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Effect of diluents on storage of ram semen

Fatemeh Soltanpour[1*] and Golamali Moghaddam[2]

1-Msc graduated of Animal Physiology, Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.
2-Professors, Department of Animal Science, Faculty of Agriculture, University of Tabriz, Iran

Corresponding Author: Golamali Moghaddam

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ABSTRACT

Semen collection and sperm evaluation and addition of preservatives for increasing storage of sperm are essential for successful artificial insemination. This study was conducted on 4 rams (2 Ghezel Merinos and 2 Merinos Moghani) rams to evaluate the effect of 2 liquid diluters (1-egg yolk- citrate, 2-milk) in Khalat poshan research station. Average age of rams was 2-3 years. After rams were trained to serve the artificial insemination, semen samples were collected weekly. It was started from October 2011 to June 2012. After collected semen was mixed with liquid diluents and was stored in a refrigerator for 8 days. Semen after diluting and storage were assessed for pH, viability and progressive motion. Effect of diluent and storage day on pH, viability and progressive motion of sperm were significant ($P< 0.01$). With the increasing storage day pH, viability and motility progressive of sperm decreased. The success of diluent of milk has been attributed to its protein fraction, which may act as a buffer against changes in pH and as a chelating agent against any heavy metals present. The success of citrate has been attributed that may act as a buffer against changes in pH. The egg-yolk providing protection of sperm against cold shock. This study showed Diluent of citrate-egg yolk had a better sperm protection ability than milk based extenders following 8 day of storage – according to sperm motility, pH and sperm viability

Keywords: *Semen, Diluter, Liquid, Viability, Progressive motility.*

INTRODUCTION

An artificial insemination is probably the most important single technique devised to facilitate the genetic improvement of animals. The wide spread use of AI in sheep may allow accurate genetic evaluation and rapid dissemination of genetic merit on a national and international basis to bring benefit to the breeder and consumer (salmon and Maxwell, 2000). Appropriate diluents provide nutrients for the spermatozoa, buffer against changes in pH, and provide an isotonic environment. In addition they should protect spermatozoa against cold shock when cooled and stored for a longer period of time compared to fresh semen.(Evance and Salamon, 1987). However, AI with frozen-thawed ram semen has not been widely adopted for sheep mainly due to the very poor fertility obtained after cervical insemination compared with the pregnancy rate after cervical insemination with fresh semen or that obtained with laparoscopic intrauterine insemination (Donovan, 2004; Donovan, 2001; Eppleston and Maxwell, 1993; Byrne, 2000; Paulenz, 2002). It is essential to inseminate and optimal time in heat to achieve an acceptable fertility result. Further it is very important to preserve and store the semen under optimal conditions. Milk and sodium citrate based extenders supplemented with egg yolk have been used widely for the storage of liquid semen. The recommended maximum storage time without impaired fertility after AI has traditionally been said to be as short as 6-12h. Extenders have been designed usually on an empirical basis, to protect and maintain spermatozoa during the processing and storage of semen. For instance, extenders based on Tris plus egg-yolk has been widely used in several farms animals, including the ram, (Salamon and Maxwell, 1995). 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 essays those focusing on sperm viability, such as motility parameters, are considered to be most reliable (Amann, 1989).

The objective of the present study was to describe the changes in daily pH, viability and progressive motility of ram sperm that was diluted with sodium citrate based egg-yolk extender and milk and stored at 4 ◦C for 8 days.

MATERIALS AND METHODS

Animals

Two Ghezel-Merinose and two merinos- 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 ram per day. Water was 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, 2000). To evaluate the wave motion, a drop of undiluted semen was placed on a pre-warmed slide 37 ◦C without a coverslip and examined under phase contrast microscope (100×) (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, 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× 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 (Salamon and Maxwell, 1995). The stain was prepared as: eosin-Y 1.67 g, nigrosin10 g, sodium citrate 2.9 g, 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. Only ejaculates including sperm progressive motility greater than 70% were used in the study.

