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THE EFFECT OF FREEZING METHOD ON THE SURVIVAL OF RAM SPERMATOZOA

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OPSOMMING: DIE INVLOED VAN BEVRIESINGSMETODE OP DIE OORLEWING VAN RAM SPERME

Die oorlewing van ram sperme na verskillende bevriesingsmetodes is ondersoek in vyf faktoriaal eksperimente. Semen is bevries in die vorm van korrels ("pellets") of in strooitjies, beide op droë ys en in vloeibare stikstof. Korrelbevriesing op droë ys het beter resultate gelewer as enige ander metode. Oorlewingsresultate was swak met korrelbevriesing direk in vloeibare stikstof, veral as toegelaat is dat die korrels sink in dié medium Die leefbaarheid van sperme was egter aansienlik hoër as die korrels na 5 sekondes oorgeplaas is na droë proefbuise in stikstofdamp. Met hierdie metode lewer 2% gliserol (in die 1:4 verdunde semen) beter resultate as 8% gliserol. Oorlewing van sperme was betreklik onbevredigend na bevriesing in strooitjies op droë ys, terwyl beter resultate behaal is met bevriesing in stikstofdamp by -80 of -160°C. Tris-glukose was 'n beter verdunningsmedium as raffinose-sitraat en die resultate was veral onbevredigend na verdunning met laasgenoemde medium sonder die byvoeging van Tris-fruktose na ontdooiing. Die beste alternatiewe metode van bevriesing in vergelyking met korrelbevriesing op droë ys, was die gebruik van strooitjies in stikstofdamp. Bevredigende resultate is behaal na verdunning (1:4) en bevriesing in Tris (300 mM)-sitroensuur (94,7 mM)-glukose (27,75 mM)-eiergeel (15%, v/v)-gliserol (5%, v/v), met of sonder die byvoeging van Tris-fruktose by die ontdooide semen.

SUMMARY

Five factorial experiments were conducted to examine the effects of different freezing methods on the survival of ram spermatozoa following the freeze-thawing procedures Semen was frozen in pellet form and in straws on either dry ice or in liquid nitrogen. Freezing of semen in pellet form on dry ice gave better results than pelleting in liquid nitrogen or freezing in straws. Recovery of spermatozoa was poor when the diluted, cooled semen was dropped directly into liquid nitrogen and the pellets allowed to sink in the freezing fluid. Cell survival was substantially improved by allowing the semen droplets to float for only five seconds in the nitrogen before transferring into dry tubes held in liquid nitrogen vapour. Best results with this method were obtained when the diluted semen contained only 2% glycerol. Increasing the glycerol concentration to 8% had a deleterious effect on cell survival, particularly when freezing in liquid nitrogen with complete sinking of the pellets. Freezing ram semen in straws on dry ice gave poor results, but recovery and survival of spermatozoa was improved by freezing the straws in liquid nitrogen vapour at -80 or -160°C Tris-glucose was a more suitable freezing medium than raffinose-citrate and results were particularly low when the semen frozen in the latter diluent was thawed in dry tubes and a solution was not added prior to incubation. The best alternative procedure to pellet-freezing on dry ice, was to freeze the semen in straws in liquid nitrogen vapour. When applying this method recovery and survival of cells was satisfactory following dilution (1:4) and freezing in Tris (300 mM)-citric acid (94,7 mM)-glucose (27,75 mM)-egg yolk (15%, v/v)-glycerol (5%, v/v) diluent, with or without addition of Tris-fructose solution after thawing.

The freezing of semen in pellet form usually involves two freezing agents. The pellets are first frozen on dry ice and then transferred to liquid nitrogen for storage. Attempts to overcome the disadvantage of using two freezing agents have been reported for semen of the bull (Goossens & Kamps, 1966; Seifert & Beller, 1966; Uwland, Pool & Baan, 1967; Juščenko, Semakov & Levin, 1968; Korotkov, 1968; Nagase & Tomizuka, 1968) and of the ram (Salamon, 1968, 1970). These workers have frozen the semen by pelleting directly into liquid nitrogen or on various surfaces cooled in liquid nitrogen. The investigations by Salamon using ram semen met with little success, but further preliminary trials in this laboratory showed some encouraging results. Consequently, a series of experiments were designed to investigate more fully the possibility of using only liquid nitrogen as freezing agent for freezing ram semen in pellet form and in straws.

