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Deep Freezing of Angora Goat Semen: Effects of Diluent Composition and Method and Rate of Dilution on Survival of Spermatozoa

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Abstract

Five factorial experiments were conducted to examine the effects of concentration of tris(hydroxymethyl)aminomethane (Tris), type and concentration of sugar in the diluent, rate and method of dilution on the survival of goat spermatozoa after freezing by the pellet method.

Spermatozoa tolerated a relatively wide range in concentration of Tris, but the cell survival depended on the type of sugar included in the Tris diluent. Glucose and fructose were more suitable components than lactose or raffinose. Survival of spermatozoa after thawing was better for three- to fivefold than twofold prefreezing dilution. There was interaction between method of semen dilution (one-step, two-step), holding time at 5°C, and glycerol concentration. The best result was obtained after one-step dilution at 30°C (Tris 375 mM–glucose 41·625 mM–citric acid 124 mM), 1·5 h holding at 5°C, and with 4% (v/v) glycerol concentration in the diluted semen.

Introduction

There are a number of reports on freezing goat semen in sodium citrate, spermasol, IVT, glucose, lactose, raffinose and milk diluents (reviewed by Corteel 1973; Bonfert 1974). Semen of the bull (Foote 1970*a*, 1970*b*), ram (Salamon and Visser 1972) and boar (Rohloff 1967; Visser and Salamon 1974*c*) has been successfully frozen in Tris-based diluents and there are also reports on their use for freezing goat semen (Samouilidis 1970; Hahn 1972; Fougner 1974, 1976, 1979; Van der Westhuysen 1978; Drobnis *et al.* 1980; Iritani 1980). However, Tris as the main component in the diluent has not been examined at various concentrations or in combination with other agents.

The experiments reported here were conducted to examine the effects of Tris concentration, type and concentration of sugar in the freezing diluent, dilution rate, and method of dilution on the survival of Angora buck semen after the freeze–thawing procedure.

Materials and Methods

Semen was collected during March–May (breeding period) from mature Angora bucks by use of an artificial vagina. In preliminary tests the bucks were examined for 'coagulation' of their semen (Ritar and Salamon 1982) in the presence of 6% (v/v) egg yolk after threefold dilution [300 mM Tris, 30 mM glucose, 9% (v/v) egg yolk] and incubation at 37°C for 6 h. Only bucks whose semen did not exhibit coagulation were used in the experiments. Ejaculates from three different bucks were pooled and regarded as replicates. Before dilution, the replicates had a concentration of $3{\cdot}3\times10^9$–$4{\cdot}7\times10^9$ spermatozoa per millilitre and the proportion of progressively motile cells was 75–85%.

In experiments 1, 2, 3 and 4 aliquots of semen were diluted at 30°C by a single addition of the glycerol-containing diluent (one-step dilution) then cooled to 5°C in 1·5 h and frozen. Experiment 5

compared one-step and two-step methods of dilution, incorporating also different storage periods (equilibration) at 5°C after cooling to this temperature as described fully under Experimental Details and Results. The pH of the Tris-based diluents was adjusted to 6·8 with an appropriate amount of citric acid. The final dilution rate before freezing was 1 : 2 (semen : diluent), except in experiments 2 and 3 where different dilution ratios were examined. The concentrations of egg yolk and of glycerol in the diluted semen were 6% (v/v) and 4% (v/v) respectively, except when both varied according to the prefreezing dilution rate (expt 2) or when different glycerol concentrations were examined (expt 5).

The semen was frozen in pellet form (0·06–0·07 ml) on dry ice (Nagase and Niwa 1964) and the frozen pellets were transferred into liquid nitrogen and stored for 2–7 days before thawing for examination. The 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 at 37°C for 4 h (0, 2, 4 h; expt 1) or 6 h (0, 2, 4, 6 h; all other experiments). Motility estimates were to the nearest 5%. The assessor did not know the identity of the samples, all of which were presented for evaluation in random order. The tonicity (in kiloPascals) of the diluents (non-glycerolated) in experiment 4 was measured with a Fiske Osmometer (Uxbridge, Mass. U.S.A.) at 273°K.

The experiments were of factorial design and the data, after angular transformation, were examined by analyses of variance for a split-plot experiment, post-thawing incubation being the subplot. Where a significant first-order interaction was revealed between replicates and other factors, the interaction mean square was used to test the relevant main effect.

