Aust. J. Biol. Sci., 1982, **35**, 305–12

Effects of Seminal Plasma and of Its Removal and of Egg Yolk in the Diluent on the Survival of Fresh and Frozen–Thawed Spermatozoa of the Angora Goat

A. J. Ritar and S. Salamon

Department of Animal Husbandry, University of Sydney, N.S.W. 2006.

Abstract

After twofold dilution with Tris–glucose medium, buck seminal plasma was a poorer milieu than ram seminal plasma for the maintenance of viability of freshly collected spermatozoa of both species. Egg yolk (9% v/v) in the diluted buck seminal plasma caused coagulation of the medium coupled with death of spermatozoa after 2 h incubation at 37°C.

Removal of seminal plasma by centrifugation (washing) of buck semen was beneficial for the survival of spermatozoa after freeze–thawing, but the effect depended on the intensity of washing. When the semen was diluted 6- or 11-fold, double washing was more effective than single washing. However, the efficiency of the latter method, after 21-fold dilution, was similar to that of double washing at 11-fold extension.

Survival of washed spermatozoa was better when the resuspending–freezing medium contained 1·5–12% (v/v) egg yolk than no egg yolk. Egg yolk concentrations higher than 1·5% (v/v) depressed the post-thawing survival of non-washed spermatozoa.

Introduction

Storage of semen of the goat in media containing egg yolk is problematical due to the presence in the seminal plasma of an enzyme (phospholipase A) which originates in the Cowper's glands. The enzyme hydrolyses the lecithin of the egg yolk to fatty acids and lysolecithin which are toxic to spermatozoa and cause coagulation of the storage medium (Roy 1957; Iritani *et al.* 1961, 1964; Iritani and Nishikawa 1963, 1964, 1972; Aamdal *et al.* 1965).

The toxic effect is substantially reduced after removal of the seminal plasma by washing the spermatozoa (Iritani *et al.* 1961), or when semen obtained from cowperectomized bucks is extended with the egg yolk-containing diluent (Iritani and Nishikawa 1972). Removal of the seminal plasma (Corteel 1974, 1975) or of the Cowper's glands (Corteel 1980) is beneficial also when the spermatozoa are frozen in a diluent containing no egg yolk.

In our previous study (Salamon and Ritar 1982) Tris–glucose–egg yolk was used for freezing non-washed semen from bucks whose ejaculates did not exhibit coagulation in the presence of egg yolk. The recovery of spermatozoa after freeze–thawing was satisfactory, but notable variation was observed between the semen samples regarding post-thawing survival of spermatozoa. The experiments presented in this paper examined the effects of seminal plasma, methods of washing the spermatozoa, and of egg yolk in the Tris-based diluent on the survival of fresh and frozen–thawed spermatozoa of the Angora buck.

Materials and Methods

Semen was collected during the months of August–October from Angora bucks by use of an artificial vagina and from Merino rams (expt 1) by electro-ejaculator. The bucks' semen was previously not tested for the phenomenon of coagulation with egg yolk. Either one (expt 2) or two ejaculates (expts 3, 4) were collected from each buck. In the latter case the two ejaculates from individual bucks were pooled before use. The concentration of spermatozoa in the ejaculates was $3{\cdot}1 \times 10^9$–$4{\cdot}4 \times 10^9$ per millilitre and the proportion of progressively motile cells was 75–85%.

In experiment 1 the buck or ram seminal plasma was obtained in each case from pooled ejaculates of three or four animals. After collection, the semen of each species was placed in ice water for 10 min (to immobilize the spermatozoa), then centrifuged at 1400 *g* for 30 min. The seminal plasma was removed and extended 1 : 1 with Tris–glucose medium containing no or 18% (v/v) egg yolk. To obtain motile spermatozoa of each species free of seminal plasma, further pooled ejaculates from five bucks and four rams were submitted to double washing. For washing the spermatozoa, the semen was diluted 1 : 5 (semen : washing solution, expts 1, 2) or at higher rates (expts 3, 4) and centrifuged at 900 *g* for 10 min. When double washing was involved, the process was repeated, using the same amount of washing solution as at first centrifugation. The loss of spermatozoa after the washing procedure was not more than 6% (based on haemocytometer counts). After washing and removal of the supernatant, the spermatozoa were resuspended and when washing was not involved the semen was diluted with Tris-based diluent to a cell concentration of $1{\cdot}0 \times 10^9$ per millilitre. The diluted semen or the resuspending medium contained 250 mM Tris, 27·75 mM glucose and egg yolk at concentrations described under Experimental Details and Results. In experiment 1 the resuspending medium also contained 50% (v/v) buck or ram seminal plasma. The pH of the washing, resuspending and diluting media was adjusted to 6·8 with an appropriate amount of citric acid.

