of semen, they should be buffered against changes in pH and tonicity and contain an energy source. In addition they should contain an agent to protect the cell membrane during cooling and a cryoprotective agent which protects the spermatozoa against membrane damage during freezing (Evance and Salmon, 1987). Many factors can affect the maintenance of spermatozoa function during freezing and thawing such as freezing method equilibration periods and cooling rate. Moreover composition of extender may also affect the freeze ability of spermatozoa and their fertilizing ability (Salamon and Maxwell, 1995). Glycerol is the most widely used cryopreservation agent for spermatozoa (Salamon and Maxwell, 2000). It can be added to the semen in a separate diluent fraction (two-step dilution) with the addition of the glycerolated diluent in the second phase, or by a single addition of diluent containing glycerol (one-step method) (Salamon and Maxwell, 1995). The level of glycerol included in diluents for frozen storage of ram semen is ultimately limited by its toxicity (Hammerstedt and Graham, 1992) which in turn depends on the cooling and freezing rates (Fisher and Fairfull, 1984), diluents composition (Pontbriand et al., 1989) and method of adding the glycerol (Colas, 1975). However, all new methods for processing ram semen need to be tested before practical application in the field. *In vitro* assays to test the effectiveness of this method are needed. Among this assays those focusing on sperm viability, such as motility parameters, are considered to be most reliable (Salamon and Maxwell, 2000). The objective of the present study was to describe the changes in daily pH, viability and progressive motility of ram sperm that was diluted and comparison of two diluents.

MATERIAL AND METHOD

Animals

Two Ghezel-Merinose and two Merino- Moghani rams (approximately 3 years old) were used in this study. They were maintained under uniform feeding, housing and lighting conditions. The experimental animals were subjected to the same feeding program of the farm. Animals were fed twice daily in order to achieve a predetermined feed intake of 2.5% body weight (on dry matter basis) per day. Levels of nutrition remained equal and without changes as each ram's daily diet consisted of 20% concentrate (75% barley, 25% corn, soya, bran, supplement and lime) and 80% alfalfa hay. All the rams had free access to salty stones twice or three times a day. Water was introduced *ad libitum*.

Evaluation of microscopic sperm parameters

Semen was collected by artificial vagina once a week during the non-breeding season. Immediately after collection, each ejaculate was immersed into a water bath maintained at 37 ◦C prior to evaluation. The semen samples were evaluated for volume, wave motion, sperm concentration, pH, progressive motility and viability. The volume of ejaculate was determined by graduated tube (Shamsuddin et al., 2000). To evaluate the wave motion, a drop of undiluted semen was placed on a pre-warmed slide at 37 °C without a cover slip and examined under phase contrast microscope (100 times) (Nikon, Eclipse, E200, Japan). The wave motion was scored 0 = no motility, 1 = few sperm with weak movement (<20%), 2 = some motile spermatozoa (20–40%) without wave movement, 3 = slow wave movement (40–60%) with motile spermatozoa, 4 = rapid wave movement without whirlpool (60–80%) with motile spermatozoa and 5 = very rapid wave movement with clear whirlpools (>80%) motile spermatozoa (Avdi et al., 2004).The sperm progressive motility was estimated subjectively by preparing a wet mount of diluted semen by placing a 1-5 drop of fresh semen under coverslip at magnification of about 200 times under phase

contrast microscope. At least 200 spermatozoa, selected randomly from a minimum of four microscopic fields, were examined. The mean of four successive estimations was recorded as the final motility. The sperm concentration was determined by means of a haemocytometer. Sperm viability of the samples was assessed by means of the nigrosin–eosin staining (Evance and salmon, 1987). The stain was prepared as: eosin-Y 1.67g, nigrosin10g, sodium citrate 2.9g, dissolved in 100 ml distilled water. The sperm suspension smears was prepared by mixing a drop of the semen sample with 2 drops of the stain on a warm slide and spreading the stain with a second slide immediately. The viability was assessed by counting 200 cells under the phase-contrast microscope at magnification 400×. Sperm showing partial or complete purple coloring was considered non-viable and only sperm showing strict exclusion of the stain were considered to be alive.

