Artificial Insemination...Deep Freezing Bull, Goat and Stallion Semen in Concentrated Pellet Form

H. NAGASE

Chief, 3rd Laboratory of Animal Reproduction, Animal Reproduction Division, National Institute of Animal Industry

A technique of freezing semen in concentrated pellet form using sugar solutions as a freezing medium was developed. The advantages of such a technique would allow a convenient and economical storage of semen, freezing in a solution of sugars may be less harmfull than are electrolyte solutions (Lovelock and Polge, 1954)[1] and allow for reconstitution with an electrolytes solution before use. The laboratory and field trials for test the effect of this technique on survival and fertility of spermatozoa were conducted on bull, goat, and stallion semen in Japan and U. S. A.

Bull Semen

Procedures of pellet freezing of semen

As shown in Fig. 1, [2),6)] the collected semen was extended at the ratio of 1 : 2-3 using an extender which composed of 20 parts of eggyolk and 74.5 parts of sugar solutions (7.5% glucose or 10.5% lactose or 18% raffinose) containing 5-5.5ml, glycerol and cooled to 5℃. Following a glycerol equilibration for 5-10 hrs., the semen was placed directly on dry ice in small drops (0.05-0.2 ml.) using a micro syringe.

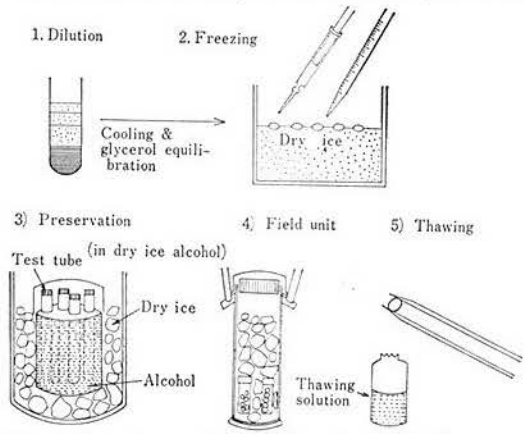


Fig. 1 Procedures of pellet freezing of bull semen.

After leaving the droplet on dry ice for 10 min., it was transfered into the containers and stored in dry ice or liquid nitrogen until use.

At thawing the pellet was added to the thawing solution. The thawing media were composed of following ingredients :

Glucose 2.4 gm., Sodium carbonate 0.31 gm., Sodium citrate dihydrate 1.4 gm., Potassium carbonate 0.17 gm., P-Aminomethylbenzen sulfonamide 0.2 gm., water (distilled) up to 100 ml.

The pH of this soution was adjusted to 7.0 using 5.5% citric acid media. The solution was added to the same volume of skim milk containing non or 6% glycerol.

In general, inseminations were carried out immediately after thawing.

Comparisons with the other freezing methods of semen in ampoules or plastic tubes using conventional extenders. Experimests were conducted on the survival[2),6)] and fertility of the spermatozoa.[3),4),8)] The results of fertility trials are shown in Table 1 and 2. These tables indicated that the pellet freezing of semen was better than the others tested. The differences were statistically significant.

Effects of glycerol levels on the spermatozoan survival

As shown in Fig. 2 and 3, experimental results, which compared to pellet size, glucose concentration and glycerol levels, indicated that there was no difference in the survival rate after freezing between pellet sizes when frozen in yolk-glucose, however, a significant differences was shown when yolk-citrate was used as the freezing medium.[2),6)] No difference in the survival rates was shown between the extender

Table 1. Fertility of the pellet and ampoule freezing semen.

Method of freezing	No. of cows inseminated	No. of cows conceived	Fertility
2 step	45	18	40.0 %
I step	47	28	59.6
Pellet	55	42	76.3
Total	147	88	59.8
		Chi-square value	16.4

Table 2. Semen of normally Iow conception bulls frozen in pellet concentrated form in a yolk-carbohydrate extender conpared to conventionally prepared semen frozen in ampoules on split samples.