Each ejaculate was divided into 2 equal aliquots. After completion of microscop evaluation, semen sample was diluted with egg-yolk citrate(each 100ml diluter contained 20ml egg-yolk, 80ml 2.9% sodium-citrate buffer, penicillin 1000IU/ml, streptomycin 100mg/ml) (group1) and milk(ultra milk 0.7% fat, penicillin 1000IU/ml, streptomycin 100mg/ml) (group 2).fixed the sperm concentration at 5×10^8 . Diluted semen samples were aspirated into 0.25ml French straws. The aliquot after cooling for 1.5-2h was stored at 4ºC in a refrigerator. The diluted fresh semen at 0^{st}, 1st, 2^{nd}, 3^{rd}, 4^{rd}, 5rd, 6^{rd}, 7^{rd} and 8^{rd} days of storage were evaluated for pH, sperm viability and progressive motility.

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 considered as random effect.

RESULTS AND DISCUSSION

Results

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

Table1. Descriptive statistics of group 1(egg-yolk & citrate)

Storage time	Variable	number	mean	SD	min	max
	pH	56	6.9	0.07	6.7	7
0	viability	56	87.44	4.37	77	96
	progressive	56	83.42	4.37	73	92
	pH	56	6.72	0.22	6	6.9
1	viability	56	81.39	5.15	68	91
	progressive	56	76.71	5.21	63	86
	pH	56	6.56	0.19	6	6.8
2	viability	56	78.03	4.34	68	88
	progressive	56	73.67	4.28	64	84
	pH	56	6.43	0.21	5.9	6.8
3	viability	56	73.42	4.42	61	82
	progressive	56	69.14	4.41	57	78
	pH	56	6.23	0.23	5.7	6.7
4	viability	56	67.72	5.36	52	78
	pH	56	6.05	0.2	5.8	6.5
5	viability	56	58.64	8.19	32	78
	progressive	56	53.94	8.38	28	73
	pH	56	5.97	0.18	5.5	4.6
6	viability	56	48.14	8.01	33	71
	progressive	56	43.41	7.85	29	66
	pH	56	5.53	0.39	5	6
7	viability	56	35.83	7.49	15	62
	progressive	56	31.1	7.36	10	58
	pH	56	5.45	0.29	5	5.9
8	viability	56	25.57	5.65	15	50
	progressive	56	22.73	5.61	11	45

Table2. Descriptive statistics of group 2 (milk)

Storage time	Variable	number	mean	SD	min	max
	pH	56	6.82	0.06	6.7	6.9
0	viability	56	80.96	3.57	74	88
	progressive	56	76.89	3.62	70	84
	pH	56	5.93	.21	5.5	6.4
1	viability	56	70	5.53	52	84
	progressive	56	65.92	6.14	48	80
	pH	56	5.79	.15	5.5	6.1
2	viability	56	57.94	3.6	50	66
	progressive	56	53	3.44	46	62
	pH	56	5.67	0.16	5.3	6
3	viability	56	47.89	6.13	30	60
	progressive	56	43.32	6.06	25	56
	pH	56	5.6	0.26	5.2	6.3
4	viability	56	39.67	8.08	15	65
	progressive	56	35.1	8.12	10	61
	pH	56	5.36	0.22	5	5.9
5	viability	56	30.17	5.7	16	40
	progressive	56	25.57	5.64	11	36
	pH	56	5.18	0.2	4.9	5.8
6	viability	56	20.8	4.55	7	32
	progressive	56	15.92	4.67	2	28
	pH	56	4.89	0.15	4.5	5.1
7	viability	56	8.87	4.28	0	25
	progressive	56	3.53	4.21	0	20
	pH	56	4.8	0.1	4.6	5.1
8	viability	56	0.51	1.61	0	7
	progressive	56	0	0	0	0

Effect of diluent and storage time on sperm pH

Effect of diluent and storage time on sperm pH has been shown in table 3. Diluent and storage time had a significant effect on sperm pH. Reversely with the increasing storage time sperm decreased. And the diluent citrate had a better performance than milk.

Table3. Effect of diluent and storage time on sperm pH

Storage time	number	Diluent 1) SE± Mean(	Diluent 2) SE± Mean(
0	56	0.7^{a}±6.89	0.06^{ab}±6.81
1	56	0.22^{b}±6.72	0.21^{ef}±5.92
2	56	0.19^{c}±6.55	0.15^{fg}±5.79
3	56	0.21^{c}±6.42	0.16^{gh}±5.67
4	56	0.23^{d}±6.22	0.26^{h}±5.6
5	56	0.2^{e}±6.04	0.26^{i}±5.3
6	56	0.18^{e}±5.96	0.2^{k}±5.17
7	56	0.32^{hi}±5.52	0.15^{l} ±4.88
8	56	0.29^{ij}±5.44	0.1^{l}±4.8

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. Diluent and storage time had a significant effect on sperm viability. In this table it is obvious that with enhancement in storage time sperm viability was reduced regardless to type of the extender. Sperm Viability of citrate diluent had better than milk.