Procedure

Semen was collected from mature Merino rams by artificial vagina. Aliquots of semen were diluted at 30°C by a single addition of the glycerol-containing diluent. The pre-freezing dilution rate was varied in Experiment 1 and was 1:4 (semen: diluent, v/v) in all other experiments. Raffinose-citrate and Tris-glucose were used as freezing diluents and the concentration of the components were adjusted for the different dilution ratios in Experiment 1. Thus, in all experiments the diluted semen contained 12% (v/v) egg yolk and either 133,2 mM raffinose – 54,4 mM sodium citrate or 240 mM Tris-75,8 mM citric acid-22,2 mM glucose. The glycerol concentration in the diluted semen was 4% (v/v) in Experiments 1, 4 and 5, while different concentrations were examined in Experiments 2 and 3.

The diluted semen was cooled to 5°C in 2 to $2\frac{1}{2}$

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hours and then frozen as pellets or in straws. The pellets were frozen either on dry ice (CO_2) or in liquid nitrogen (N_2). In the former case the pellet volumes were 0,03 ml in Experiments 1, 2 and 3 and 0,06 ml in Experiments 4 and 5. When pelleting directly into liquid nitrogen, semen droplets of 0,03 ml were pipetted into the compartments of a wire-mesh basket held in the freezing medium. The pellets were kept in the nitrogen for 5, 10 or 20 seconds (complete immersion) and then quickly transferred into dry test tubes held in liquid nitrogen vapour at -150°C. After 15 to 20 minutes the tubes containing the pellets were completely immersed in the liquid nitrogen and stored for 24 to 48 hours before thawing for examination. The "medium" straws containing 0,25 ml semen were frozen by placing horizontally either on dry ice or in liquid nitrogen vapour at -80°C (Expts. 4, 5) or -160°C (Expt. 5). After 10 to 15 minutes the straws were transferred into liquid nitrogen and stored until thawing and examination.

The frozen pellets were thawed either in dry test tubes (Expts. 4, 5) or in tubes containing a thawing solution (Expt. 1, 2, 3) and held in a waterbath at 37°C (thawing dilution 1:3, pellets: thawing solution, v/v). The semen frozen in straws was thawed by plunging the straws in a waterbath at 37°C. The thawed semen was then pressed out from the straws into dry tubes for incubation with or without addition of a solution (dilution rate 1:3, semen: solution, v/v). The composition of the solution added to the thawed semen (for both pellet- and straw-freezing) was Tris (300 mM)-citric acid (94,7 mM)-fructose (55,5 mM). In addition to this solution inositol (210 mM)-sodium citrate (40 mM) was also used in Experiment 2.

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.

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). All results are presented as the re-transformed values of the means for the transformed data.

Results

Experiment 1

This experiment was of 4x2x3x3 factorial design, incorporating method of freezing, diluent, pre-freezing dilution rate, and rams. The results are presented in Table 1. The mean percentage of motile spermatozoa was higher after pelleting on dry ice than into liquid nitrogen (on dry ice v. liquid nitrogen, $P<0,001$). Floating of the pellets on the surface of liquid nitrogen for 5 seconds resulted in better cell survival than floating for 10 seconds ($P<0,05$), and both these methods were superior to submersion of the pellets into the freezing medium (floating for 5 and 10 seconds v. 20 seconds, submersion, $P<0,001$).

Table 1

Experiment 1: Interaction of diluent with method of pelleting and dilution rate (Mean percentage of motile spermatozoa during post-thawing incubation)

Freezing diluent	Method of pelleting				Dilution rate			Means
	On dry ice	Into N_2 and floating for:						
		5 secs	10 secs	20 secs*	1:1,5	1:4	1:9	
Raffinose-citrate	26,2	20,3	19,9	16,2	18,2	22,6	20,9	20,5
Tris-glucose	29,0	22,8	18,7	14,5	25,6	18,4	19,2	21,0
Means	27,6	21,6	19,3	15,3	21,8	20,5	20,1	

* Submersion in liquid nitrogen (N_2).