Experimental Details and Results

Experiment 1

The factors included in the experiment ($3 \times 4 \times 3 \times 3$) were Tris concentration, type and concentration of sugar in the diluent, and three replicates. The semen was diluted 1 : 2 (semen : diluent).

The results are summarized in Table 1. The mean percentages of motile spermatozoa differed for Tris concentration ($P < 0{\cdot}05$) and for type of sugar in the diluent ($P < 0{\cdot}001$). The mean results for Tris–glucose and Tris–fructose were similar, but better than for Tris–lactose or Tris–raffinose extenders. The concentration of Tris and type and concentration of sugar interacted ($P < 0{\cdot}001$). With different concentrations of Tris and of glucose or fructose a higher number of successful combinations were achieved than with Tris–lactose or Tris–raffinose.

The best survival rates within each sugar type were obtained with 450 mM Tris. The most suitable combinations of this Tris concentration were with 41·625 or 83·250 mM of glucose and lactose, and with 83·250 or 124·825 mM concentrations of fructose. Raffinose performed best at 41·625 mM combined with 450 mM Tris.

The analysis of variance detected two further second-order interactions (Tris concentration × sugar type × incubation time, $P < 0{\cdot}01$; Tris concentration × sugar concentration × incubation time, $P < 0{\cdot}001$). Nevertheless, the survival of spermatozoa during the post-thawing incubation period was best maintained when the sugars and the sugar concentrations were combined with 450 mM Tris in the diluent.

There was a significant difference between the three replicates ($P < 0{\cdot}05$).

Experiment 2

The factors involved in this experiment ($3 \times 3 \times 4 \times 3$) were concentrations of Tris and of glucose in the diluent, dilution rate, and three replicates.

The results are presented in Table 2. The mean percentages of motile spermatozoa during post-thawing incubation were affected by glucose concentration ($P < 0{\cdot}001$)

and rate of dilution ($P < 0{\cdot}01$). There was a second-order interaction involving concentration of Tris and glucose, and dilution rate ($P < 0{\cdot}01$). When the semen was diluted three-, four-, or fivefold, increasing concentrations of glucose within each Tris level had a depressing effect on the survival of spermatozoa, and this became more pronounced as the dilution ratio and the Tris concentration increased.

Table 1. Effects of concentration of Tris, sugar and type of sugar in the diluent on the percentage of motile spermatozoa during post-thawing incubation (experiment 1)

The semen was diluted 1 : 2 (semen : diluent)

Type and concentration (mM) of sugar in diluent	Motile spermatozoa (%) for concentrations of Tris in diluent (mM) of: 300	450	600	Mean
Glucose				
41·625	28·7	41·4	25·8	31·7
83·250	33·1	43·8	29·6	35·4
124·825	34·1	34·6	26·2	31·6
Mean	31·9	39·9	27·2	32·9
Fructose				
41·625	33·4	37·5	28·5	33·0
83·250	31·6	40·8	29·9	34·1
124·825	33·0	41·0	20·8	31·5
Mean	32·7	40·0	26·3	32·9
Lactose				
41·625	29·2	38·7	13·6	26·4
83·250	34·5	38·6	25·5	32·7
124·825	37·0	36·7	10·5	26·9
Mean	33·5	38·0	16·1	28·6
Raffinose				
41·625	15·2	36·7	9·7	19·4
83·250	30·8	10·6	6·7	14·7
124·825	24·9	18·6	6·9	16·0
Mean	23·3	21·0	7·7	16·7
Overall mean	30·3	34·4	18·5	

In the case of twofold dilution, increasing concentrations of glucose improved the results when combined with 300 or 450 mM Tris, but the situation was reversed for combinations of 375 mM Tris with increasing concentrations of glucose. The best survival rates (40·4 and 40·8%) were observed after three- and fourfold dilution with the diluent containing 450 mM Tris and 41·625 mM glucose.

The semen of the three replicates differed in resistance to the freeze–thawing procedure ($P < 0{\cdot}001$).

Experiment 3

In this experiment ($4 \times 4 \times 3$) the four Tris concentrations in the diluted semen were constant for all four dilution rates. There were three replicates.

All factors included in the experiment had significant effects ($P < 0{\cdot}001$) on the mean percentage of motile spermatozoa following thawing and incubation. There was an interaction between Tris concentration and dilution rate ($P < 0{\cdot}01$, Table 3).

