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THE EFFECT OF PELLET VOLUME, DILUTION RATES PREFREEZING AND AT THAWING, AND OF THAWING TEMPERATURE ON THE SURVIVAL AND ACROSOME-MORPHOLOGY OF FROZEN RAM SPERMATOZOA

D. Visser*

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Dept. Animal Husbandry, University of Sydney, Sydney, N.S.W. 2006, Australia

OPSOMMING: DIE INVLOED VAN KORREL-GROOTTE, GRAAD VAN VERDUNNING VOOR BEVRIESING EN MET ONTDOOIING EN VAN TEMPERATUUR VAN ONTDOOIING OP DIE OORLEWING EN AKROSOOM-MORFOLOGIE VAN BEVRORE RAM SPERME

Die invloed van korrel-grootte, verdunningsgrade en ontdooiingstemperature op die leefbaarheid en akrosoom-morfologie van bevrore ram sperme is ondersoek in drie faktoriaal eksperimente. Beide lae en hoë verdunningsgrade voor bevriesing het 'n nadelige effek op die oorlewing van sperme gehad; optimum resultate is behaal met 3- tot 8-voudige verdunning. Toenemende graad van verdunning met ontdooiing was voordelig na die gebruik van lae grade van verdunning voor bevriesing; die omgekeerde geld egter met hoë verdunning voor bevriesing. Verdunning van 3-voud en hoër was nodig voor bevriesing ten einde bevredigende oorlewing van sperme te verseker na ontdooiing in droë proefbuise. Korrel-grootte, ontdooiingstemperatuur en graad van verdunning met ontdooiing het 'n betekenisvolle effek op die spoed van bevriesing en ontdooiing van semen gehad. Verder het beide hoër ontdooiingstemperature en die gebruik van 'n ontdooiingsmedium ook die oorlewing en akrosoom-status van sperme gunstig beïnvloed tydens die prosedures van bevriesing, ontdooiing en inkubasie.

SUMMARY

Three experiments were conducted to examine the effects of dilution rates prefreezing and at thawing, pellet volume, and of thawing temperature on the survival and acrosome-morphology of ram spermatozoa. Semen was diluted with a Tris-glucose diluent and frozen in pellet form on dry ice. Best results were obtained with 3- to 8-fold dilution prefreezing. Increasing rate of dilution at thawing improved the results when low prefreezing rates were used, but the reverse occurred with high rates of dilution prefreezing. Recovery of spermatozoa after thawing in dry tubes was satisfactory only when the semen was diluted 3-fold or at higher rates before freezing. Post-thawing survival of spermatozoa frozen and thawed in this way also equalled survival of cells thawed and incubated in a solution. In the final experiment, temperature recordings showed that freezing and thawing velocities of pellet-frozen semen were affected significantly by pellet volume, thawing temperature and dilution rate at thawing. The latter two factors also had an influence on the survival and acrosome-morphology of spermatozoa. Both higher thawing temperatures and thawing of the pellets in a solution acted beneficially on the viability and maintenance of acrosome-integrity of the cells following the freeze-thaw-incubation procedures.

The relationships between freezing and thawing velocities and survival of spermatozoa following pellet-freezing have been examined in the bull (Rottensten, 1971; Juščenko, Semakov & Levin, 1972) and ram (Schmidt, 1968; Lightfoot & Salamon, 1969a, b). These reports outlined the important effects of prefreezing and thawing dilution rates and of thawing temperature on the freezing and thawing velocities and subsequent survival of spermatozoa. It is generally reported that a thawing solution acts beneficially on the recovery and survival of spermatozoa. With ram semen, however, the additional dilution at thawing necessitates reconcentration of the thawed semen before insemination in order to obtain satisfactory fertility (Lightfoot & Salamon, 1970a, b; Salamon & Lightfoot, 1970).

The experiments reported here were conducted to examine the effects of pellet volume, prefreezing and thawing dilution rates and of thawing temperature on the survival and acrosome-morphology of spermatozoa frozen in pellet form. In addition, attention has also been given to the development of suitable techniques for thawing of semen in dry tubes in order to avoid excessive dilution and the need for reconcentration of the thawed semen before insemination.

Procedure

Semen was collected from mature Merino rams by artificial vagina (Expts. 1, 2) or electro-ejaculator (Expt. 3) and only samples with good initial motility were used. Aliquots of semen were diluted at 30°C by a single addition of the glycerol-containing diluent.