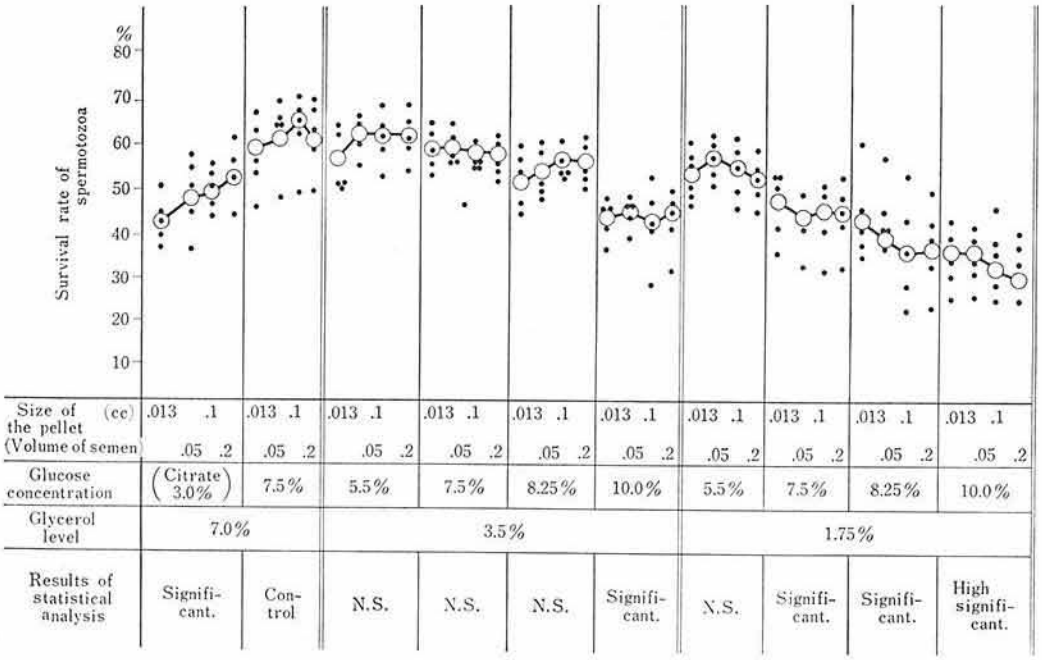
Pellet
Freezing extender yolk-glucose-lactose raffinose-7% glycerol.

No.	No. Ist services	No. Non-return	% 90-day Non-return
1	175	123	70.2
2	82	57	69.5
3	210	139	66.2
Total	467	319	68.3

$X^2=13.18$ $P>.01$

Ampoules
whole milk-7% glycerol

No.	No. Ist services	No. Non-return	% 90-day Non-return
1	177	108	61.0
2	114	74	64.9
3	254	128	50.4
Total	545	310	56.9



• Mean of 3 ejaculates from each bull
○ Mean of 15 ejaculates from 5 bulls

Fig. 2 Effect of pellet size, glucose concentrations and glycerol levels on survival rates of frozen spermatozoa.

