THE RESPONSE OF RAM SPERMATOZOA TO PREPARATIONS OF EGG YOLK IN SEMEN DILUENTS DURING STORAGE AT 5 OR −196°C

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Abstract

Egg yolk dispersed at levels between 0·1 and 3·75% (v/v) in a diluent used for the preservation of ram spermatozoa at 5°C was shown to affect the structure and survival of spermatozoa. A motility-preserving factor was studied in greater detail but a factor causing disruption of the acrosome was also detected. The substance prolonging the motile life of spermatozoa was shown to be a regular component of hen eggs, it was stable during storage of the egg up to 28 days after laying, and heating of the yolk-containing diluent to 100°C for 30 min inactivated it.

After centrifugation of the yolk-containing diluent at 35,000 *g* for 30 min, the motility-preserving factor remained in the supernatant solution. More than 50% of spermatozoa survived for 72 hr at 5°C when the supernatant solution contained 15 mg/ml of soluble egg yolk solids, in contrast with a survival of less than 20% when egg yolk was not included in the diluent.

I. INTRODUCTION

Phillips (1939) first reported that the inclusion of egg yolk in a semen extender prolonged the survival of bull spermatozoa at 10°C. It was subsequently shown that egg yolk was also beneficial for the storage of stallion, ram, and turkey spermatozoa (Lardy and Phillips 1939), and since that time egg yolk has been a common constituent of semen extenders. Several attempts have been made to isolate the factor(s) in egg yolk responsible for the protection afforded to spermatozoa (Lardy and Phillips 1941; Mayer and Lasley 1945; Phillips and Spitzer 1946; Blackshaw 1954*a*). Phospholipids and proteins have been claimed to be beneficial during cold shock and prolonged low-temperature storage. Kampschmidt *et al.* (1953) showed that both a lipoprotein fraction and a phospholipid fraction derived from egg yolk protected bull spermatozoa during cold shock, but only the lipoprotein fraction was beneficial during cold storage. All these studies involved isolation techniques using organic solvents which are now recognized as likely to alter the chemical structure of such compounds. Also, no indication has ever been given of the level of egg yolk necessary to minimize the adverse effects of cold treatments.

The experiments reported here concern the stability of substances in egg yolk which protect ram spermatozoa against the detrimental effects of cold storage and are part of a continuing study to identify these substances. Evidence is presented concerning the critical level of egg yolk necessary to provide protection, and the possible nature of the protective substances.

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II. Materials and Methods

All diluents, except that for the freezing experiment, contained 185 mM glucose, 17 mM fructose, 31 mM NaCl, 10 mM Na_2HPO_4, and 5 mM each of NaH_2PO_4 and KH_2PO_4 with 500 i.u./ml each of penicillin and dihydrostreptomycin. The freezing diluent differed in that it contained 247 mM glucose, 49 mM NaCl, and 7·5% (v/v) glycerol. Egg yolk was added at the highest concentration (v/v) in the experiment. Lower concentrations were derived by dilution of portions of the original solution with diluent containing no egg yolk but in all other respects complete.

The hen eggs were obtained from retail stores, except for those of known age used in experiment 3 which were obtained direct from a poultry research unit. Centrifugation in experiment 5 was carried out in a Sorvall RC-2B refrigerated centrifuge, running at 5°C.

Semen was obtained from Merino rams by electrical stimulation (Blackshaw 1954*b*), and only ejaculates of high initial motility were used. The semen was diluted 20-fold (except those samples to be frozen in experiment 1) at 30°C within 20 min of collection and the extended semen was cooled to 5°C at a constant rate of 0·28 degC/min. It was then stored for 72 hr at 2–5°C, as this period was found to give the greatest discrimination in response between egg yolk levels. At the end of this period spermatozoa were separated from the diluent by centrifugation at 1000 *g* for 10 min and were resuspended in diluent previously warmed to 37°C containing no egg yolk. Samples to be frozen in experiment 1 were initially diluted 10-fold at 30°C and after chilling were further diluted to 20-fold with glycerol-containing diluent. They were stored for 4 hr at 5°C, placed in sealed glass ampoules in aliquots of 1 ml, and frozen at 4 degC/min to −79°C. The ampoules were transferred to liquid nitrogen for a storage period of 72 hr after which they were thawed at 37°C for motility appraisal. The motility of all samples was assessed in a random sequence so that the observer had no knowledge of the treatment or ejaculate from which the sample came. Motility was estimated on a scale of 0–4 (Emmens 1947) and the percentage of motile spermatozoa was estimated to the nearest 10% (Martin 1963). Separate analyses of variance were calculated for the motility and percentage motile estimations, using the totals of three estimates from each sample.