The prefreezing dilution rate varied in Experiments 1 and 2 and was 1:4 (semen:diluent, v/v) in Experiment 3. When the prefreezing dilution rates varied, the diluent components were adjusted in order to have the same concentration in the diluted semen for every rate of dilution. Thus, in all experiments the diluted semen contained 240 mM Tris-75,8 mM citric acid-22,2 mM glucose-12% (v/v) egg yolk – 4% (v/v) glycerol.

The diluted semen was cooled to 5°C in 2 to $2^1/2$ hours and then pelleted on dry ice. The pellet volume was 0,06 ml in Experiments 1 and 2 and varied in Experiment 3. The semen was kept on dry ice for three minutes, after which the pellets were transferred into liquid nitrogen and stored for 24 to 48 hours before thawing for examination. The pellets were thawed in test tubes containing a thawing solution (300 mM Tris-94,7 mM citric acid-55,5 mM fruc-

* Present address: Dept. Agricultural Technical Services, Windhoek, 9100, South West Africa.

tose) or in dry tubes held in a waterbath at 37°C (Expts. 1, 2). The thawing temperatures varied in Experiment 3 and immediately when the pellets were completely thawed, the tubes were transferred to a waterbath at 37°C for incubation. The dilution rate at thawing varied in all experiments. In Experiment 3 the number of pellets thawed for each pellet volume was predetermined so that equal volumes of thawed semen were incubated for all pellet volumes (e.g. 20 x 0,03 ml = 1 x 0,60 ml).

The percentage of motile spermatozoa was assessed under coverslip on a warmstage (37°C) immediately after thawing and at two hour intervals during subsequent incubation for six hours at 37°C. In Experiment 3 the freezing and thawing velocities were measured with a Chromel-Alumel 2ABI thermocouple (+50 to -200°C) connected to a Phillips recorder. The temperature decrease during freezing was measured in the centre of the pellets. Changes in temperature during thawing were recorded by placing the thermocouple in the thawing solution or on the bottom of the test tube before the pellets were transferred into the tube. During the thawing process the tubes were shaken briskly to ensure adequate contact between the pellets and thermocouple. When more than one pellet had to be thawed (e.g. smaller pellets), they were transferred into the test tubes simultaneously.

In Experiment 3 the spermatozoa were also examined for acrosome-morphology immediately after thawing and after six hours incubation on smears stained and cleared according to Dott & Foster (1972). On each smear 100 cells were examined under a phase-contrast microscope using the yellowish-green part of a continuous interference filter (Leitz Wetzlar). The vials containing the semen for incubation and the smears were coded and presented for examination in random order, so that the observer did not know their identity.

Motility data for each experiment, following angular transformation, were examined by analyses of variance for a split-plot experiment, post-thawing incubation being the sub-plot (Cochran & Cox, 1957). Where significant first-order interaction was revealed between rams and other factors, this interaction mean square was used to test the relevant main effect. When necessary, individual means were compared with Duncan's new multiple-range test (Steel & Torrie, 1960). Data for freezing rates and acrosome-morphology in Experiment 3 were examined by standard analysis of variance. All results are presented as the re-transformed values of the means for the transformed data.

Results

Experiment 1

The effects of different rates of dilution prefreezing and at thawing on the survival of spermatozoa were examined in a 4x4x3 factorial experiment:

1. Prefreezing dilution rate: 1:1 v. 1:3 v. 1:7 v. 1:15 (semen:diluent, v/v)
2. Dilution rate at thawing: 1:0 v. 1:1 v. 1:3 v. 1:7 (pellets: thawing solution, v/v)
3. Rams: pooled ejaculates from each of three rams.

Revival of spermatozoa following freeze-thawing was highest for 1:3 to 1:7 prefreezing dilution rates. Lower and higher rates of dilution resulted in poorer cell survival (prefreezing dilution, quadratic, $P<0,01$, Table 1). Dilution rate at thawing had little effect on the mean percentage of motile spermatozoa after thawing. There was, however, an interaction between prefreezing and thawing dilution rates ($P<0,01$, Table 1). Increasing dilution at thawing improved the results for low (1:1) prefreezing dilution, but the reversed occurred with high rates of dilution prefreezing (1:7, 1:15). Thawing in dry tubes was more successful when the semen was diluted 4- to 16-fold rather than two-fold before freezing. Survival of spermatozoa during incubation was influenced by both prefreezing and thawing dilution rates ($P<0,05$, Table 1). Viability of cells declined steeper with either high prefreezing or low thawing rates, whereas combination of high dilution before freezing and at thawing resulted in low recovery and survival rates. There was a significant difference in the semen of the rams in resistance to the freeze-thawing procedures ($P< 0,01$).