There was an interaction between type of freezing diluent and method of pelleting (P < 0,05, Table 1). Tris-glucose performed better than raffinose-citrate after pelleting on dry ice or into liquid nitrogen and subsequent floating of the pellets for five seconds, while the reverse occurred when the pellets were allowed to float for 10 seconds or to submerge in the freezing medium. The diluting media also performed differently depending on the pre-freezing dilution rate (diluent x dilution rate, P<0,001, Table 1). While increasing rate of dilution had a depressing effect for Tris-glucose, it was beneficial when raffinose-citrate diluent was used. The best results were obtained by using Tris-glucose and a pre-freezing dilution ratio of 1:1,5. The semen of the three rams differed significantly in resistance to the freeze-thawing procedures (P<0,001).

Experiment 2

The factors included in this experiment (3x3x2x3 factorial) were method of pelleting, glycerol concentration, thawing solution, and rams. The semen was frozen in Tris-glucose diluent. The results are presented in Table 2. The percentage of motile spermatozoa was significantly higher after pelleting on dry ice than in liquid nitrogen. Allowing the semen pellets to float for 10 seconds on the surface of the nitrogen resulted in the poorest cell survival. Glycerol concentrations of 2 and 4% yielded similar results which were higher than for 8% glycerol in the diluted semen. The survival rates were similar after thawing in Tris-fructose or inositol-citrate solution.

The only significant interaction was between method of pelleting and glycerol concentration (P< 0,05). Semen frozen on dry ice gave the best results with 4% glycerol. There was little difference in cell survival for 2 and 4% glycerol when the semen pellets were kept in liquid nitrogen for five seconds, but 2% glycerol was the most suitable concentration for 10 seconds holding time in liquid nitrogen. This result was confirmed in Experiment 3 (Table 3).

Experiment 3

In this experiment (3x4x3 factorial) three pelleting methods and four glycerol concentrations were examined using semen from three individual rams, diluted and frozen in Tris-glucose. The analysis of variance detected an interaction between pelleting method and glycerol concentration (P<0,05, Table 3). Pelleting on dry ice yielded the best results with 4% glycerol, which is similar to findings in Experiment 2. When the semen was pelleted into nitrogen and the pellets floated for five or 10 seconds, glycerol at 2% was slightly better than at 4% concentration. Absence of glycerol or its presence at 8% yielded poorer survival of spermatozoa than 2 or 4% glycerol, particularly when the semen was pelleted into nitrogen and the pellets were floating for 10 seconds in the freezing medium.

There was a steeper decline in the survival of spermatozoa during incubation when the diluted semen contained no or 8% glycerol (glycerol concentration x time of incubation, P< 0,01) and when the semen pellets were floating in the liquid nitrogen for 10 seconds (pelleting method x time of incubation, P<0,01).

Table 2

Experiment 2: Survival of spermatozoa after pelleting on dry ice and into liquid nitrogen (Mean effects)

Pelleting method	Motile sperm (%)	Glycerol concen.* (%)	Motile sperm (%)	Thawing solution	Motile sperm (%)	Rams	Motile sperm (%)	Time of incubation (hr)	Motile sperm (%)
On dry ice	40,3	2	35,3	Inositol–	29,4	1	28,1	0	36,8
Into N_2 and		4	34,0	citrate		2	33,1	2	32,9
floating for:		8	22,8	Tris-fruc-	31,6	3	30,5	4	28,1
5 secs	32,9			tose				6	24,7
10 secs	19,5								
P:	<0,001		<0,001		n.s.		<0,05		<0,001

* Concentration in 1:4 diluted semen.

Table 3

Experiment 3: Interaction of method of pelleting and glycerol concentration (Mean percentage of motile spermatozoa during post-thawing incubation)

Freezing method	Glycerol concentration (%) *				Means
	0	2	4	8	
On dry ice	32,1	42,2	47,0	42,4	40,8
Into N_2 and floating for: 5 seconds	24,9	43,0	42,6	31,3	35,3
10 seconds	17,4	33,7	31,3	10,0	22,3
Means	24,5	39,6	40,2	26,5	

* Concentration in 1:4 diluted semen.