In experiment 1, fixed smears of diluted semen were stained with Giemsa, and acrosomal damage was estimated on a 0–3 scale of increasing disruption of the structure (Watson and Martin 1972). The unit of data for analysis was the mean score of 20 spermatozoa selected at random.

III. Results

The effect of the inclusion of egg yolk in the diluent on the survival of ram spermatozoa was examined after chilling and deep-freezing (experiment 1). The results shown in Table 1 represent means from three ejaculates. After chilling, the motility and percentage motile scores showed a beneficial effect of egg yolk continuing up to a content of 3·75%. In contrast, the acrosome score showed a reduction in acrosomal damage only up to 0·75% egg yolk. At the higher level the degree of damage increased significantly ($P < 0·001$). This difference between levels was also seen immediately after dilution. The motility of spermatozoa after freezing was improved by the presence of egg yolk in the diluent, and the percentage motile was directly related to the level of egg yolk. The overall reduction in survival and the increased acrosomal damage resulting from this more severe treatment was clearly seen.

The following experiments were concerned with the preservation of motility of spermatozoa after dilution and chilling. In experiment 2 the protective activity of eggs from different retail stores was assayed (Table 2). The experiment was completely replicated on two separate days. No significant difference in protective activity was recorded between eggs either on day 1 or day 2. Differences between ejaculates within days were highly significant ($P < 0·001$), and this variation from ram to ram is the most likely cause of the significance of the between-day contrast.

TABLE 1

EXPERIMENT 1: MOTILITY AND ACROSOMAL MORPHOLOGY OF RAM SPERMATOZOA BEFORE AND 72 HR AFTER CHILLING OR DEEP-FREEZING, SHOWING THE EFFECT OF YOLK IN THE DILUENT

Egg yolk level (%)	Motility	Percentage motile	Acrosome score	Motility	Percentage motile	Acrosome score
	30°C before chilling			**5°C+72 hr after chilling**		
0	3·17	73·33	0·98	0·50	10·00	1·32
0·15	3·67	73·33	0·77	2·33	30·00	1·25
0·75	3·33	63·33	0·52	2·67	46·67	0·83
3·75	3·50	60·00	1·15	2·67	50·00	1·20
	30°C before freezing			**−196°C+72 hr after freezing**		
0	3·50	63·33	0·60	0·83	6·67	2·03
0·15	2·83	56·67	0·75	1·67	13·33	1·98
0·75	3·50	66·67	0·48	1·33	20·00	1·68
3·75	3·00	50·00	0·77	1·50	30·00	1·43

TABLE 2

EXPERIMENT 2: MEAN MOTILITY OF RAM SPERMATOZOA STORED FOR 72 HR AT 5°C SHOWING THE RESPONSE TO EGG YOLKS DERIVED FROM DIFFERENT SOURCES

	0·1% egg yolk in diluent		0·5% egg yolk in diluent		2·5% egg yolk in diluent		Mean	
	Motility	Percentage motile	Motility	Percentage motile	Motility	Percentage motile	Motility	Percentage motile
Day 1								
Egg 1	1·29	22·50	2·00	33·33	2·38	43·33	1·89	33·05
Egg 2	1·46	25·83	1·67	30·83	2·42	43·33	1·85	33·33
Day 2								
Egg 3	2·08	33·33	2·38	42·50	2·67	51·67	2·38	42·50
Egg 4	1·96	28·33	2·33	38·33	2·54	46·67	2·28	37·78
Mean	1·70	27·50	2·10	36·25	2·50	46·25		
Diluent without egg yolk								
Day 1							1·17	20·00
Day 2							1·54	21·67

Analyses of variance

Source of variation	D.F.	Motility		Percentage motile	
		Mean square	*F*	Mean square	*F*
Levels of egg yolk	2	23·16	51·47***	12657·00	43·67***
"L"	1	46·32	102·93***	25312·50	87·20***
"Q"	1	<0·01	0·01	37·50	0·13
Days	1	22·69	50·42***	5208·33	17·94**
Within day 1					
Eggs	1	0·94	2·09	4·17	0·01
Ejaculates	3	39·59	87·98***	22570·83	77·76***
Eggs × ejaculates	3	1·09	2·42	193·06	0·67
Within day 2					
Eggs	1	0·51	1·13	1204·17	4·15
Ejaculates	3	3·95	8·78**	9837·50	33·89***
Eggs × ejaculates	3	0·90	2·00	37·50	0·13
Levels × days	2	1·83	4·07*	58·33	0·20
Levels × eggs	4	0·63	1·40	79·17	0·27
Levels × ejaculates	12	1·47	3·27*	916·67	3·16*
Residual high-order interactions (error)	12	*0·45*		*290·28*	