Experiment 2

In this experiment (3x5x3 factorial) a narrower range of prefreezing and thawing dilution rates were examined:

1. Dilution rate prefreezing: 1:2 v. 1:3 v. 1:4 (semen: diluent, v/v)
2. Dilution rate at thawing: 1:0 v. 1:1 v. 1:2 v. 1:3 v. 1:4 (pellets: thawing solution, v/v)
3. Three replicates at dilution from pooled semen of six rams.

The means for increasing dilution rates prefreezing (42,4, 43,7 and 43,4) and at thawing (41,0, 43,9, 44,0, 43,8 and 43,2) did not differ significantly. The only significant interaction detected by the analysis of variance was between dilution rate at thawing and time of incubation ($P< 0,05$, Table 2). Spermatozoa survived better during post-thawing incubation when the semen was thawed in dry tubes or when thawing dilution rates were low (1:1, 1:2) than after the use of high dilution rates at thawing (1:3, 1:4).

Experiment 3

This 4x4x5 factorial experiment examined the effects of pellet volume (0,03 v. 0,15 v. 0,30 v. 0,60 ml), thawing temperature (37 v. 42 v. 47 v. 52°C) and dilution rate at thawing (1:0 v. 1:1 v. 1:2 v. 1:3 v. 1:4, pellets: thawing solution, v/v) on (a) the thawing velocity of semen, (b) the survival of spermatozoa and (c) the acrosome-morphology of the thawed cells. The effect of pellet vo-

Table 1

Experiment 1: Interaction of dilution rate prefreezing and at thawing and time of incubation (Percentage of motile spermatozoa)

Dilution rate at thawing	Time of incubation (hr)	Prefreezing dilution rate				Means
		1:1	1:3	1:7	1:15	
1:0*	0	43,0	51,7	50,0	48,2	48,2
	2	37,9	44,8	46,5	46,5	43,9
	4	36,2	41,3	39,6	41,3	39,6
	6	29,7	37,9	36,2	36,2	35,0
	Means	36,6	43,9	43,0	43,0	41,6
1:1	0	41,3	51,7	50,0	48,2	47,8
	2	39,6	46,5	44,8	46,5	44,3
	4	36,2	39,3	41,3	36,2	38,3
	6	31,3	34,5	34,5	32,9	33,3
	Means	37,1	43,0	42,6	40,9	40,9
1:3	0	51,7	50,0	46,5	41,3	47,4
	2	50,0	43,0	41,3	37,9	43,0
	4	39,6	37,9	39,6	34,5	39,9
	6	34,5	34,5	37,9	32,9	35,0
	Means	43,9	41,3	41,3	36,6	40,8
1:7	0	50,0	46,5	46,5	39,6	45,6
	2	44,8	41,3	43,0	36,2	41,3
	4	41,3	41,3	37,9	34,5	38,7
	6	39,6	39,6	36,2	22,0	34,1
	Means	43,9	42,2	40,9	32,9	39,9
Overall means		40,3	42,6	42,0	38,3	

* Thawing in dry tubes.

Table 2

Experiment 2: Interaction of dilution rate at thawing and time of incubation (Percentage of motile spermatozoa)

Time of incubation (hr)	Dilution rate at thawing					Means
	1:0*	1:1	1:2	1:3	1:4	
0	45,3	48,2	48,8	49,4	47,7	47,9
2	42,5	45,3	45,9	46,5	46,5	45,3
4	38,5	42,5	42,3	41,3	41,9	41,3
6	37,9	39,6	39,0	37,9	36,8	38,2
Means	41,0	43,9	44,0	43,8	43,2	

* Thawing in dry test tubes.