Experiment 4

The suitability of straws for freezing ram semen was investigated in a 2x3x2x6 factorial experiment, incorporating diluent, freezing and incubation method and rams. All factors, except rams, had significant effects on the viability of spermatozoa after freeze-thawing. Tris-glucose was a more suitable diluent than raffinose-citrate. The results with the latter medium were particularly low when the thawed semen was incubated without addition of a solution after thawing (diluent x incubation method, $P<0,001$, Table 4).

Survival of spermatozoa during post-thawing incubation was influenced by both freezing and incubation methods and these factors were furthermore involved in a second-order interaction (freezing method x incubation method x time of incubation, $P<0,05$, Table 5). Post-thawing viability of spermatozoa declined steeper when frozen in straws on dry ice than in pellet form or in straws in liquid nitrogen vapour. The addition of a solution immediately after thawing improved the motility of spermatozoa with all three freezing methods. When, however, the semen frozen in pellet form was thawed and incubated with a solution, the post-thawing survival of the cells was poorer than

Table 4

Experiment 4: Interaction of freezing diluent and method of incubation (Mean percentage of motile spermatozoa during post-thawing incubation)

Method of incubation	Freezing diluent		Means
	Tris-glucose	Raffinose-citrate	
Solution*	40,3	34,8	37,6
No solution	35,7	22,7	29,0
Means	38,0	28,6	

* Tris-fructose solution added to the thawed semen.

Table 5

Experiment 4: Interaction of freezing method with method and time of incubation (Percentage of motile spermatozoa)

Time of incubation (hr)	Incubation method	Freezing method			Means
		Pellets on dry ice	Straws on dry ice	Straws in N_2 vapour*	
0	Solution+	53,5	38,7	40,5	44,2
	No solution	41,3	29,9	31,3	34,1
	Means	47,4	34,3	35,8	39,1
2	Solution	47,4	32,1	37,1	38,7
	No solution	38,7	27,2	29,7	31,8
	Means	43,0	29,6	33,3	35,2
4	Solution	44,8	28,9	33,3	35,5
	No solution	36,6	21,0	24,2	27,0
	Means	40,7	24,8	28,7	31,2
6	Solution	40,0	25,9	30,5	32,0
	No solution	32,9	17,0	21,6	23,5
	Means	36,4	21,3	25,9	27,6
Overall means		41,8	27,3	30,8	

* Straws frozen in liquid nitrogen vapour at -80°C.

\+ Tris-fructose solution added to the thawed semen.

after incubation with no added solution (pellets v. straws x solution v. no solution x linear, P<0,01.)

Experiment 5

The experiment was of 2x3x2x6 factorial design and examined freezing diluent, method of freezing and post-thawing incubation and rams. The straws were frozen in liquid nitrogen vapour at either -80 or -160°C. Spermatozoa frozen in pellet form survived the freeze-thawing procedures better than when frozen in straws (P<0,001). There was no difference between the two methods of freezing in straws. The mean percentages of motile spermatozoa following freezing by the three methods were 43,5 39,5 and 40,2 respectively.

Freezing in Tris-glucose was more successful than in raffinose-citrate diluent (P<0,001). Type of diluent further interacted with method (P<0,001) and time of incubation (P<0,01). Table 6 shows that survival of spermatozoa was similar for the two diluents when the thawed semen was incubated with a Tris-fructose solution; cell survival, however, was poorer when the semen frozen in raffinose-citrate medium was incubated without addition of the solution after thawing. Post-thawing survival of cells also declined steeper for raffinose-citrate than for Tris-glucose diluent.

Discussion

Semen of the bull has been pelleted successfully on surfaces cooled in liquid nitrogen. These included aluminium foil (Goossens & Kamps, 1966), steel grids (Seifert & Beller, 1967; Uwland *et al.*, 1967) or polymer plates (Juščenko *et al.*, 1968; Korotkov, 1968). The authors reported satisfactory survival and/or fertility for bull semen frozen by these methods. Nagase & Tomizuka (1968) reported moderate revival rates after pelleting bull semen directly into liquid nitrogen. Reports on freezing of ram spermatozoa on surfaces cooled in liquid nitrogen seem to be limited to that of Salamon (1970), who obtained satisfactory cell survival when pelleting on a stainless steel plate cooled to -100 to -140°C. Pelleting ram semen directly into liquid nitrogen resulted in very poor recovery rates of spermatozoa (Salamon, 1968, 1970).

