THE PREVENTION OF TEMPERATURE SHOCK OF BULL AND RAM SEMEN

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Summary

The decreased vitality of ram and bull spermatozoa caused by sudden cooling (cold shock) may be largely prevented by the presence of egg yolk in the diluent.

The activity of egg yolk in preventing cold shock lies in the alcohol-soluble, acetone-insoluble fraction. The phospholipid lecithin may be isolated from this and will prevent cold shock in concentrations as low as 0·12 per cent.

The protective action of lecithin for ram spermatozoa is greater at pH 6·5 than at a neutral or alkaline pH.

I. Introduction

The collection and storage of semen subjects spermatozoa to numerous environmental changes, which all tend to reduce the number of living and active cells. Sudden changes in temperature affect the vitality of spermatozoa; Milovanov (1934) calls the harmful effect of sudden cooling, "temperature or cold shock." Thus, Birillo and Puhaljskii (1936) observed that a fall in temperature from 40 to 0°C in 30-60 min caused temperature shock to ram and bull spermatozoa. Cooling over 2 hr caused less shock and if it was prolonged to 3 or 4 hr, cold shock did not occur. However, positive results with ram semen were only obtained with artificially buffered specimens. Gladcinova (1937) reported that rapid cooling of horse, bull, and ram semen from body temperature to 5 or 10°C, caused reduced activity which could not be restored by rewarming. Cooling to 20 or 30°C did little harm. It was also noted that temperature-shocked spermatozoa rapidly died at 37°C.

Ram semen was investigated by Gunn, Saunders, and Granger (1942), who found no advantage in slow cooling, but placed the semen in a container at 4°C as soon as collected. A cooling rate of not more than 5°C an hour was recommended by Easley, Mayer, and Bogart (1942) for bull semen, but semen diluted with egg yolk could be cooled rapidly, although sudden cooling to 0°C caused significant motility loss. Earlier, Phillips and Lardy (1940) had recommended the use of egg yolk as a protective agent against cold shock and for the storage and transport of semen.

Epididymal spermatozoa of the bull and boar have been shown to be very resistant to temperature shock, but after ejaculation the cells have little resistance (Lasley and Bogart 1944; Lasley and Mayer 1944). The addition of egg yolk to the semen before cooling restores resistance.

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A factor, which greatly increases the number of spermatozoa resistant to cold shock, has been isolated from egg yolk (Mayer and Lasley 1944). The factor was acetone, alcohol, and ether insoluble, and gave a positive ninhydrin reaction. An acetone-insoluble, alcohol-soluble fraction was toxic, killing the spermatozoa within a few minutes at room temperature. Mayer and Lasley (1945) also showed that ram spermatozoa did not acquire much resistance from the factor; maximum survival after cold shock did not exceed 30 per cent. On the other hand, Walton (1947) observed that the ether-soluble or fatty fraction of egg yolk completely protected bull spermatozoa from cold shock.

TABLE 1

EFFECT OF EGG YOLK ON THE DEGREE OF COLD SHOCK WITH BULL AND RAM SPERMATOZOA

½ Hr motility scores

Bull Number	Bull				Ram Number	Ram			
	20 Per Cent Egg Yolk Citrate		Buffered Glucose Sodium Chloride			20 Per Cent. Egg Yolk Citrate		Buffered Glucose Sodium Chloride	
	Control	Shocked	Control	Shocked		Control	Shocked	Control	Shocked
1	3·3	3·3	3·5	2·5	1	3·8	3·8	3·3	2·0
2	2·5	2·5	2·8	1·5	2	3·5	3·0	3·5	1·5
3	3·0	2·5	2·8	1·0	3	3·0	2·8	3·3	1·0
4	3·3	3·3	3·5	2·3	4	3·3	3·0	3·3	1·8
5	3·5	3·5	3·5	2·3	5	3·5	3·0	3·5	0·5
6	3·0	3·0	3·0	1·5	6	4·0	3·5	4·0	1·5
7	3·3	3·3	3·0	1·0					
Totals	21·9	21·4	22·1	12·1		21·1	19·1	20·9	8·3
Mean score	3·1	3·0	3·2	1·7		3·5	3·2	3·5	1·4

II. MATERIALS AND METHODS

Fresh bull and ram ejaculates of good initial motility were used in the tests. Bull semen was collected by means of an artificial vagina and ram semen by electrical ejaculation (Gunn 1936). Precautions were taken to avoid sudden changes in temperature and the ejaculates were used as soon after collection as possible.

The diluents used were (i) 3 per cent. glucose, 0·26 per cent. sodium chloride containing 20 ml of 0·1M phosphate buffer per 100 ml. The usual pH was 7·2; variations are recorded when necessary, (ii) 3 per cent. sodium citrate containing phosphate buffers as above.