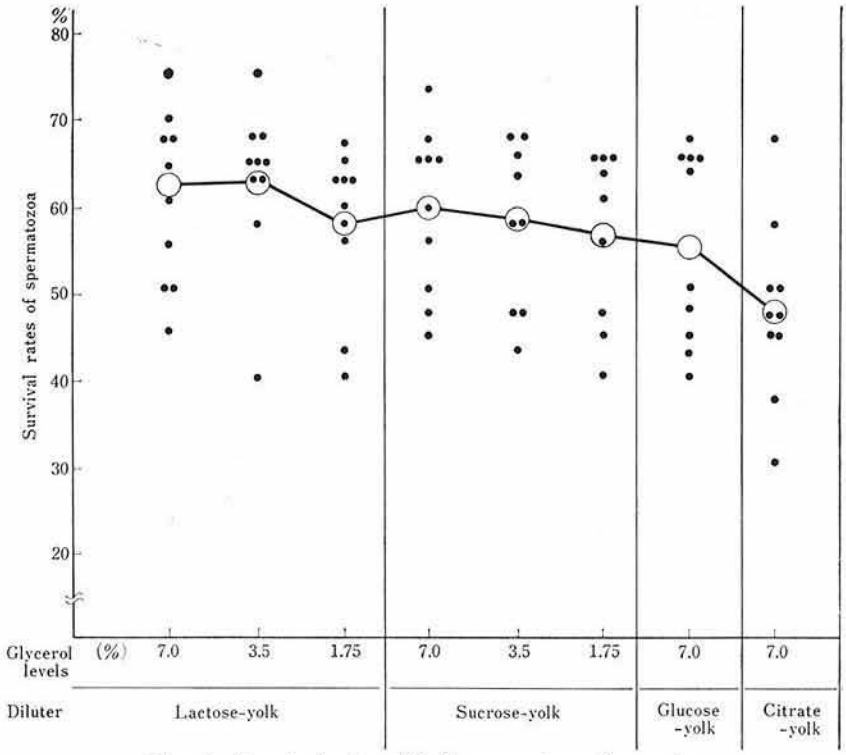


Fig. 3 Survival rates of bull spermatozoa frozen in disaccharide extenders.

Table 3. Effects of glycerol levels on the fertility of pellet frozen semen (60-90 day non-return)

Glycerol level %	Yolk-glucose			Yolk-lactose			Total
	No. of cows inseminated	No. of N. R.	% of N. R.	No. of cows inseminated	No. of N. R.	% of N. R.	% of N. R.
7	36	20	55.5	28	13	46.4	51.5
3.5	34	23	67.6	44	33	75.0	71.7
1.75				47	33	70.2	70.2
Total	70	43	61.4	119	79	66.3	64.5

Table 4. Effect of glycerol levels on fertility of spermatozoa frozen in pelleted form. (60-90 day N. R.)

Final concentration of glycerol %	Trial I. 2 Bulls	Trial II. 2Bulls	Trial III. 4 Bulls	Trial IV. 3 Bulls	Total
	Basic solution				
	Yolk-lactose	Yolk-glucose-lactose	Yolk raffinose	Yolk lactose	
7.0 No. lst serv.(%N. R.)	172 (74.1)	227 (74.3)	260 (76.5)		658(74.9)
5.0 No. lst serv.(%N. R.)			271 (72.7)	180 (72.7)	451(72.7)
3.5 No. lst serv.(%N. R.)	164 (78.0)	232 (75.4)	176 (72.1)	171 (69.0)	743(75.4)
1.75 No. lst serv.(%N. R.)	143 (75.5)	252 (70.6)		170 (68.2)	565(71.2)
1.0 No. lst serv.(%N. R.)			236 (68.2)	175 (68.5)	411(69.8)
0 No. lst serv.(%N. R.)			59 (59.3)	32 (50.0)	91(56.0)

containing a 7.5% glucose 7.0% glycerol and these various concentration of glucose containing 3.5% glycerol except when the concentration of glucose was 10%. Also no difference was shown at the isotonic solution of glucose using only 1.75% glycerol (Fig. 2). No difference was shown in the survival rates when frozen in yolk-disaccharides media containing 1.75, 3.5 and 7.0% glycerol, respectively (Fig. 3). Fertility rates in testing the effects of glycerol levels are shown in Table 3 and 4. It was indicated that reducing the glycerol levels of the freezing extender from 7.0 to 3.5 or 1.75% had no effect on fertility.[5),8)]

In trials involving no glyerol extenders a significant difference was shown.

Protective effects of sugars to the spermatozoa during freezing and thawing

The protective effects of various sugars in pellet freezing are shown in Fig. 4 and 5. It was indicated that the survival rates of spermatozoa were increased when sugars of high molecular weight were used.[5),7),8)]

These results did not conform to those reported by Polge and Soltys (1960).[16)] They had reported only sugars of small molecular weights such as xylose and glucose showed better protective effects than did sucrose. Fertility results using yolk-lactose or yolk-raffinose media containing no glycerol showed good fertility which was the same as that of non frozen fresh semen (Table 5).[12)]

Fffects of different freezing methods on spermatozoan survival

The tests were made using sugar solutions containing various glycerol levels as freezing media (Fig. 6).[7)]

Freezing in the pellet form resulted in greater survival of spermatozoa than the other freezing at the lower glycerol levels. In general the slow freezing rate required an increasd concentration of glycerol. The results may be explained as follows. In pellet freezing the material passed through the critical temperature range faster than in regular freezing and super cooling or rebound phenomenon in the freezing curve were negligible (Fig. 7).