Semen processing

One ejaculate of each ram was divided in the 2 treatments. Ejaculates including sperm progressive motility greater than 70% were used in the study. After microscopic evaluation, one of the aliquot semen samples was diluted 1:3 with 3.786 g Tris, 2.172 g citric acid and 1 g fructose in 100 ml distilled water. The diluent was supplemented with 5.0% (v/v) glycerol 5% egg yolk, penicillin (100,000 IU) and streptomycin (100mg) (group 1) and another aliquot was diluted 1:3 with 2.71 g Tris, 1 g citric acid and 1.4 g fructose in 100 ml distilled water. Then 70ml from this solution mixed with 7% (v/v) glycerol 20% egg yolk; penicillin (100,000 IU) and streptomycin (100 mg) (group 2). The sperm concentration was fixed at 5×10^8. Diluted semen samples were aspirated into 0.25ml French straws. The aliquots after cooling were frozen in NL_2. Thawed semen samples were evaluated for pH, viability and progressive motility at 0, 1, 2 and 3 day after storage.

Statistical analyses

The progressive motility and viability for semen samples were analyzed using Proc MIXED of SAS (SAS, Version 9, Carry, NC) in an initial model with fixed effects for antioxidants, storage time, counting storage time and antioxidants. Animal was considered as random effect.

RESULTS

Characteristics of diluted sperm have been shown in Table 1 and 2.

Table1. Descriptive statistics of group 1

Storage time(day)	Variable	Number	mean	SD	min	max
0	pH	24	6.8	0.08	6.6	7
	Viability (%)	24	60.33	2.79	55.23	65.43
	Progressive motility (%)	24	57.2	2.76	53.2	61.2
1	pH	24	6.7	0.06	6.5	6.9
	Viability (%)	24	50.33	2.49	45.26	55.4
	Progressive motility (%)	24	47.29	2.74	43.1	51.48
2	pH	24	6.68	0.1	6.5	6.8
	Viability (%)	24	46.3	3.17	41.23	51.43
	Progressive motility (%)	24	43.03	3.2	38.06	48

	pH	24	6.2	0.26	5.7	6.7
3	Viability (%)	24	34.25	3.22	28.15	40.35
	Progressive motility (%)	24	30.2	3.35	24.2	36.2

Table2. Descriptive statistics of group 2

Storage time(day)	Variable	Number	mean	SD	min	max
	pH	24	6.9	0.07	6.8	7
0	Viability (%)	24	65.5	2.44	62	69
	Progressive motility (%)	24	61.45	2.43	57.5	65.4
	pH	24	6.8	0.24	6.7	6.9
1	Viability (%)	24	55.33	4.33	51.42	59.24
	Progressive motility (%)	24	51.75	4.38	48.18	55.32
	pH	24	6.7	0.3	6.6	6.8
2	Viability (%)	24	51.37	3.6	44.49	58.25
	Progressive motility (%)	24	48.37	3.63	42.48	54.26
	pH	24	6.4	0.28	6.1	6.7
3	Viability (%)	24	44.13	3.39	41.1	47.16
	Progressive motility (%)	24	40.04	3.58	37.06	43.02

Effect of diluent and storage time on sperm pH

Effect of diluents and storage time on sperm pH has been shown in Table 3. Diluent and storage time had a significant effect on sperm pH. PH with the increasing storage time sperm decreased. The diluents of group 2 had better performance than group 1.

Table3. Effect of diluents and storage time on sperm pH

Storage time(day)	Number	Mean(±SE) Diluent 1	Mean(±SE) Diluent 2
0	24	6.85 ± 008^{a}	6.91 ± 0.07^{a}
1	24	6.7 ± 0.062^{c}	6.8 ± 0.24^{b}
2	24	6.68 ± 0.1^{d}	6.7 ± 0.13^{cd}
3	24	6.17 ± 0.26^{e}	6.4 ± 0.28^{e}

Least squares means with different letters are significantly different ($P< 0.05$).