The resuspended spermatozoa and the diluted semen were cooled to 5°C in 1·5 h, then pellet-frozen (0·06–0·07 ml) on dry ice and stored in liquid nitrogen until thawing for examination. The frozen pellets were thawed in dry test tubes held in a water-bath at 37°C. The percentage of progressively motile spermatozoa was assessed under a coverslip on a warm stage (37°C) after thawing, and at intervals of 2 h during subsequent incubation for 6 h at 37°C (0, 2, 4, 6 h). In experiment 1 in which only incubation at 37°C was involved, the assessments were made at intervals of 1 h during incubation for 5 h. Motility estimates were to the nearest 5%. The assessor did not know the identity of the samples, all of which were presented for examination in random order.

The data were examined by analyses of variance after angular transformation, except for experiment 1, as described previously (Salamon and Ritar 1982).

Experimental Details and Results

Experiment 1

This experiment examined the survival of fresh buck and ram spermatozoa during incubation at 37°C in Tris–glucose diluent containing 50% (v/v) seminal plasma of either species and no or 9% (v/v) egg yolk.

The results are presented in Fig. 1. Survival of buck and ram spermatozoa during incubation was poorer when the incubating medium contained buck rather than ram seminal plasma. Incubation in the medium containing buck seminal plasma and egg yolk had a dramatic effect on the spermatozoa of both species which did not survive beyond 2 h.

Viability of ram spermatozoa was similar when egg yolk was present or absent in ram plasma–Tris–glucose, but the egg yolk in this incubating medium had a depressing effect on the survival of buck spermatozoa.

Experiment 2

The experiment ($3 \times 2 \times 8$) examined the effect of method of washing on the survival of fresh or frozen–thawed spermatozoa using semen of eight individual bucks. The concentrations of egg yolk and of glycerol were 6% (v/v) and 4% (v/v) respectively

after resuspension of washed spermatozoa and in the diluted semen when washing was not involved. The ratio of semen to washing solution was 1 : 5.