In other fast freezing, drop into liquid nitrogen and plunge into dry ice alcohol, the spermatozoan survival in low glycerol levels was better than that of 7 % glycerol. These results suggest that the main factor affecting freezing rates of bull spermatozoa may be the dehydration of the cell during the freezing in preference to "cold shock" (Polge and Lovelock, 1952).[15)]

Effects of thawing solution on fertility of the pellet freezing semen

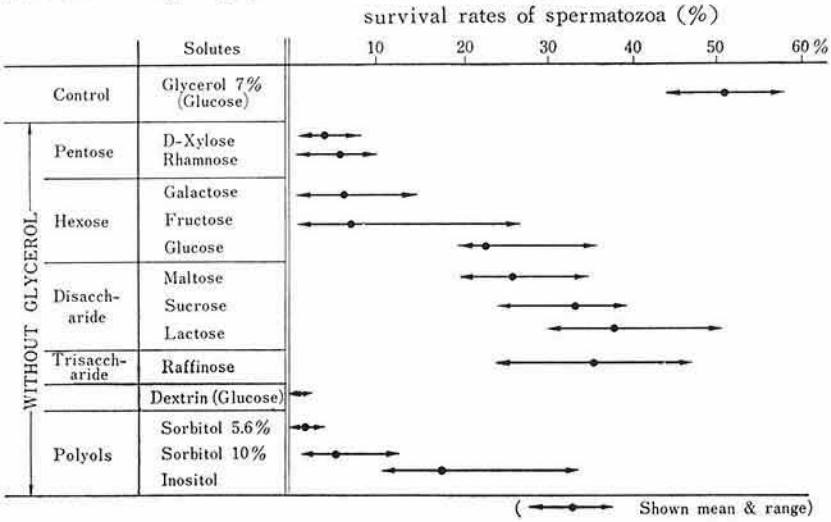


Fig. 4 Protective action of various sugars on survival of frozen bull spermatozoa.

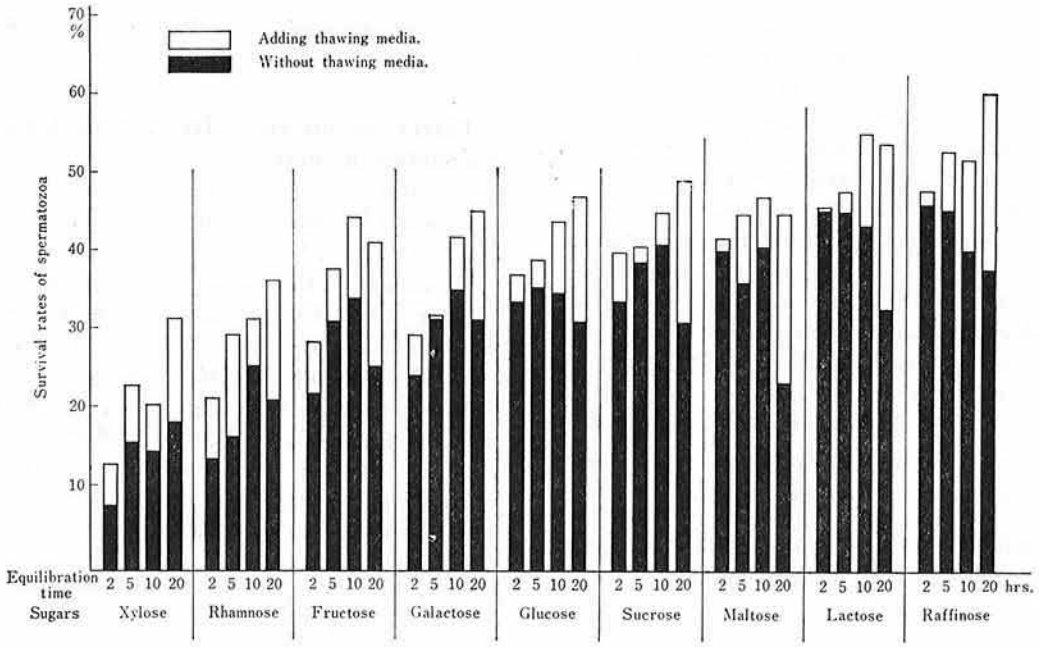


Fig. 5 Protective action of sugars on spermatozoan survival in various equilibration time.