Effect of diluent and storage time on sperm viability

Effect of diluent and storage time on sperm viability has been shown in Table 4. Diluents and storage time had a significant effect ($P< 0.01$) on sperm viability. In this table it is obvious that with

enhancement in storage time sperm viability was reduced regardless of type of the extender. Sperm viability of group2 was better than group1.

Table 4.Effect of diluents and storage time on sperm viability

Storage time(day)	Number	Mean(±SE) Diluent 1	Mean(±SE) Diluent 2
0	24	60.33 ± 2.79^{b}	65.5 ± 2.44^{a}
1	24	50.33 ± 2.49^{d}	55.79 ± 4.33^{c}
2	24	46.2 ± 3.17^{e}	51.37 ± 3.6^{d}
3	24	34.25 ± 3.22^{f}	44.13 ± 3.39^{f}

Least squares means with different letters are significantly different ($P< 0.05$).

Effect of diluent and storage day on sperm progressive motility

Effect of diluent and storage time on sperm viability has been shown in Table 5. Sperm storage time and diluent had a significant effect (P< 0.01). on sperm progressive motility. Reversely with the increasing storage time sperm progressive motility decreased. Diluents of group2 performed better than diluent group1.

Table 5. Effect of diluent and storage day on sperm progressive motility

Storage time(day)	number	Mean(±SE) Diluent 1	Mean(±SE) Diluent 2
0	24	57.2 ± 2.76^{b}	61.45 ± 2.43^{a}
1	24	47.29 ± 2.74^{d}	51.75 ± 4.38^{c}
2	24	43.3 ± 3.2^{e}	48.37 ± 3.63^{d}
3	24	30.2 ± 3.35^{f}	40.04 ± 3.58^{f}

Least squares means with different letters are significantly different ($P< 0.05$).

DISCUSSION

Cryopreservation is an extensively used technique for the long-term storage of semen (Purdy, 2006). This study involved the evaluation of the effect of diluent and storage time on sperm pH, viability and progressive motility. Diluent content of 7% glycerol and 20% egg-yolk were found to have better sperm protection ability than milk based extenders following storage – regarding sperm motility, pH and viability. The success of this diluent has been attributed to the amount of percentage of glycerol and egg-yolk.Glycerol is the most commonly used protective substance in diluents for freezing ram semen. For semen frozen by the slow ‘‘conventional’’ method, and using mainly hypertonic diluents, most investigators found that the optimal glycerol concentration was within the range of 6–8 % (Graham et al., 1987) and glycerol protect the spermatozoa against membrane damage during freezing (Evance and Salmon, 1987). Egg yolk is a main component in extenders for storage and cryopreservation of semen in most mammalian species including bull, ram, goat, pigs, and even humans. The cryoprotectant fraction of egg yolk is related to the low-density lipoprotein (Moussa et al., 2002; Jeyendran et al., 2008; Waston, 1981) and egg-yolk protects the cell membrane during cooling. (Evance and Salmon, 1987). Ritar and Salmon (1982) reported that tris may act as a buffer against changes in pH and tonicity and fructoseisan energy source. Some investigation found that adding of 20% egg- yolk and 7% glyceol to the diluent was claimed to improve the viability and progressive motility of spermatozoa during storage (Salamon and

Lightfoot, 1969). Storage time had a significant effect on progressive motility, viability and pH up to 3 days post collection. Diluent and storage time had a significant effect on sperm pH. Reversely with the increasing storage time sperm decreased. Increasing of storage time led to accumulation of lactic acid. It reduced the pH. This finding contradicts to the ram semen study of Paulenz et al., (2002). Diluent and storage time had a significant effect on sperm viability. By enhancement in storage time sperm viability was reduced. Increasing of storage time led to accumulation of CO_2. This reduced the sperm viability. This finding contradicts the ram semen study of Preservage2009. Sperm storage time and diluent had a significant effect on sperm progressive motility. Reversely with the increasing storage time sperm progressive motility decreased. Increased co2 concentration results in reduced progressive motility.

Conclusion

The achieved results showed that diluent containing of 7% glycerol 20% egg-yolk had better sperm protection ability.

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