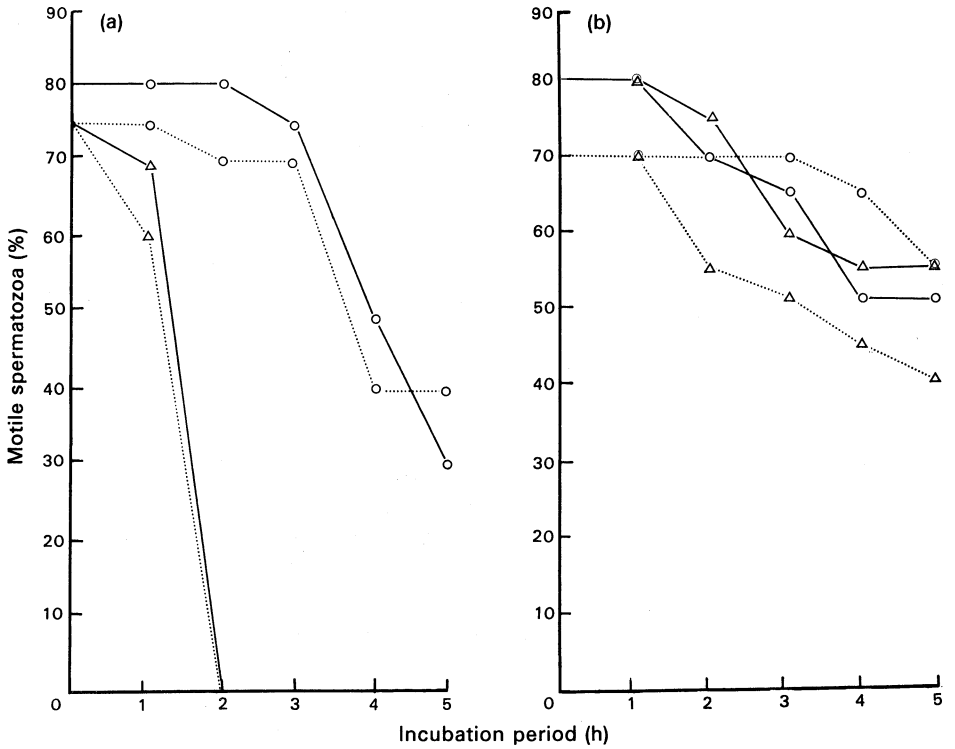

Fig. 1. Survival of buck (· · · ·) and ram (——) spermatozoa in the incubating medium containing 50% (v/v) buck (*a*) or 50% (v/v) ram (*b*) seminal plasma and no egg yolk (○) or 9% (v/v) egg yolk (△).

Table 1. Effect of method of washing on the percentage of surviving fresh and frozen–thawed spermatozoa during incubation (expt 2)

The dilution ratio at washing was 1 : 5 (semen : washing solution)

Washing method	Spermatozoa	Spermatozoa surviving (%) after incubation for: 0 h	2 h	4 h	6 h	Mean	Overall mean
No washing	Fresh	77·0	35·3	22·5	1·3	34·0	29·5
	Frozen–thawed	41·2	35·9	19·2	3·4	24·9	
Single washing	Fresh	68·8	65·9	38·6	25·1	49·6	41·9
	Frozen–thawed	39·1	40·3	37·1	20·2	34·2	
Double washing	Fresh	73·3	65·1	54·5	36·1	57·3	47·8
	Frozen–thawed	39·9	39·1	39·2	34·5	38·2	
Mean		56·6	46·9	35·2	20·1		

All factors examined had significant effects ($P < 0{\cdot}001$). The mean survival rate was higher for washed than non-washed spermatozoa (single and double washing *v.*

no washing, $P < 0{\cdot}001$), but within washing treatments the viability of cells during incubation depended on whether fresh or frozen–thawed spermatozoa were used (washing method × type of spermatozoa × incubation time, $P < 0{\cdot}01$; Table 1). The pattern of decline in survival of non-washed fresh and frozen–thawed spermatozoa during incubation was similar but steeper than after single or double washings. After both methods of washing, however, the decline in survival was steeper for fresh than for frozen–thawed spermatozoa, and in the latter case the spermatozoa survived better during incubation when they were doubly rather than singly washed before freezing.

The viability of spermatozoa of individual bucks differed in their survival during the 6-h incubation period, depending on whether the sperm cells were singly, doubly or not washed (washing method × bucks × incubation time, $P < 0{\cdot}05$). The spermatozoa of those bucks which had poor survival during incubation in the milieu containing their own seminal plasma (non-washed semen) survived better when they were previously singly washed, and there was a further improvement in viability after double washing.

Experiment 3

The experiment (5 × 2 × 4) included the following factors:

(1) Method of washing the spermatozoa: no washing
v. single washing 1 : 5*
v. single washing 1 : 10*
v. double washing 1 : 5*
v. double washing 1 : 10*

(2) Egg yolk in diluted semen or after resuspension of washed spermatozoa: 0 *v.* 6% (v/v)

(3) Buck: two pooled ejaculates from each of four bucks.

The glycerol concentration after resuspension of washed spermatozoa or in the diluted semen (non-washed) was 4% (v/v).