Table 5. Fertility of pellet frozen bull semen diluted with non-glycerol extenders. (30~60 days. N. R.)

Extenders \ Name of bull		J. D. B. D.	H. R. R.	G. A. P.	Total
Yolk-lactose-glycerol (3.5%) (Control)	No. of cows Inseminated	14	50	20	84
	No. of cows Conception	10	38	15	63
	Fertility (%)	71.4	76.0	75.0	75.9 %
Yolk-lactose	No. of cows Inseminated	14	53	20	87
	No. of cows Concepted	10	36	10	56
	Fertility (%)	71.4	67.9	59.0	64.3 %
Yolk-raffinose	No. of caws Inseminated	13	54	19	86
	No. of cows Conception	12	34	13	59
	Fertility (%)	92.3	62.9	67.4	68.6 %
4℃ storage (Control)	No. of cows Inseminated	21	49	18	88
	No. of cows Conception	16	34	95	59
	Fertility (%)	76.1	69.3	50.0	67.0 %

Field trials were conducted using the electrolytes and non-electrolytes media.[5]

The results are given in Table 6. No difference was shown between thawing solutions in trial I, III and IV. A difference existed, however, in trial II amomg buffered glucose milk, 3% buffered glycine.

In the trial, electrolytes, especially buffered milk was better. Further investigations will be needed. The problems of thawing media

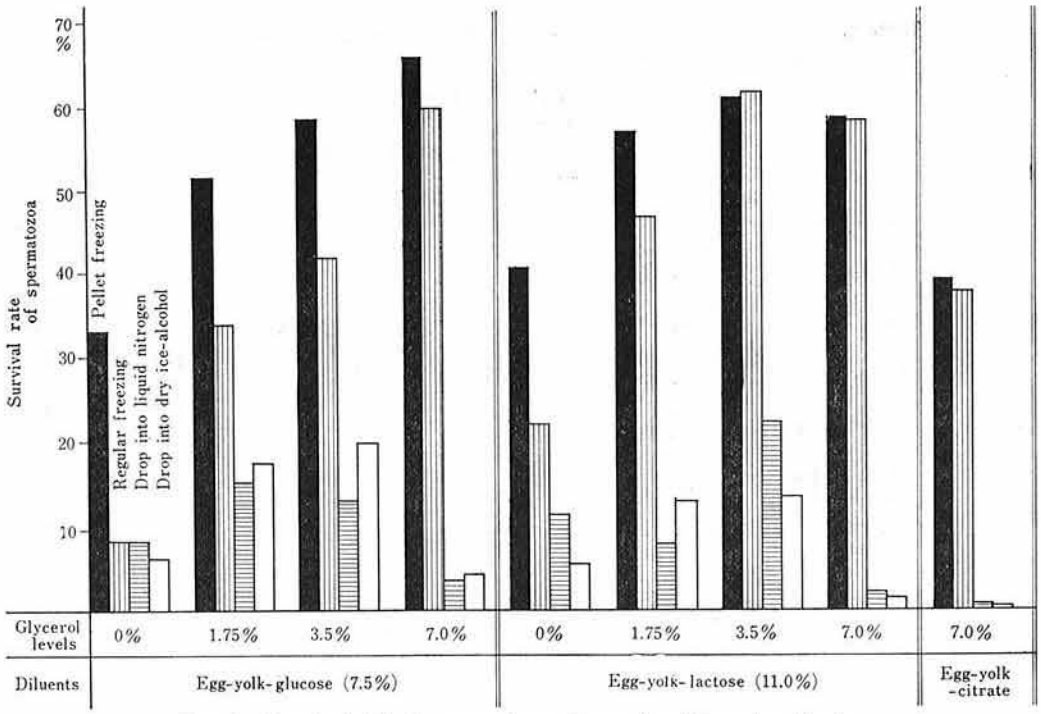


Fig. 6 Survival of bull spermatozoa frozen by different methods.