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

Storage time	number	Diluent 1) SE± Mean(	Diluent 2) SE± Mean(
0	56	4.37^{a}±87.44	3.57^{b}±80.96
1	56	5.15^{b}±81.39	5.53^{cd}±70
2	56	$4.^{34b}$±87.03	3.6^{e}±57.94
3	56	4.42^{c}±73.42	6.13^{f}±47.89
4	56	5.36^{d}±67.82	8.08^{g}±39.67
5	56	8.19^{e}±58.64	5.7^{h}±30.17
6	56	8.01^{f}±48.17	4.55^{i}±20.8
7	56	7.49^{g}±35.83	4.28^{j}±8.87
8	56	5.65^{h}±27.57	1.61^{k}±0.51

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 on sperm progressive motility. Reversely with the increasing storage time sperm progressive motility decreased. And the diluent citrate had a better performance than milk.

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

Storage time	number	Diluent 1) SE± Mean(	Diluent 2) SE± Mean(
0	56	4.37^{a}±83.42	3.62^{b}±76.89
1	56	5.21^{b}±76.71	6.14^{cd}±65.92
2	56	4.28^{b}±73.67	3.44^{e}±53.48
3	56	$4.^{44c}$±69.14	6.06^{f}±43.32
4	56	6.28^{d}±63.1	8.12^{g}±35.1
5	56	8.38^{e}±53.94	5.64^{h}±25.57
6	56	7.85^{f}±43.41	4.67^{i}±15.92
7	56	7.36g±31.1	4.2^{j}±3.53
8	56	5.61^{h}±22.73	0.01^{j}±0.04

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

Discussion

The liquid storage of ram semen has been extensively reviewed in recent years (Paulenz et al., 1995; Revell and Mrode, 1994). This study involved the evaluation of the effect of diluent and storage time on sperm pH, viability and progressive motility. Diluent of citrate-egg yolk were found to have a better sperm protection ability than milk based extenders following 8 day of storage – regarding sperm motility, pH and viability. Milk has also been used for many years as extenders for ram semen. The success of this diluent has been attributed to its protein fraction, which may act as a buffer against changes in pH and as a chelating agent against any heavy metals present (Salamon, and Maxwell, 2000). It may also partially protect spermatozoa during reduction of temperature for storage. Cows' milk has been preferable to the milk of other species (Salamon and Lightfoot, 1969). Milk should be heated because of inactivate the lactenin in the protein fraction which is toxic to spermatozoa. Ultra-heat-treated ''long-life'' milk has proved a satisfactory diluent for fresh semen and also for maintaining the viability of spermatozoa during

liquid storage (Flipse, 1954).The success of citrate has been attributed that may act as a buffer against changes in pH (Evanc and Salmons, 1987). The egg yolk component and more likely its high molecular weight low density lipoprotein fraction (Leboeuf, 2000). Apart from providing protection against cold shock, has been shown to reduce the loss of acrosome enzymes and to prevent degenerative changes in the acrosome during liquid storage (Farshad and Holtz, 1994). Some investigation found that adding of 20% egg- yolk to the citrate was claimed to improve the viability and motility progressive of spermatozoa during storage (Kasimanickam, 2011; Paulenz, 2002).Storage time had a significant effect on progressive motility sperm and viability and pH up to 8 day 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 became accumulation of lactic acid. It reduced the pH. This is the contradictory to the ram semen study of palenz, (2002). Diluent and storage time had a significant effect on sperm viability. Enhancement in storage time sperm viability was reduced. Increasing of storage time became accumulation of CO_2. It reduced the sperm viability. This is the contradictory to the ram semen study of preservage, (2009). Sperm storage time and diluent had a significant effect on sperm progressive motility. Reversely with the increasing storage time sperm progressive motility decreased. Decreasing of viability due to co_2 concentration became the decreasing of progressive motility. This is the contradictory to the ram semen study of preservage, (2009).

Conclusion

This study showed Diluent of citrate-egg yolk had a better sperm protection ability than milk based extenders following 8 day of storage – according to sperm motility, pH and sperm viability

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