The results are summarized in Table 2. Washing was beneficial for the survival of spermatozoa during post-thawing incubation and the viability improved with more intensive washing (increase in number and rate of washing). The effect, however, depended on whether the spermatozoa were frozen in the presence or absence of egg yolk (washing method × egg yolk × incubation time; $P < 0{\cdot}001$). When there was no egg yolk in the freezing medium the pattern of decline in the survival rate was similar for both washed and non-washed spermatozoa, although a higher proportion of the former cells survived during the post-thawing incubation period. When, however, egg yolk was included in the freezing medium the non-washed spermatozoa did not survive beyond 2 h, and only a few 1 : 5 singly washed sperm cells remained alive after this period of incubation. The viability of spermatozoa in the presence of egg yolk was prolonged after more intensive washing (1 : 10 single and 1 : 5 double washings) and the best survival during the 6-h post-thawing incubation was obtained after double washing at a rate of 1 : 10.

The spermatozoa of individual bucks responded differently to the washing methods (buck × washing method, $P < 0{\cdot}05$), and varied also in their survival during post-thawing incubation (buck × incubation time, $P < 0{\cdot}05$).

* Ratio of semen to washing solution.

Experiment 4

The factors incorporated in this experiment ($3 \times 5 \times 5$) were methods of washing the spermatozoa, concentrations of egg yolk before freezing and ejaculates from each of five bucks. The glycerol concentration after resuspension of washed spermatozoa or in the diluted semen (non-washed) was 4% (v/v).

Table 2. Effect of method of washing and of egg yolk on the percentage of motile spermatozoa during post-thawing incubation (expt 3)

The glycerol concentration after resuspension of washed spermatozoa or in the diluted semen (non-washed) was 4% (v/v)

Time of incubation (h)	Motile spermatozoa (%) for washing regimes: No washing	Single washing 1 : 5[A]	Single washing 1 : 10	Double washing 1 : 5	Double washing 1 : 10	Mean[B]
		No egg yolk at freezing				
0	35·0	33·7	38·7	41·2	45·0	38·7
2	36·2	34·9	38·7	36·2	42·5	37·7
4	26·1	31·2	32·2	36·2	31·0	31·3
6	19·9	27·3	27·5	29·9	29·9	26·8
Mean	29·0	31·7	34·2	35·8	37·0	33·5
		6% (v/v) egg yolk at freezing				
0	37·5	43·6	48·7	50·0	48·7	45·7
2	27·5	41·3	47·8	46·2	47·8	41·9
4	0	1·3	28·6	27·3	41·2	21·7
6	0	0	1·9	13·5	37·3	10·5
Mean	16·3	21·3	31·8	36·8	43·8	30·0

[A] Dilution ratios at washing (semen : washing solution).

[B] Overall means 42·2, 39·8, 25·5 and 14·4 for 0, 2, 4 and 6-h incubation respectively.

The analysis of variance revealed a second-order interaction involving method of washing, egg yolk concentration and post-thawing incubation time ($P < 0{\cdot}001$, Table 3). After freezing the spermatozoa in absence of egg yolk, the recovery rates upon thawing (0 h) for non-washed and washed spermatozoa were similar, although lower than when egg yolk was present in the freezing medium. The decline in the survival rates during post-thawing incubation was also similar for all egg yolk concentrations within single or double washings, but it became progressively steeper with the increase in egg yolk levels in non-washed semen. While egg yolk concentrations higher than 1·5% (v/v) depressed the survival of non-washed spermatozoa, concentrations of between 1·5 and 12% (v/v) were equally well tolerated by 1 : 20 singly washed or 1 : 10 doubly washed spermatozoa.

The spermatozoa of the five bucks differed in resistance to the freeze–thaw procedure ($P < 0{\cdot}001$), but there was no interaction between bucks and other factors.

Discussion

In experiment 1 of this study the concentration of buck or ram seminal plasma in the Tris–glucose medium was 50% (v/v), i.e. the plasma of each species was extended twofold. After such extension, the buck seminal plasma was poorer milieu than the

ram plasma for the maintenance of viability of spermatozoa of both species. When egg yolk was included in the diluted buck seminal plasma, hydrolysis of lecithin led to further deterioration of the environment, and as a consequence the spermatozoa did not survive beyond 2 h at which time coagulation of the incubating medium was also noticeable. The early occurrence of the latter phenomenon, coupled with the death of spermatozoa, was attributable to the relatively high (9% v/v) egg yolk concentration and to the incubation temperature of 37°C which was close to the optimum (40°C) for the activity of the 'egg yolk coagulation enzyme' in the buck