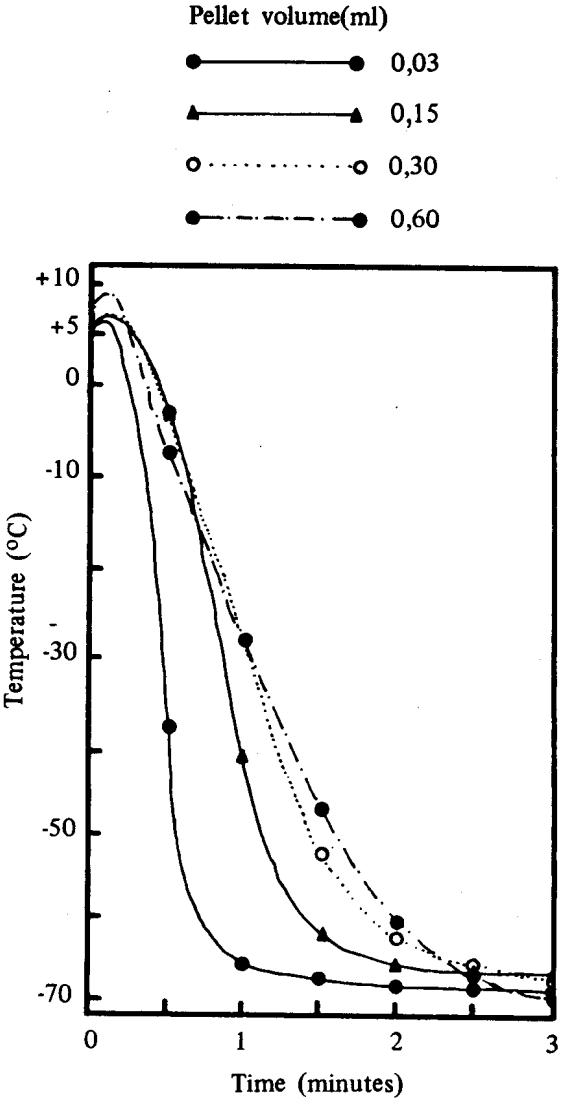

Fig. 1. *Experiment 3: The effect of pellet volume on the freezing rate of pellet-frozen semen*

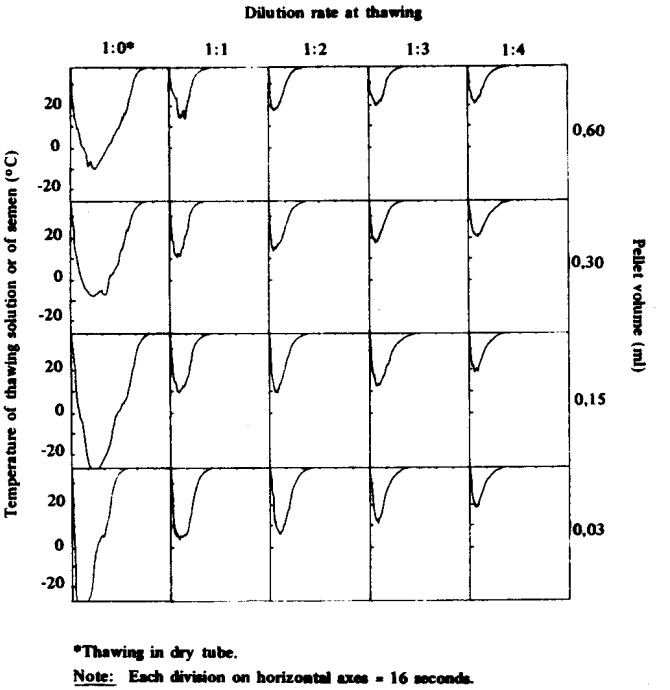

Fig. 2. *Experiment 3: The effect of pellet volume, thawing temperature and dilution rate on the thawing velocity of pellet-frozen semen*
Thawing temperature 37°C

lume on the freezing rate of semen was also determined. Recording for each pellet volume was replicated five times.

(a) **Freezing and thawing rates of semen:**

The freezing curves for different pellet volumes are shown in Fig. 1. An initial small rise in temperature was caused by the transfer of the semen with a pasteur pipette from the waterbath at 5°C to the surface of the dry ice. It follows therefore that the bigger pellets showed the greatest rise in temperature, because of the longer exposure of the cooled pipette to air temperature (± 20°C). The freezing rates (°C/minute) between + 5 and -60°C differed significantly for the pellet volumes used (P<0,001). Comparison of individual means showed that the freezing rate for 0,03 ml pellets (93,1°C/min) was significantly greater than for 0,15, 0,30 and 0,60 ml pellets (48,0, 37,3 and 33,7°C/min respectively), but there was no difference in freezing rate for the larger volumes.