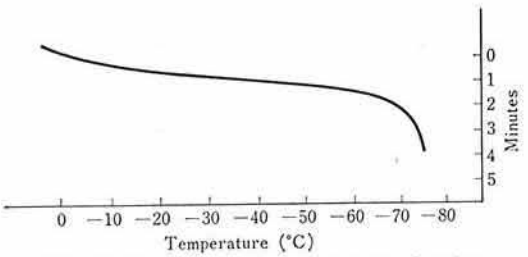


Fig. 7 Freezing curve of 0.2 ml. droplet directly on dry ice.

may be the most important item, not only in the pellet freezing of semen but in fresh semen storage and also in lyophilizing of spermatozoa.

Fertility of spermatozoa at 5 °C storage after thawing

It is well known that the fertilizing capacity of spermatozoa decreased rapidly after freezing and thawing in the conventional method. Results of several laboratory experiments indicated good longevity of spermatozoa at 5°C storage after thawing by this method.

In fertility trials, the pellet freezing semen was thawed in a small flask kept at 35°C, without the thawing media. And after cooling to 5°C, the the thawed semen was filled separately with thawing media in plastic tubes, called "straw". Inseminations were carried out after mixing the semen and the thawing media in the "stra".

Fertility results are given in Table 7. It was clear the fertilizing capacity of the spermatozoa, preserved at 5°C after freezing and thawing, was improved markedly.[13)]

Fertility of spermatozoa in pellet freezing at routine works

Several reports on the fertility in this method came from Japan and overseas. These results are given in Table 8 and 9.

A comment in the report of the Milk Marketing Board (1965) was as follows; "It is interesting to note that the semen of two of the Friesian bulls which have given satisfactory results in this trial has been difficult to freeze ia ampoules, and non-return rates have been low".

Stallion Semen

Procedures of pellet freezing semen

Table 6. Effect of thawing solution on fertility of the spermatozoa frozen in pelleted form. (13 bulls)

			Trial I 3 Bulls	Trial II 2 Bulls	Trial III 3 Bulls	Trial IV 5 Bulls	Total
			(60–90 day N. R.)				
Electrolytes	B. G. Milk	No. 1st serv.	452	93			545
		% N. R.	77.6	80.1			78.1
	Minn. Go	No. 1st serv.	433				433
		% N. R.	77.8				77.8
	3% Sod. citrate	No. 1st serv.		88	196	217	501
		% N. R.		68.1	70.9	77.9	73.5
	Milk	No. 1st serv.				183	173
		% N. R.				76.0	76.0
	0.9 % NaCl	% N. R.				180	180
		No. 1st serv.				70.0	70.0
Non Electrolytes	3% Glycine (Buffered)	No. 1st serv.		83	194	203	480
		% N. R.		61.4	68.0	79.4	69.2
	10.5 % Lactose	No. 1st serv.			202		202
		% N. R.			69.3		69.3
	Tris puffer	No. 1st serv.				199	199
		N. R.				71.4	71.4
	Total	No. 1st serv.	885	264	592	982	2,723
		% N. R.	77.7	70.4	69.4	73.8	73.8
X^2 * (P>.05)			0.01 NS	7.82*	0.38NS	1.36NS	

Table 7. Fertility of spermatozoa at 5°C storage after freezing and thawing.

Experimental area	Days after thawing	1	2	3	4	Total
Ibarki Pref.	No. of cows inseminated	16	54	42	55	168
	fertility (%)	50.0	46.0	48.0	46.0	47.0
Saitama Pref.	No. of cows inseminated	58	87	78	9	232
	fertility (%)	48.0	28.0	30.0	33.3	34.0
Gumma Pref.	No. of cows inseminated	22	15	57	4	136
	fertility (%)	54.5	28.3	26.3	0	30.8
Total	No. of cows inseminated	96	191	177	68	536
	fertility (%)	50.0	33.3	33.0	41.0	37.0

Table 8. Fertility of spermatozoa in pellet freezing at routine work in Japan.