In the present study survival of spermatozoa was best when the semen was frozen by pelleting on dry ice. Pelleting

Table 5

Experiment 4: Interaction of freezing method with method and time of incubation (Percentage of motile spermatozoa)

Time of incubation (hr)	Incubation method	Freezing method			Means
		Pellets on dry ice	Straws on dry ice	Straws in N_2 vapour*	
0	Solution+	53,5	38,7	40,5	44,2
	No solution	41,3	29,9	31,3	34,1
	Means	47,4	34,3	35,8	39,1
2	Solution	47,4	32,1	37,1	38,7
	No solution	38,7	27,2	29,7	31,8
	Means	43,0	29,6	33,3	35,2
4	Solution	44,8	28,9	33,3	35,5
	No solution	36,6	21,0	24,2	27,0
	Means	40,7	24,8	28,7	31,2
6	Solution	40,0	25,9	30,5	32,0
	No solution	32,9	17,0	21,6	23,5
	Means	36,4	21,3	25,9	27,6
Overall means		41,8	27,3	30,8	

* Straws frozen in liquid nitrogen vapour at -80°C.

\+ Tris-fructose solution added to the thawed semen.

after incubation with no added solution (pellets v. straws x solution v. no solution x linear, P<0,01.)

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The experiment was of 2x3x2x6 factorial design and examined freezing diluent, method of freezing and post-thawing incubation and rams. The straws were frozen in liquid nitrogen vapour at either -80 or -160°C. Spermatozoa frozen in pellet form survived the freeze-thawing procedures better than when frozen in straws (P<0,001). There was no difference between the two methods of freezing in straws. The mean percentages of motile spermatozoa following freezing by the three methods were 43,5 39,5 and 40,2 respectively.

Freezing in Tris-glucose was more successful than in raffinose-citrate diluent (P<0,001). Type of diluent further interacted with method (P<0,001) and time of incubation (P<0,01). Table 6 shows that survival of spermatozoa was similar for the two diluents when the thawed semen was incubated with a Tris-fructose solution; cell survival, however, was poorer when the semen frozen in raffinose-citrate medium was incubated without addition of the solution after thawing. Post-thawing survival of cells also declined steeper for raffinose-citrate than for Tris-glucose diluent.

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Semen of the bull has been pelleted successfully on surfaces cooled in liquid nitrogen. These included aluminium foil (Goossens & Kamps, 1966), steel grids (Seifert & Beller, 1967; Uwland *et al.*, 1967) or polymer plates (Juščenko *et al.*, 1968; Korotkov, 1968). The authors reported satisfactory survival and/or fertility for bull semen frozen by these methods. Nagase & Tomizuka (1968) reported moderate revival rates after pelleting bull semen directly into liquid nitrogen. Reports on freezing of ram spermatozoa on surfaces cooled in liquid nitrogen seem to be limited to that of Salamon (1970), who obtained satisfactory cell survival when pelleting on a stainless steel plate cooled to -100 to -140°C. Pelleting ram semen directly into liquid nitrogen resulted in very poor recovery rates of spermatozoa (Salamon, 1968, 1970).

In the present study survival of spermatozoa was best when the semen was frozen by pelleting on dry ice. Pelleting

Table 6

Experiment 5: Interaction of type of freezing diluent with method and time of incubation (Percentage of motile spermatozoa)

Freezing diluent	Incubation method		Time of incubation (hr)				Means
	No solution	Solution*	0	2	4	6	
Raffinose-citrate	31,3	44,8	43,5	40,7	37,3	30,3	37,9
Tris-glucose	43,3	45,3	49,0	46,1	43,5	38,6	44,3
Means	37,2	45,0	46,2	43,4	40,4	34,4	

* Tris-fructose solution added to the thawed semen.

into liquid nitrogen with complete submersion of the pellets was detrimental to cell recovery rates. When, however, the pellets were transferred to dry, cooled tubes after five seconds floating in the freezing medium, the results were substantially improved. An immediate explanation for this cannot be offered. It was not possible to measure the temperature of the pellets at the moment of transfer, which could have given some information on whether a "critical" temperature was involved in the freezing process.