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

Figure 1 shows dose–response curves for three replicates of experiment 3 in which the activity of yolk from eggs stored for 14 or 28 days was compared with that of new-laid eggs. During storage eggs were kept in a humidity-controlled room at

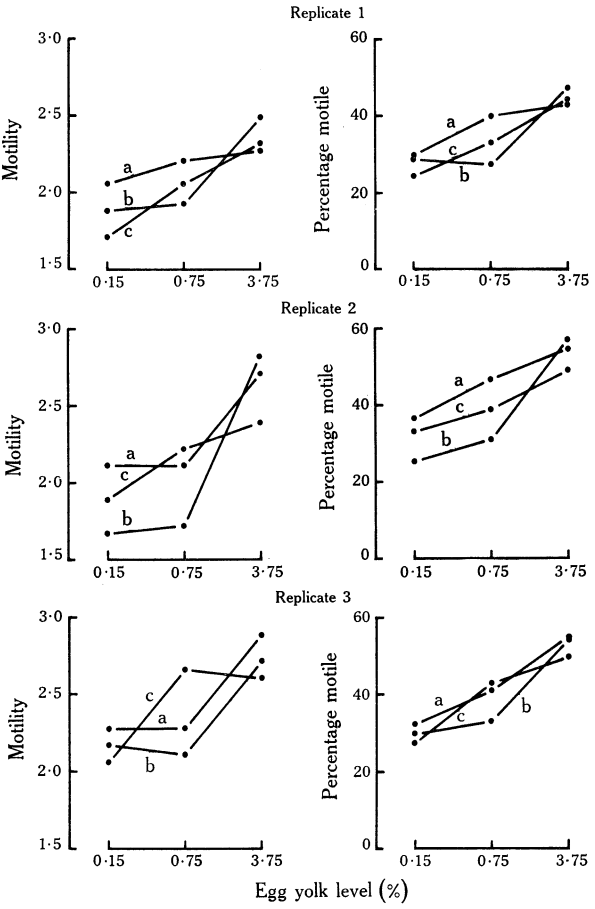

Fig. 1.—Mean survival of ram spermatozoa after storage for 72 hr at 5°C in diluents containing yolk from new-laid eggs (*a*), eggs 14 days after laying (*b*), and eggs 28 days after laying (*c*). (Mean scores of three ejaculates in each replicate.)

13°C. The analyses of variance of these data are shown in Table 3. A highly significant linear effect of level of egg yolk was seen for both the motility and percentage motile scores ($P < 0{\cdot}001$) and a significant quadratic effect of level of egg yolk for motility ($P < 0{\cdot}05$). The effect of the age of the egg on the survival of spermatozoa was not significant. There were significant ejaculate differences in replicates 1 and 2.

To test the heat stability of the active substance, samples of diluent containing 3·75% egg yolk were heated in a water-bath before preparation of the higher dilutions for storage experiments with spermatozoa. In a preliminary experiment, heating for 15 min at 100°C had no significant effect on the protective nature of the diluent. In the experiment reported here (experiment 4) heating was continued for 30 min at 50 or 100°C. Some protein was presumed to have been denatured when a low-density flocculent layer was observed on the surface of the solution after treatment at the higher temperature. The results and analyses of variance of spermatozoal survival in these diluents are shown in Table 4. While no effect on

TABLE 3

EXPERIMENT 3: SUMMARY OF ANALYSES OF VARIANCE OF SURVIVAL OF RAM SPERMATOZOA IN DILUENTS CONTAINING YOLK OF EGGS OF VARYING AGE (SEE FIG. 1)

Source of variation	D.F.	Motility		Percentage motile	
		Mean square	*F*	Mean square	*F*
A. Egg yolk levels	2	23·73	41·63***	27186·42	62·56***
L	1	44·46	78·00***	52890·74	121·71***
Q	1	2·99	5·25*	1482·10	3·41
B. Replicates	2	6·31	11·07**	2579·01	5·93*
C. Age of egg (replicate 1)	2	0·44	0·77	311·11	0·72
(replicate 2)	2	1·19	2·09	1300·00	2·99
(replicate 3)	2	0·48	0·84	292·59	0·67
D. Ejaculates (replicate 1)	2	4·75	8·33**	6033·33	13·88***
(replicate 2)	2	15·36	26·95***	13877·78	31·93***
(replicate 3)	2	0·90	1·58	1114·81	2·57
A×B	4	0·68	1·19	208·64	0·48
Pooled interaction terms					
A×C	12	1·20	2·11	608·64	1·40
A×D	12	1·21	2·12	866·05	1·99
C×D	12	0·69	1·21	532·72	1·23
Pooled error term					
A×C×D	24	*0·57*		*434·57*	