Table 2. Effects of dilution rate and of concentration of Tris and of glucose in diluent on the percentage of motile spermatozoa during post-thawing incubation (experiment 2)

The diluents used for all rates of dilution contained 9% (v/v) egg yolk and 6% (v/v) glycerol. Concentrations of glucose in diluted semen (mM) given in parentheses for each dilution rate

Dilution rate (semen: diluent)	Tris concn in diluent (mM)	Tris concn in diluted semen (mM)	Motile spermatozoa (%) for glucose concentrations in diluent (mM) of: 41·625	83·250	124·825	Overall mean
			(20·8)	(41·6)	(62·4)	
1 : 1	300	(150)	25·2	29·9	26·8	27·6
	375	(187·5)	33·1	27·4	26·7	
	450	(225)	24·2	26·3	29·4	
			(27·75)	(55·50)	(83·25)	
1 : 2	300	(200)	35·0	35·2	32·4	34·8
	375	(250)	39·1	35·6	30·6	
	450	(300)	40·4	35·1	30·1	
			(31·2)	(62·4)	(93·7)	
1 : 3	300	(225)	38·6	33·9	33·5	35·2
	375	(281·3)	38·7	39·1	31·4	
	450	(337·5)	40·8	39·4	22·6	
			(33·3)	(66·6)	(99·9)	
1 : 4	300	(240)	37·0	35·4	28·3	32·0
	375	(300)	35·2	37·0	22·5	
	450	(360)	39·8	34·7	19·8	
Overall mean			35·5	34·0	27·7	

The optimum concentration of Tris in the diluted semen was 250 mM after two-, three-, and fourfold dilution, but 300 mM Tris was preferred when the semen was diluted fivefold. Concentrations of 300 and 350 mM Tris in the twofold-diluted semen and of 350 mM Tris in the fourfold- and fivefold-diluted semen had depressing effects on the survival of spermatozoa.

The decline in viability of spermatozoa during the 6-h post-thawing incubation was steeper for 350 mM than for lower Tris concentrations in the diluted semen (Tris concentration × incubation time, $P < 0{\cdot}05$). Spermatozoa of the three replicates differed in viability during post-thawing incubation (replicate × incubation time, $P < 0{\cdot}001$).

Experiment 4

In this experiment (4 × 4 × 3) each concentration of Tris in the diluent (300, 375, 450, 525 mM) was combined with a narrower range (0, 20·813, 41·625, 62·438 mM) of glucose than in experiment 2. Three replicates were used. The semen was diluted 1 : 2 (semen : diluent).

Table 4 shows that the survival of spermatozoa during the post-thawing incubation was influenced by both the concentration of Tris ($P < 0{\cdot}001$) and concentration of glucose ($P < 0{\cdot}05$) in the diluent. At the 0 h (immediate post-thawing) assessment, there was no difference between 300, 375 and 450 mM Tris concentrations, although these treatments gave better results than 525 mM Tris. During subsequent incubation,

Table 3. Relationship between dilution rate and Tris concentration in diluted semen on the percentage of motile spermatozoa during post-thawing incubation (experiment 3)

Each concentration combined with 27·75 mM glucose, 6% (v/v) egg yolk, and 4% (v/v) glycerol in the diluted semen

Dilution rate (semen : diluent)	Motile spermatozoa (%) for Tris concentrations in diluted semen (mM) of: 200	250	300	350	Mean
1 : 1	32·4	34·9	26·0	20·1	28·1
1 : 2	33·7	36·6	31·8	31·9	33·5
1 : 3	31·9	34·1	33·5	26·0	31·3
1 : 4	32·3	33·2	35·1	26·3	31·6
Mean	32·6	34·7	31·5	25·9	

however, the superiority of 375 mM Tris became apparent. The viability of spermatozoa during post-thawing incubation was better when the glucose concentration in the diluent was 20·813 or 41·625 mM than when no sugar or 62·438 mM sugar was present. The decline in survival rate of spermatozoa during the post-thawing incubation differed for the three replicates (replicate × incubation time, $P < 0{\cdot}001$).