An illustration of the effects of different pellet volumes and thawing dilution rates on thawing velocity is presented in Fig. 2 (thawing temperature 37°C). Two features of these curves are indicative of the thawing velocity, namely the minimum temperature recorded in the thawing process and the total thawing time of the pellets. The differences in thawing velocity for the various treatments were tested by analysis of variance. Tables 3 and 4 show that the thawing velocity was increased by increasing thawing temperature and thawing dilution rate, or by decreasing the pellet volume. The time of thawing for the different pellet volumes was influenced by the dilution rate at thawing (pellet volume x dilution rate at thawing, P<0,001, Table 5). When thawing in dry test tubes, the bigger pellets thawed considerably slower than the smaller ones, but there was little effect of pellet volume when thawing was performed in a solution (dry tube v. solution x 0,03 v. rest, P<0,001; x linear, P<0,001; x quadratic, P<0,05).

(b) **Survival of spermatozoa:**

The survival of spermatozoa after the freeze-thawing procedures was influence by both thawing temperature (P<0,01) and dilution rate at thawing (P<0,001), whereas pellet volume had no significant effect on the viability of the cells (Table 6). There was no significant interaction between any of the factors.

(c) **Acrosome-morphology of the thawed cells:**

The effects of pellet volume, thawing temperature and dilution rate at thawing on acrosome-morphology are summarised in Table 7. Pellet volume and thawing temperature had relatively little influence on the acrosome of the thawed cells. Rate of dilution at thawing and time of incubation, however, had significant effects on the proportion

Table 3

Experiment 3: The effect of pellet volume, thawing temperature and dilution rate on the minimum temperature reached during the thawing process

Pellet volume (ml)	Minimum temperature* (°C)	Thawing temperature (°C)	Minimum temperature* (°C)	Dilution rate at thawing	Minimum temperature* (°C)
0,03	6,6	37	7,0	1:0+	−15,0
0,15	9,4	42	9,3	1:1	10,6
0,30	13,5	47	12,9	1:2	15,1
0,60	14,5	52	14,8	1:3	20,3
				1:4	23,9
P 0,03 v. rest, <0,001 linear, <0,001 quadratic, n.s.			linear, <0,001 residual, n.s.		0 v. rest, <0,001 linear, <0,001 residual, n.s.

* Temperature of thawing solution or of semen.

\+ Thawing in dry test tubes.

Table 4

Experiment 3: The effect of pellet volume, thawing temperature and dilution rate on the thawing time of pellet-frozen semen

Pellet volume (ml)	Thawing time* (sec)	Thawing temperature (°C)	Thawing time* (sec)	Dilution rate at thawing	Thawing time* (sec)
0,03	25,1	37	43,1	1:0+	53,3
0,15	28,1	42	28,8	1:1	23,8
0,30	28,5	47	22,5	1:2	22,3
0,60	31,7	52	18,9	1:3	21,1
				1:4	21,2
P 0,03 v. rest, <0,001 linear, <0,05 quadratic, n.s.			linear, <0,001 quadratic, <0,001 cubic, n.s.		0 v. rest, <0,001 residual, n.s.

* Time required by semen to reach 37°C.

\+ Thawing in dry test tubes.

Table 5

Experiment 3: Interaction of pellet volume and dilution rate at thawing (Thawing time of pellets, seconds)

Pellet volume (ml)	Dilution rate at thawing					Means
	1:0+	1:1	1:2	1:3	1:4	
0,03	36,3	24,0	23,5	21,0	20,5	25,1
0,15	51,8	24,0	22,3	22,0	20,5	28,1
0,30	54,3	22,0	22,3	21,0	22,8	28,5
0,60	71,0	25,0	21,0	20,3	21,0	31,7
Means	53,3	23,8	22,3	21,1	21,2	

+ Thawing in dry test tubes.

Table 6

Experiment 3: The effect of pellet volume, thawing temperature and dilution rate on the viability of spermatozoa during post-thawing incubation

Pellet volume (ml)	Motile sperm (%)	Thawing temperature (°C)	Motile sperm (%)	Dilution rate at thawing	Motile sperm (%)
0,03	40,7	37	40,9	1:0*	49,1
0,15	42,6	42	40,6	1:1	42,9
0,30	42,6	47	43,6	1:2	41,4
0,60	43,2	52	43,9	1:3	39,7
				1:4	38,3
P	n.s.		linear, <0,001 residual, n.s		0 v. rest, <0,001 linear, <0,001 residual, n.s.