Table 3. Relationship between method of washing and egg yolk concentration at freezing on the percentage of motile spermatozoa during post-thawing incubation (expt 4)

Glycerol concentration at freezing was 4% (v/v)

Washing method	Incubation time (h)	Motile spermatozoa (%) for egg yolk concn (% v/v) at freezing of: 0	1·5	3·0	6·0	12·0	Mean
No washing	0	37·0	40·0	43·0	43·0	39·0	40·4
	2	36·0	39·0	36·0	25·0	25·0	32·0
	4	35·0	33·5	15·0	11·0	1·3	18·9
	6	34·0	31·5	13·0	0·0	0·0	15·4
Mean		35·5	36·0	26·8	19·8	15·3	26·7
Single washing (1 : 20)	0	38·0	41·0	41·0	42·0	45·0	41·4
	2	38·0	39·0	41·0	41·0	41·0	40·0
	4	34·0	36·0	34·0	37·0	38·0	35·8
	6	31·0	33·0	33·0	31·0	32·0	32·0
Mean		35·3	37·3	37·3	38·3	39·0	37·3
Double washing (1 : 10)	0	38·0	43·0	42·0	39·0	39·0	40·2
	2	34·0	40·0	41·0	39·0	39·0	38·6
	4	28·0	36·0	36·0	34·0	37·0	34·2
	6	27·0	34·0	35·0	33·0	34·0	32·6
Mean		31·8	38·3	38·5	36·3	37·3	36·4
Overall mean		34·2	36·9	34·2	31·5	30·5	

seminal plasma (Iritani and Nishikawa 1961). These investigators found that at the optimum temperature a concentration of only 4% (v/v) seminal plasma in the egg yolk–citrate incubating medium caused coagulation, and that the activity of the enzyme was about four times higher in the extract of Cowper's glands than in seminal plasma. Our further experiments (not presented here) revealed that 2·5% (v/v) buck seminal plasma in the egg yolk-containing incubating medium was also sufficient to cause coagulation. Moreover, in the present study the depression of the viability of buck spermatozoa in the presence of egg yolk in the diluted ram seminal plasma indicated that even double washing did not completely remove the enzyme which still had some action upon the egg yolk, although coagulation of the resuspending medium was not observed. For the occurrence of this phenomenon, probably an incubation period longer than 5 h would have been necessary, as indicated by Iritani *et al.* (1961) who also reported that double washing of buck spermatozoa was not fully effective in removing the enzyme.

In the present study, as in the work by other investigators (Iritani *et al.* 1961; Corteel 1974; Drobnis *et al.* 1980) washing of buck spermatozoa was clearly beneficial for their survival during incubation shortly after resuspension (fresh sperm) or after freeze–thawing (Table 1). The beneficial effect, however, depended on the intensity of the washing procedure, i.e. dilution ratio of semen before washing and number of washings (centrifugation). After 6- or 11-fold dilution double washing was more effective than single washing, and the efficiency of each method increased for the higher dilution ratio (Tables 2 and 3). Further, it has been shown that the number of washings can be reduced by extension of semen at a higher rate (21-fold) before single centrifugation, without affecting the survival of resuspended and frozen–thawed spermatozoa (Table 3).

It is noteworthy that in experiment 2 (Table 1) the spermatozoa previously subjected to washing survived better when they were incubated after freeze–thawing than after immediate resuspension (fresh spermatozoa). No explanation can be offered for this and the possibility of deactivation of the enzyme (not removed by washing) during freezing can also be excluded, as in our further investigation fresh or frozen (−196°C)–thawed seminal plasma had similar depressing effects on the viability of doubly washed spermatozoa. Apparently the buck spermatozoa killed during the freeze–thawing procedure also had no toxic effect on the live cells, a phenomenon reported for dead bull spermatozoa due to their amino acid oxidase activity (Shannon and Curson 1972).