The findings in Experiments 2 and 3 indicate that ram spermatozoa survived the freeze-thawing procedures satisfactorily when a low concentration of glycerol was used in the diluent (2,5%,v/v). A high percentage of motile cells was also recovered after freezing in diluent containing no glycerol (Expt. 3: pelleting on dry ice or in liquid nitrogen with five seconds floating). The interaction between glycerol concentration and method of freezing (Table 3, Expt. 3) showed, however, that more studies are necessary in order to develop suitable diluents for different methods of freezing of ram semen. Nagase & Tomizuka (1968) also showed that recovery of bull spermatozoa after pelleting into liquid nitrogen was higher when inositol rather than sorbitol, xylitol, adonitol or erythritol was included as cryoprotectant in the freezing diluent. Cell recovery was very poor when glycerol or ethylene glycol were used in media with this method of rapid freezing, even though these two agents were found to be suitable for slow freezing (in glass tubes, 5 to 7°C/minute) or pellet-freezing methods (on dry ice).

Bull semen frozen in synthetic straws is used routinely for artificial insemination since the development of this technique by Cassou (1964). Salamon (1967) and Loginova & Zeltobrjuh (1968) found no difference in the fertility of ram spermatozoa frozen in straws, ampoules or in pellet form, but the overall lambing rates in both studies were low. Salamon (1968) reported more satisfactory revival rates for spermatozoa frozen in straws and also found liquid nitrogen vapour superior to dry ice as freezing agent. Subsequent reports (Lunca, 1968; Colas & Brice, 1970; Samouilidis, 1970; Colas, Brice, Courot & Cottier, 1971; Colas, 1972; Andersen & Aamdal, 1972; Linge, 1972; Andersen, Aamdal & Fougner, 1973) indicated that ram semen can be frozen successfully in straws, and fertility results were in general similar to that obtained with semen frozen in ampoules or in pellet form.

The results of this study showed that revival of ram spermatozoa following freezing in straws was only slightly lower than after pelleting on dry ice (Expt. 5). Freezing the straws on dry ice was, however, less successful, similar to findings of Salamon (1968). It is also note-worthy that addition of an "incubating" solution to the semen thawed in dry test tubes had a beneficial effect on the survival of the cells (Expts. 4, 5). This effect was evident for both pellet- and straw-freezing methods. A thawing solution is generally reported to have a beneficial effect on the recovery and post-thawing survival of ram spermatozoa (Lightfoot & Salamon, 1969; Salamon & Brandon, 1971; Salamon & Visser, 1972). The beneficial effect of the solution added after thawing cannot be attributed to a change in thawing velocity, since it was only added after thawing was completed. It can, nevertheless, be assumed that the "incubating" medium acted similar to a thawing solution in providing a more suitable milieu for the maintenance of viability of the cells during post-thawing incubation. It should also be mentioned that redilution of the thawed semen and subsequent ease of microscopical estimates of the motility of cells, could have contributed to the better survival rates observed when an "incubating" medium was used.

The experiments reported here showed that ram spermatozoa can be frozen successfully by methods involving only one freezing agent (liquid nitrogen). Pelleting directly into the nitrogen was, however, more tedious than pelleting on dry ice, and the former method appears to hold only limited promise for practical application. Results following straw-freezing were more encouraging and this technique offers a suitable alternative to pellet-freezing on dry ice. There is, nevertheless, a need for further research to

improve results with this method, especially on diluent composition and thaw-incubation procedures.

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References

ANDERSEN, K. & AAMDAL, J., 1972. Artificial insemination with frozen semen in sheep in Norway. *Wld Rev. Anim. Prod.* 8, 77.

ANDERSEN, K., AAMDAL, J. & FOUGNER, J.A., 1973. Intra-uterine and deep cervical insemination with frozen semen in sheep. *Zuchthyg.* 8, 113.

CASSOU, R., 1964. La méthode des paillettes en plastique adaptée á la generalisation de la congelation. *Proc. Vth int. Congr. Anim. Reprod., Trento.* 4, 540.

COCHRAN, W.G. & COX, G.M., 1957. *Experimental designs.* 2nd edn. New York: John Wiley & Sons, Inc.