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

TABLE 4

EXPERIMENT 4: MEAN MOTILITY OF RAM SPERMATOZOA AFTER STORAGE FOR 72 HR AT 5°C SHOWING THE EFFECTS OF HEATING OF THE DILUENT FOR 30 MIN

Treatment	0·15% egg yolk in diluent		0·75% egg yolk in diluent		3·75% egg yolk in diluent		Mean	
	Motility	Percentage motile	Motility	Percentage motile	Motility	Percentage motile	Motility	Percentage motile
1. Control (unheated)	1·75	26·67	2·33	38·33	2·69	50·00	2·26	38·33
2. Heated to 50°C	1·94	28·83	2·47	41·11	2·64	47·22	2·35	38·89
3. Heated to 100°C	1·69	24·44	1·78	25·56	2·08	31·11	1·85	27·04
Mean	1·79	26·48	2·19	35·00	2·47	42·78		
Diluent without egg yolk							1·56	22·22

Summary of analyses of variance

Source of variation	D.F.	Motility		Percentage motile	
		Mean square	*F*	Mean square	*F*
A. Treatments	2	11·46	10·32***	7246·30	13·00***
1 *v.* 2 and 3	1	2·68	2·41	3114·81	5·59*
2 *v.* 3	1	20·25	18·24***	11377·78	20·41***
B. Levels of egg yolk	2	18·70	16·85***	10762·96	19·31***
L	1	37·01	33·34***	21511·11	38·59***
Q	1	0·39	0·35	14·81	0·03
C. Ejaculates	5	6·90	6·22**	3055·00	5·48**
A × B	4	1·46	1·32	1146·30	2·06
A × C	10	2·17	1·96	984·07	1·77
B × C	10	1·14	1·03	534·07	0·96
A × B × C (error)	20	*1·11*		*557·41*	

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

activity was seen when the diluent was heated to 50°C, activity declined significantly as a result of heating the diluent to 100°C for 30 min. Again, significant ejaculate differences were recorded.

TABLE 5

EXPERIMENT 5: MEAN MOTILITY OF RAM SPERMATOZOA COOLED TO 5°C AND STORED 72 HR IN FRACTIONS OF EGG YOLK DILUENT SEPARATED BY CENTRIFUGATION AT 35,000 *g*

Treatment	0·15% egg yolk in diluent		0·75% egg yolk in diluent		3·75% egg yolk in diluent		Mean	
	Motility	Percentage motile	Motility	Percentage motile	Motility	Percentage motile	Motility	Percentage motile
1. Control (uncentrifuged)	1·69	23·89	2·31	40·00	2·81	50·00	2·27	37·96
2. Supernatant	1·69	23·33	2·28	36·67	2·89	57·22	2·29	39·07
3. Precipitate	1·36	20·56	1·25	19·44	2·08	28·33	1·56	22·78
Mean	1·58	22·59	1·95	32·04	2·59	45·18		
Diluent without egg yolk							1·64	19·44

Analyses of variance

Source of variation	D.F.	Motility		Percentage motile	
		Mean square	*F*	Mean square	*F*
A. Treatments	2	27·46	23·08***	13429·63	30·74***
1 *v.* 2 and 3	1	12·68	10·66**	5348·15	12·24**
2 *v.* 3	1	42·25	35·50***	21511·11	49·24***
B. Levels	2	42·37	35·61***	20857·41	47·75***
L	1	82·51	69·34***	41344·44	94·64***
Q	1	2·22	1·87	370·37	0·85
C. Ejaculates	5	9·97	8·38***	5641·85	12·91***
A×B	4	2·34	1·97	2760·19	6·32**
A×C	10	1·99	1·67	722·96	1·65
B×C	10	0·74	0·62	390·74	0·89
A×B×C (error)	20	*1·19*		*436·85*	

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

The behaviour of the protective substance was followed during centrifugation of the egg yolk diluent at 35,000 *g* for 30 min (experiment 5). The precipitate was resuspended in diluent containing no egg yolk by gentle agitation with a glass rod. The dose–response curves of the supernatant and the precipitated portion were compared with that of an uncentrifuged portion, and the protective activity was found to be entirely associated with the supernatant portion (Table 5). Some slight protective activity was associated with the highest concentration of the precipitate. This probably represents contamination with supernatant solution since no attempt was made to wash the precipitate before resuspension. The analyses of variance show a significant treatment × levels interaction for the scores of percentage motile which was attributable to a departure from parallelism of the response lines for the supernatant and precipitate fractions, indicating that the precipitate contains little activity. Significant differences between ejaculates were again recorded.