In the last experiment the intensively washed spermatozoa tolerated a relatively wide range (1·5–12% v/v) in egg yolk concentration in the resuspending and freezing medium, and such egg yolk levels improved the immediate post-thawing recovery and survival of spermatozoa during subsequent incubation. The egg yolk in the freezing medium also had a protective effect on the non-washed spermatozoa, as judged by post-thawing recovery, but the effect of toxic products during incubation became apparent as the egg yolk concentration increased (Table 3). It seems that an egg yolk concentration of 1·5% (v/v) is about the 'safe margin', at least in the Tris-based diluent, for freezing buck spermatozoa without the removal of seminal plasma. Egg yolk at 1·5% (v/v) in the diluted semen (non-washed) had no depressing effect on viability of spermatozoa, even of those bucks that were 'sensitive' to higher egg yolk levels.

Acknowledgment

One author (A. J. Ritar) was recipient of the University Post-graduate Research Studentship.

References

Aamdal, J., Lyngset, O., and Fossum, K. (1965). Toxic effect of lysolecithin on sperm. *Nord. VetMed.* **17**, 633–4.

Corteel, J. M. (1974). Viabilité des spermatozoïdes de bouc conservés et congelés avec ou sans leur plasma séminal: effet du glucose. *Ann. Biol. Anim. Biochem. Biophys.* **14**, 741–5.

Corteel, J. M. (1975). Effet du 'lavage' sur la conservation des spermatozoïdes de bouc à basse température. *Ann. Biol. Anim. Biochem. Biophys.* **15**, 525–8.

Corteel, J. M. (1980). Effets du plasma séminal sur la survie et la fertilité des spermatozoïdes conservés in vitro. *Reprod. Nutr. Devel.* **20**(4A), 1111–23.

Drobnis, E. Z., Nelson, E. A., and Burrill, M. J. (1980). Effect of several processing variables on motility and glutamic oxalacetic transaminase levels for frozen goat semen. II. Washing. *J. Anim. Sci.* **51** (*Suppl. 1*), 439.

Iritani, A., and Nishikawa, Y. (1961). Studies on the egg yolk coagulating factor in goat semen. II. Properties of the coagulating factor and influential conditions for coagulation. Proc. Silver Jubilee Lab. Anim. Husb. Kyoto Univ. pp. 97–104.

Iritani, A., and Nishikawa, Y. (1963). Studies on the egg-yolk coagulating factor in goat semen. III. Release of some acids accompanied by the coagulating phenomena. *Jap. J. Anim. Reprod.* **8**, 109–12.

Iritani, A., and Nishikawa, Y. (1964). Studies on the egg-yolk coagulating enzyme in goat semen. VI. On the chemical properties of the ejaculated semen and the secretion of accessory sexual organs in the goat. *Jap. J. Anim. Reprod.* **10**, 44–51.

Iritani, A., and Nishikawa, Y. (1972). Studies on the egg-yolk coagulating enzyme (phospholipase) in goat semen. IX. Enzyme concentration in the semen collected from the Cowper's gland-removed goat. *Mem. Coll. Agr. Kyoto Univ.* **101**, 57–63.

Iritani, A., Nishikawa, Y., and Fukuhara, R. (1961). Studies on the egg-yolk coagulating factor in goat semen. I. Localization of the coagulating factor and decline of pH following coagulation. Proc. Silver Jubilee Lab. Anim. Husb. Kyoto Univ. pp. 89–96.

Iritani, A., Nishikawa, Y., and Nagasawa, S. (1964). Studies on the egg-yolk coagulating enzyme in goat semen. VII. Variations in the enzyme activity of the semen between breeding season and non-breeding season, and in each ejaculate collected three times successively. *Jap. J. Anim. Reprod.* **10**, 52–6.

Roy, A. (1957). Egg-yolk coagulating enzyme in the semen and Cowper's gland of the goat. *Nature (London)* **179**, 318–19.

Salamon, S., and Ritar, A. J. (1982). Deep freezing of Angora goat semen: effects of diluent composition, method and rate of dilution on survival of spermatozoa. *Aust. J. Biol. Sci.* **35**, 295–303.

Shannon, P., and Curson, B. (1972). Toxic effect and action of dead sperm on diluted bovine semen. *J. Dairy Sci.* **55**, 614–20.

Manuscript received 5 October 1981, accepted 21 April 1982