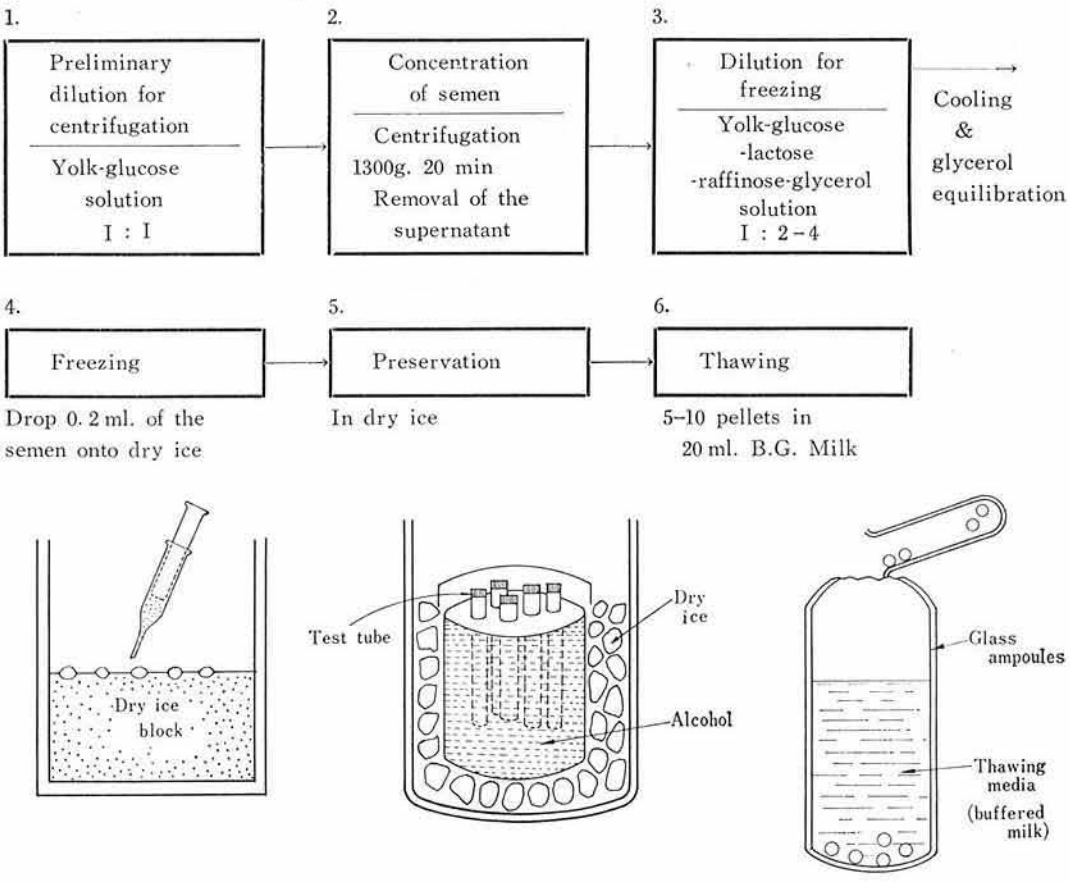
Area	No. of Inseminations	No. of cows conceived	fertility	Remarks
Saitama Pref.	4,208	2,731	64.9 %	1964, 1965
Gumma Pref.	1,344	856	63.7	1964, 1965
Niigata Pref.	959	614	64.0	1965
Total	6,511	4,201	64.5	

Table 9. Fertility of pellet frozen bull semen with routine work in Europe

Country	No. of inseminations	No. of cows conceived	fertility	Remarks
England	2,182	1,729	79.2 %	Milk Marketing Board, 1965
West Germany	1,215	836	68.8	Zentral Rinderbesamungsgen, Rheinland
Denmark	304	221	72.6	Dr. H. Wibling's comment

As shown in Fig. 8, [9),10)] the method for stallion semen was same as that of bull semen except that in stallion concentrating semen was used. The results of laboratory trials were shown in Fig. 9. The thawing medium was same as that of bull semen except containing non glycerol. No difference was found on spermatozoan survival between 1.75, 3.5 and 7.0% glycerol in the diluters.[10)] Fertility of the pellet freezing stallion semen are shown in Table 10.[11)]