Experiment 6 was conducted to assess the proportion by weight of egg yolk removed by centrifugation from a diluent containing 3·75% (v/v) egg yolk. Two drying methods, an oven at 35°C and a desiccator at room temperature, and two

egg yolks were investigated. All samples were conducted in duplicate. Weighing was repeated for 12 days at which time the weight change of the samples in a 48-hr period was less than 1%. Table 6 summarizes the results and shows that nearly 25% of the total egg yolk solids were removed. Only small differences were recorded between eggs and between drying methods.

TABLE 6

EXPERIMENT 6: DRY WEIGHT OF EGG YOLK REMOVED FROM 25 ML OF 3·75% EGG YOLK DILUENT BY CENTRIFUGATION AT 35,000 *g* FOR 30 MIN

Drying method	Mean dry wt. of 1 ml egg yolk (mg)	Total egg yolk solids in 25 ml diluent (mg)	Mean dry wt. of precipitated solids (mg)	Yolk solids removed (%)
Oven, 35°C				
Egg 1	517	485	116	23·9
Egg 2	537	503	123	24·5
Desiccator				
Egg 1	532	499	120	24·1
Egg 2	536	502	130	25·9
Mean	531	497	122	24·6

IV. DISCUSSION

The results of experiment 1 indicated that egg yolk has at least two effects on ram spermatozoa. It contains a substance or substances which preserve the motility during chilling and deep-freezing, and in the experiment the response increased with increasing levels of egg yolk. This motility-preserving factor may also protect cell membranes against damage. A second factor, hitherto unrecognized, appeared to be detrimental to the acrosomal membranes and its effect was only detected at higher egg yolk levels when the degree of damage resulting from the treatment was minimal. Hence at lower levels of egg yolk and after freezing only a protective effect was seen. The second factor may in part account for the reduction in fertility of semen associated with egg yolk in the diluent (Shannon 1972). The other experiments reported here concern the motility-preserving factor. Isolation of this factor may simultaneously eliminate the detrimental factor.

No differences were detected between individual egg yolks in their ability to protect ram spermatozoa during chilling and prolonged storage at 5°C. This indicates that the protective substance is a regular component of egg yolk. When the egg yolk level was reduced to 0·15% (v/v) a response was still detectable. Those constituents of egg yolk which occur in small and variable quantities may therefore be discounted as the protective substance.

The motility-preserving factor in egg yolk is stable during storage of the egg at least up to 28 days after laying. This is in close agreement with an earlier report by Meglioli *et al.* (1955) who found no obvious effect on the motility of bull semen in a diluent containing yolk from eggs up to 20–25 days old.

The reduction in activity of the egg yolk diluent when heated for 30 min at 100°C suggests that a protein is involved. However, no significant deterioration in protection

was detected in a preliminary experiment after heating for 15 min at 100°C. Heating to 50°C had no effect at all. Such resistance to heat inactivation may indicate that the activity does not depend solely on a specific protein configuration but on the bond between a protein and a non-protein moiety.

Experiments 5 and 6 showed that no activity was present in the 25% of total solids removed by centrifugation at 35,000 *g* for 30 min. Centrifugation did not alter the protective nature of the supernatant fraction which is in agreement with the results of Rikmenspoel (1957) who found that the supernatant from ultracentrifugation of an egg yolk diluent was still protective for bull spermatozoa. The resultant optically clear solution was also advantageous for the microscopic observation of spermatozoa. From the results of experiment 6 the concentration of egg yolk at which a definite and repeatable protective activity was evident can be calculated as about 0·6 mg/ml of egg yolk solids, and more than 50% of spermatozoa survived for 72 hr at 5°C if the diluent contained 15 mg/ml of egg yolk solids.

The experimental conditions of prolonged storage of spermatozoa at 5°C following slow cooling from 30°C closely resemble the type of stress associated with processing of semen for artificial insemination. Lightfoot and Restall (1971) have demonstrated the importance of the motility of spermatozoa in the penetration of the cervix of the ewe following artificial insemination. Thus scores of motility of spermatozoa after storage in these experiments could be related to their ability to penetrate the female reproductive tract.

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