Table 4. Effects of concentration of Tris and of glucose in diluent on the percentage motile spermatozoa during post-thawing incubation (experiment 4)

The diluent contained 9% (v/v) egg yolk, 6% (v/v) glycerol, and the semen was extended 1 : 2 (semen : diluent)

Tris or glucose concn in diluent (mM)	Range in tonicity (kPa)	Motile spermatozoa (%) for incubation periods (h) of: 0	2	4	6	Mean
Tris 300	687–851	41·6	39·5	35·2	32·2	37·1
375	848–1023	41·6	40·4	39·1	34·0	38·8
450	994–1175	40·4	39·5	32·7	28·1	35·1
525	1177–1329	28·7	26·9	23·5	12·9	22·7
Glucose 0	687–1177	37·8	37·3	30·6	25·3	32·6
20·813	737–1226	36·5	35·6	33·9	28·1	33·5
41·625	789–1270	37·8	38·2	33·8	26·5	34·0
62·438	851–1329	39·9	34·8	31·7	25·3	32·8
Mean		38·0	36·5	32·5	26·3	

The relationship between the mean percentage of motile spermatozoa during the post-thawing incubation time and tonicity of diluents, within the range (687–1329 kPa) examined, was curvilinear (quadratic regression, $P < 0{\cdot}001$; $Y = -33{\cdot}8053 + 0{\cdot}1662X - 0{\cdot}000097X^2$, Y = percentage of motile spermatozoa, X = tonicity in

kiloPascals). The best result was obtained with the diluent that had a tonicity of 948 kPa.

Experiment 5

The factors examined in this experiment ($3 \times 3 \times 3 \times 3$) were the following:

(1) Method of semen dilution:
   (i) one-step: one addition of glycerol-containing diluent at 30°C (1 : 2 final dilution);
   (ii) two-step: addition of non-glycerolated diluent at 30°C (1 : 1 dilution) followed 10 min later by the addition at 30°C of glycerol-containing diluent (1 : 2 final);
   (iii) two-step: addition of non-glycerolated diluent at 30°C (1 : 1) and of glycerol-containing diluent after cooling to 5°C (1 : 2 final).
(2) Time of storage at 5°C after cooling: 0 *v.* 1·5 *v.* 3·0 h
(3) Glycerol concentration in diluted semen: 2·5 *v.* 4·0 *v.* 5·5%(v/v)
(4) Three replicates.

The diluent used consisted of 375 mM Tris, 41·625 mM glucose, 9% (v/v) egg yolk and an appropriate amount of glycerol, depending on the method of dilution.

Table 5. Relationship between method of dilution, storage of diluted semen at 5°C and glycerol concentration on the percentage of motile spermatozoa during post-thawing incubation (experiment 5)

The diluent consisted of 375 mM Tris, 41·625 mM glucose, 9% (v/v) egg yolk, and an appropriate amount of glycerol, depending on the method of dilution

Method of dilution (final rate 1 : 2)	Storage time at 5°C (h)	Motile spermatozoa (%) for glycerol concentration (% v/v) in diluted semen of: 2·5	4·0	5·5	Mean
(i) One-step at 30°C	0	26·3	31·7	26·8	28·3
	1·5	28·7	38·5	26·9	31·4
	3·0	26·2	26·9	19·7	24·3
Mean		28·1	32·4	25·5	28·5
(ii) Two-step at 30°C	0	21·6	35·0	13·2	23·3
	1·5	29·8	37·6	22·2	29·9
	3·0	30·8	30·4	18·3	26·5
Mean		27·4	34·3	17·9	26·6
(iii) Two-step: first at 30°C, second at 5°C	0	25·0	25·1	20·2	23·4
	1·5	30·4	26·1	22·2	26·2
	3·0	33·3	27·3	23·2	27·9
Mean		29·6	26·2	21·9	26·1
Overall mean		28·4	30·7	21·7	

The results are summarized in Table 5. The survival of spermatozoa after thawing varied depending on the method of dilution, time of storage at 5°C, and glycerol concentration in the diluted semen ($P < 0·05$). When the semen was diluted in one step (method i), storage at 5°C for 1·5 h was beneficial, but prolongation of the storage period to 3·0 h had a depressing effect for all glycerol concentrations. After the two-step dilution at 30°C (method ii), the results for 2·5% (v/v) glycerol concen-

tration improved as the storage period increased, but with 4·0 or 5·5% (v/v) glycerol concentrations storage time of only 1·5 h was more beneficial. In the case of two-step dilution with addition of the glycerol-containing diluent at 5°C (method iii), a 3-h storage period was beneficial for all three glycerol concentrations.