* Thawing in dry test tubes.

Table 7

Experiment 3: Interaction of pellet volume, dilution rate at thawing and thawing temperature (Percentage of spermatozoa with normal acrosomes)

Thawing temperature (°C)	Dilution rate at thawing	Pellet volume (ml)				Means
		0,03	0,15	0,30	0,60	
37	1:0*	28,1	28,1	17,2	31,3	26,0
	1:1	26,5	38,7	30,5	21,3	29,1
	1:2	41,3	35,4	32,9	32,1	35,4
	1:3	43,9	41,3	32,9	28,1	36,4
	1:4	37,1	38,7	37,1	36,2	37,3
	Means	35,2	36,4	29,8	29,7	32,7
42	1:0	26,5	21,3	28,1	22,8	24,6
	1:1	19,9	20,6	27,3	27,3	23,7
	1:2	46,5	37,1	41,3	33,7	39,6
	1:3	42,2	32,9	36,2	28,1	34,8
	1:4	35,4	33,7	27,3	35,4	32,9
	Means	33,7	28,9	31,9	29,3	30,9
47	1:0	28,1	32,1	28,1	22,8	26,3
	1:1	33,7	23,5	23,5	29,7	27,5
	1:2	38,7	37,1	22,8	43,0	35,2
	1:3	37,9	33,7	49,1	46,5	41,7
	1:4	31,3	33,7	47,4	36,2	37,1
	Means	33,9	31,9	33,7	34,2	33,4
52	1:0	21,3	38,7	32,9	22,8	28,7
	1:1	30,5	32,9	40,5	33,7	34,3
	1:2	32,9	22,0	35,4	34,5	31,1
	1:3	44,8	43,9	43,9	37,9	42,6
	1:4	37,9	36,2	39,6	35,4	37,3
	Means	33,2	34,5	38,4	32,7	34,7
Overall means		34,0	32,9	33,4	31,5	

* Thawing in dry test tubes.

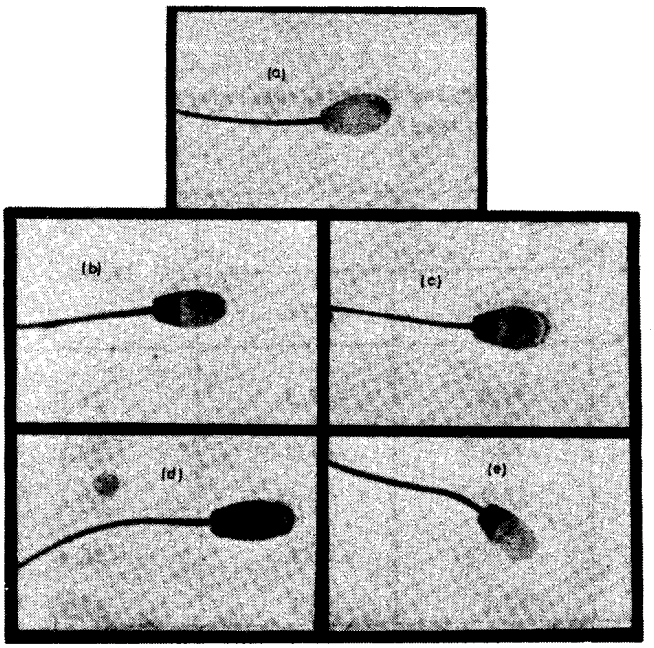

Fig. 3. *Acrosome-morphology of ram spermatozoa after freeze-thawing procedures*
(a) Normal, undamaged (Type I).
(b) Slight damage (Type II).
(c) Medium damage (Type III).
(d) Severe damage (Type IV).
(e) Complete loss of acrosome (Type V).

of cells with normal acrosomes. In general, low dilution rates and six hours post-thawing incubation decreased the proportion of cells with normal or with only slightly damaged acrosomes, and concomittantly increased the incidence of severely damaged cells and of those with loss of acrosome.