COLAS, G., 1972. Ewe fertility following insemination with liquid or deep frozen semen in the course of oestrusses induced by progestins during the breeding season. *Proc. VIIth int. Congr. Anim. Reprod., München.* 2, 925.

COLAS, G. & BRICE, G., 1970. Fertility of fluorogestone acetate-treated ewes inseminated with frozen semen: Preliminary results. *Annls. Zootech.* 19, 353.

COLAS, G., BRICE, G., COUROT, M. & COTTIER, M., 1971. Artificial insemination in the plan of intensification of ovine production: Present state and prospectives. *Bull. Tech. Inform.* No. 257, 1.

GOOSSENS, J.M.M. & KAMPS, W.J.G., 1966. The deep freezing of bovine semen in pellet form. *Tijdschr. Dieregeneesk.* 91, 273.

JUSCENKO, N.P., SEMAKOV, V.G. & LEVIN, K.L., 1968. Pellet freezing of bull semen without dry ice. *Moloch. myas. Skotov., Mosk.* 13(5), 36.

KOROTKOV, A.I., 1968. The pelleting of bull semen in liquid nitrogen. *Vest. Sel'.-khoz. Nauki, Mosk.* 13(8), 67. *(Anim. Breed. Abstr.* 37, 386).

LIGHTFOOT, R.J. & SALAMON, S., 1969. Freezing ram spermatozoa by the pellet method. III. The effects of pellet volume, composition of the thawing solution, and reconcentration of the thawed semen on survival of spermatozoa. *Aust. J. biol. Sci.* 22, 1561.

LINGE, F., 1972. Field trials with frozen semen. Fårskötsel 52, 12. *(Anim. Breed. Abstr.* 41, 1679).

LOGINOVA, N.V. & ZELTOBRJUH, N.A., 1968. Evaluation of various methods of freezing semen. *Ovtsevodstvo, Mosk.* 14(9), 22. *(Anim. Breed. Abstr.* 37, 582).

LUNCA, N., 1968. Some aspects of freezing ram semen. *Proc. VIth int. Congr. Anim. Reprod., Paris.* 2, 1615.

NAGASE, H. & TOMIZUKA, T., 1968. Protective effects of polyols against freezing injury of bull spermatozoa. II. Polyols effects in different freezing methods. *Proc. VIth int. Congr. Anim. Reprod., Paris.* 2, 1107.

SALAMON, S., 1967. Observations on fertility of ram semen frozen by different methods. *Aust. J. exp. Agric. Anim. Husb.* 7, 559.

SALAMON, S., 1968. Deep freezing of ram semen: Recovery of spermatozoa after pelleting and comparison with other methods. *Aust. J. biol. Sci.* 21, 351.

SALAMON, S., 1970. The survival of ram spermatozoa following pellet freezing below-79°C. *Aust. J. biol. Sci.* 23, 459.

SALAMON, S. & BRANDON, M.R., 1971. Effect of composition of thawing solution on survival of ram spermatozoa frozen by the pellet method. *Aust. J. biol. Sci.* 24, 355.

SALAMON, S. & VISSER, D., 1972. Effect of composition of Tris-based diluent and of thawing solution on survival of ram spermatozoa frozen by the pellet method. *Aust. J. biol. Sci.* 25, 605.

SAMOUILIDIS, S., 1970. *Ein Beitrag zur Tiefkühlkonservierung von Schaf-und Ziegenbocksamen mit Hilfe des Pellet-bzw. Paillette-verfahrens.* Vet.-med. Diss., Tierärztl. Fak., Ludwig-Maximillians Univ., München. *(Anim. Breed. Abstr.* 39, 1408).

SEIFERT, H. & BELLER, K.A., 1966. Pelleting with liquid nitrogen. A new and inexpensive method of freezing and storing large quantities of bull semen. *Proc. Vth. Pan.-Am. Congr. Vet. med. Zootech., Venezuela.* 1, 433.

STEEL, R.G.D. & TORRIE, J.H., 1960. *Principles and Procedures of Statistics, with special Reference to biological Sciences.* New York, Toronto & London: McGraw-Hill Book Co., Inc.

UWLAND, J., POOL, H. v.d. & BAAN, A., 1967. A new method for deep freezing cattle semen in pellets. *Tijdschr. Dieregeneesk.* 92, 1177.