Fig 8. Procedures of pellet freezing of stallion semen



Goat Semen

Procedures were the same as freezing of bull semen with the exception that the pellet

Fig. 9 Effect of glycerol level, glycerol equilibration time and sugars on survival rates of stallion spermatozoa frozen in the pellet form (A korean ponny, Ave. of 7 ejaculates)

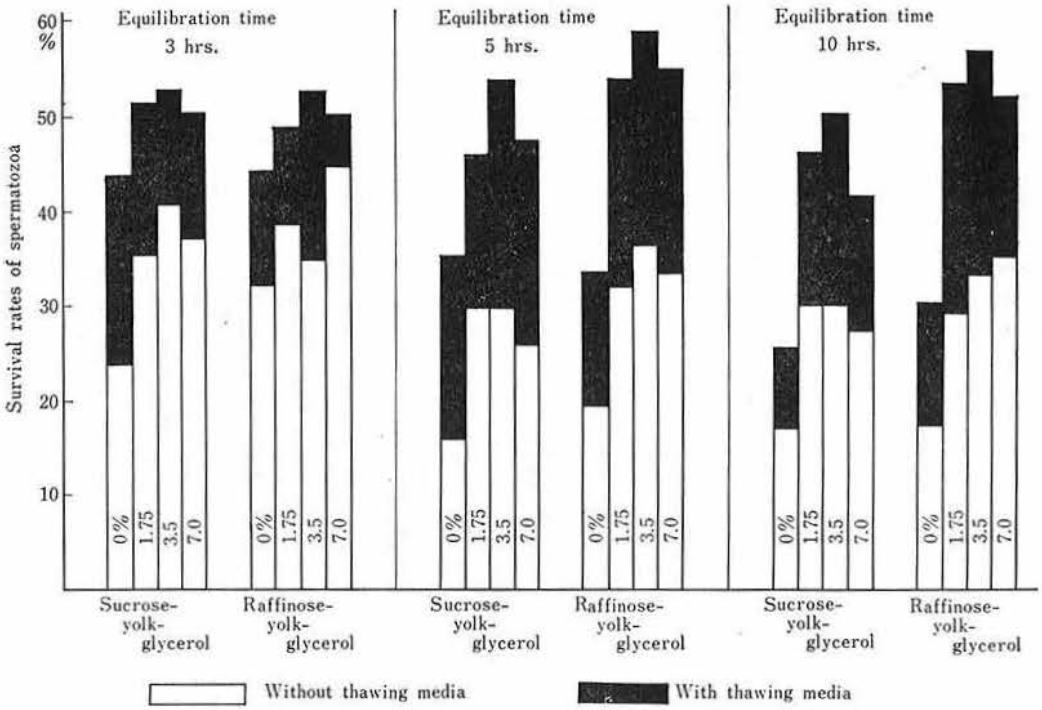


Table 10. Fertility of pellet frozen stallion semen (using liquid nitrogen)(May-July, 1966)

Name of stallion	No. of inseminations	No. of mares conceived	Fertility
Gōko (Percheron hybrid)	18	12	66.7 %
Kōsan (Korean ponny)	10	4	40.0
Kozakura (Hokkaido island ponny)	23	8	34.8
Total	51	24	47.1

Table 11. Fertility of pellet frozen goat semen (using dry ice)

Years	No. of inseminations	No. of conceptions	Fertility	Remarks
1962	78	58	74.3 %	Storage period 1–30 days
1964	144	113	78.4	Storage period 1–90 days

(Fertility rates of frozen goat semen glycerolated with 3.5 and 7.0 % levels were 82.9 (29/35) and 91.2 % (31/34), respectively.)

size 0.1 ml and glycerol equilibration time was 1.5–3.0 hours. Fertility results are given in Table 11.[14)]

Summary

Many experiments were conducted to study the pellet freezing of bull, goat and stallion semen. Frozen bovine semen in concentrated pellet form, with solutions containing egg-yolk, sugars and glycerol increased fertilizing capacity, over that of other methods tested and of low efficiency sires. In this form semen can be frozen at semi-rapid rates with less glycerol. This method was also effective in preserving the viability and fertility of stallion and goat spermatozoa at high level.

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