Thawing temperature interacted with pellet volume ($P<0,01$) and dilution rate at thawing ($P<0,001$) and these factors were further involved in a second-order interaction ($P<0,001$, Table 7). When thawing at 37 and 42°C small pellets tended to perform better than large ones, whereas larger pellets (0,30 and 0,60 ml) performed better than smaller ones (0,03 and 0,15 ml) at 47 and 52°C. Low dilution rate at thawing (1:0, 1:1) was more harmful to the acrosome than higher rates, especially for larger pellets and when thawing at lower temperatures.

Discussion

Nagase & Niwa (1964) and Juščenko *et al.* (1972) reported that increasing the volume of pellets from 0,013 to 0,20 ml and from 0,1 to 0,5 ml respectively, had no effect on the revival of bull spermatozoa after pellet-freezing. Similar results were reported for ram semen by Lightfoot & Salamon (1969b) when pellet volumes were increased from 0,03 to 0,86 ml. In the present study, pellet volumes of 0,03 to 0,60 ml had no effect on survival and acrosome-morphology of ram spermatozoa frozen by the pellet method on dry ice. Freezing and thawing rates were, however, influenced by the pellet volume; large pellets (0,30 and 0,60 ml) had lower freezing and thawing velocities than small pellets (0,03 and 0,15 ml). The freezing curves and rates of cooling for different pellet volumes were similar to those reported by Lightfoot & Salamon (1969b) and Nagase (1972). The findings of these authors were also confirmed that no rebound-phenomenon occurred during the freezing of the pellets, contrary to results of Schmidt (1968) who observed a slight rise in temperature between 0 and -3°C.

Velocity of thawing, measured either in the pellets or in the thawing solution, is reported to be dependent on pellet volume and thawing temperature (Schmidt, 1968; Juščenko *et al.*, 1972). Smaller pellets and higher thawing temperatures increased the velocity of thawing. The results presented here are in agreement with these findings. In addition it was found that pellet volume, thawing temperature and dilution rate at thawing acted additively in their effect on thawing velocity: Increasing any one of these resulted in faster thawing, while a combined increase in two or three of the factors further increased the thawing velocity. It is also noteworthy that the dilution rate at thawing had an influence on the minimum temperature reached inside the test tubes during thawing. These temperatures dropped below 0°C only when the pellets were thawed in dry tubes (Table 3). The crystallization and recrystallization zones (0 to -15°C and below -15°C respectively; Behrman, 1971) present a harmful environment to cells during the thawing process and this could explain the greater membrane damage observed after "dry" thawing in Experiment 3.

Lightfoot & Salamon (1969a) investigated the effects of freezing and thawing dilution rates on survival of ram spermatozoa pellet-frozen in a raffinose-citrate diluent. They found that increasing prefreezing dilution required lower dilution rates at thawing for optimal revival of cells. Lightfoot & Salamon (1969b) also indicated the importance of a thawing solution in providing a suitable milieu for the recovery and post-thawing survival of spermatozoa. The results presented here substantiated these findings and also confirmed results with Tris-based media reported by Salamon & Visser (1972). An important feature of this study was the observation that ram spermatozoa pellet-frozen in Tris-diluent can be thawed successfully in dry tubes, provided that the prefreezing dilution rate was between 4- and 8-fold. Both the recovery and survival rates of cells treated in this way were comparable to results obtained after thawing in a solution. Motility of spermatozoa was, however, not correlated with their acrosome-morphology (Experiment 3, Tables 6 and 7). Substantially more acrosome-damage occurred after thawing in dry tubes than when the semen was thawed and incubated in a solution. The successive stages of acrosome-damage (Fig. 3) observed in this study were similar to those reported by Healy (1969), Nath (1972) and Watson & Martin (1972). The proportion of cells with normal appearing acrosomes after freeze-thawing was, however, higher than the values reported by these workers and by Varnavskiĭ (1970) and Smorag (1971).

This discrepancy can most probably be accounted for by the more optimal conditions of freezing and thawing employed in the experiments reported here.

In conclusion, the results of this study showed that ram semen can be frozen successfully in pellet volumes up to 0,60 ml and that after adequate prefreezing dilution, can be thawed in dry tubes without serious detrimental effect on cell recovery and survival. The harmful effect of "dry" thawing on acrosome-morphology of the spermatozoa should, however, be borne in mind when evaluating frozen-thawed semen which is to be used for insemination. Loss of acrosome-integrity can be assumed to have a depressing effect on the fertility of frozen-thawed cells and adjustment of inseminate volumes will be necessary to compensate for this loss of intact cells.

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