There was no benefit in diluting the semen in two steps, and the survival of spermatozoa was best after one-step dilution at 30°C with 1·5-h storage time at 5°C and 4·0% (v/v) glycerol in the diluted semen.

As in the previous experiments, there was a significant difference between the three replicates ($P < 0·001$).

Discussion

In this study the reason for using only bucks whose semen did not exhibit coagulation in the presence of egg yolk was to reduce (or eliminate) the occurrence of this 'complicating' phenomenon during examination of the parameters included in the experiments.

It has been found that, as in the ram (Salamon and Visser 1972) and boar (Visser and Salamon 1974*c*), spermatozoa of the buck can tolerate a relatively wide range of Tris concentration in the freezing diluent. The type of sugar included in the Tris medium had an effect on the survival of spermatozoa, and of the sugars examined glucose or fructose were more suitable components than lactose or raffinose (Table 1). When different concentrations of glucose were examined, it became evident that the amount of sugar required in the Tris medium was relatively small (Table 4). The tonicity of diluents obtained by combinations of Tris and glucose concentrations varied from 687 to 1329 kPa. The tonicity range of 737–1112 kPa was well tolerated by the spermatozoa, but the best survival rate was obtained at 948 kPa with the diluent that consisted of 375 mM Tris, 41·625 mM glucose and 124 mM citric acid. This diluent was hypertonic to buck semen (tonicity of semen 715 kPa).

In the published reports on frozen storage of buck semen generally a fivefold or higher (10- to 20-fold) prefreezing dilution of the whole or washed (seminal plasma removed) semen was adopted. In the present study whole semen was used and a narrow (two- to fivefold) range in dilution ratio was examined, with the aim of obtaining thawed semen of satisfactory concentration of surviving spermatozoa for insemination (at least $0·4 \times 10^9$ motile spermatozoa per millilitre). The survival of spermatozoa after thawing was better for three-, four-, and fivefold than for twofold prefreezing dilution, and this was evident when the diluent components were varied in the semen diluted at various rates (expt 2), or when the diluent components in the diluted semen were held constant (expt 3). It is unlikely that the assessment of motility was affected by the prefreezing dilution rate, as in our preliminary work and also in additional examinations in this study the assessed values for motile spermatozoa were similar for the thawed semen samples adjusted for cell concentration before examination (by extension to fivefold rate) and for non-adjusted samples. The interaction detected between dilution rate and either composition of diluent (Table 2) or diluent components in the diluted semen (Table 3) indicate that care should be taken in selection of a diluting medium suitable for use within a range or for a certain rate of extension.

In the studies of freezing ram semen in raffinose–citrate diluent (Lightfoot and Salamon 1969) the post-thawing survival of spermatozoa progressively improved

with the increase in the prefreezing dilution rate from twofold to 16-fold when the frozen pellets were thawed in dry test tubes. When, however, the semen pellets were thawed in a thawing solution, the survival rates were similar for all prefreezing dilution rates. Subsequently, Visser and Salamon (1973, 1974*a*, 1974*b*) reported that ram semen pellet-frozen in Tris-based diluent can be successfully thawed without a thawing solution. Our further experiments with buck semen (not presented here) also showed that when the semen was diluted at two- to fivefold prefreezing rates, thawing of the frozen semen pellets in a thawing solution—which could increase the velocity of thawing—was not advantageous.

Buck semen has generally been diluted in two steps by addition of the non-glycerol-containing diluent at room temperature or higher, and of the glycerolated medium after cooling at 5°C, followed by equilibration periods of 2–3 h (Lyngset *et al.* 1965; Corteel 1975; Fougner 1979), 5 h (Samouilidis 1970), 6–18 h (Waide *et al.* 1977). Van der Westhuysen (1978) claimed that after one-step dilution of Angora buck semen by a Tris-based diluent, a period of 30 min cooling to 5°C was sufficient, but an equilibration time of 4 h was required at that temperature. In the present study, as in the work with ram semen (Salamon 1968; Lightfoot and Salamon 1969), dilution of Angora buck semen by a single addition of the glycerol-containing diluent at 30°C (one-step) was better than by the two-step method of dilution. Method of dilution, time of holding the diluted and cooled semen at 5°C ('equilibration'), and glycerol concentration interacted. Nevertheless the best post-thawing survival rate of spermatozoa was obtained after one-step dilution, 1·5-h holding time at 5°C, and with 4% (v/v) glycerol concentration in the diluted semen.

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