A standard procedure was used to produce cold shock. Before dilution, the buffers were brought to the temperature of the semen, usually that of the

room, 0·2 ml of semen was added to 2·0 ml of diluent and gently mixed. The suspension was then split into equal parts, one was kept at room temperature, the other rapidly chilled by immersion in a bath of ice and water. After 10 min the chilled specimens were removed and allowed to return to room temperature. Observations of motility were made on the control tubes as soon after mixing as possible. After half an hour both control and chilled tubes were examined for motility. In some experiments observations were made for 2 or 3 hr but this was abandoned in the later tests.

Estimations of motility were made as described by Emmens (1947) in which full activity is scored as four, complete immotility as zero.

TABLE 2

EFFECT OF EGG YOLK AND SOME YOLK CONSTITUENTS ON THE OCCURRENCE OF COLD SHOCK

½ Hr motility scores

Diluent	Bull Ejaculates					Ram Ejaculates						
Control	1	2	3	4	Total	1	2	3	4	5	6	Total
(1) Glucose-sodium chloride Cold shocked	2·5	3·3	3·0	2·5	11·3	3·0	1·5	3·3	3·5	3·3	3·5	18·1
(2) Glucose-sodium chloride	1·0	2·5	0·5	0·5	4·5	0·5	1·0	1·0	2·0	1·0	1·0	6·5
(3) 1% Freeze-dried egg yolk	2·3	3·5	2·5	2·0	10·3	2·5	1·5	3·0	2·8	3·0	3·0	15·8
(4) 1% Acetone-insoluble residue of (3)	2·3	3·5	2·5	2·5	10·8	2·5	1·0	2·8	3·0	2·8	3·0	15·1
(5) 0·5% Acetone-soluble extract of (3)	1·0	2·0	0·5	0·5	4·0	1·5	0·0	2·0	3·0	1·8	1·0	9·3
(6) 0·5% Alcohol extract of (4)	2·3	3·0	3·0	2·0	10·3	2·5	1·5	2·8	3·3	3·0	2·8	15·9
(7) 0·5% Acetone precipitate of (6)	2·3	3·5	3·0	2·5	11·3	3·0	2·3	3·0	3·3	3·0	2·8	17·4
(8) 1% Acetone, alcohol, ether-insoluble residue of (3)	1·8	3·0	1·0	0·5	6·3	1·0	1·0	1·3	2·0	1·0	1·3	7·6

The data of Tables 1, 2, 5, and 6 were examined by the analysis of variance and in those cases where a *t*-test was later used, *t* was calculated using the error mean square and the mean of the differences between the treatments being compared. The degrees of freedom for the error mean square were used to determine probability levels.

When *t*-tests alone were used (Tables 3 and 4), *t* was calculated using the mean of the difference between treatment pairs. In the text the degrees of freedom are given in brackets after *t*.

III. Results

Preliminary experiments were made with 20 per cent. egg yolk in sodium citrate diluent to determine the protection afforded bull and ram spermatozoa against cold shock (Table 1). Analyses of the data showed significant treatment effects, $F = 48{\cdot}1$, $DF = 3$ and 18, $P < 0{\cdot}01$ (bull), and $F = 63{\cdot}9$, $DF = 3$ and 15, $P < 0{\cdot}01$ (ram). The two groups which had been cold shocked were compared using a t-test and $t_{(18)} = 9{\cdot}1$, $P < 0{\cdot}01$ for bull spermatozoa, while $t_{(15)} = 10{\cdot}1$, $P < 0{\cdot}01$ for ram spermatozoa. There were clearly no differences between the control groups and the cold-shocked group containing egg yolk, which shows the efficiency of egg yolk in preventing cold shock.

Table 3

EFFECT OF 0·5 PER CENT. CRUDE LECITHIN ON THE DEGREE OF COLD SHOCK

½ Hr motility scores

Ejaculates	Bull				Ram			
	Buffered Glucose Sodium Chloride		0·5 Per Cent. Crude Lecithin		Buffered Glucose Sodium Chloride		0·5 Per Cent. Crude Lecithin	
	Control	Shocked	Control	Shocked	Control	Shocked	Control	Shocked
1	2·5	0·5	2·5	2·3	3·5	2·5	3·5	3·5
2	3·5	2·0	3·5	3·0	2·8	2·0	2·8	2·8
3	3·0	1·5	3·0	3·0	4·0	2·3	4·0	3·0
4	3·0	2·0	3·0	3·0	2·5	1·5	2·5	2·0
5	2·8	0·0	2·8	2·5	3·5	2·0	3·5	2·8
6	2·5	1·8	2·5	1·8	2·8	1·5	2·8	2·5
7	2·5	0·5	2·8	3·3	3·5	1·5	3·3	2·8
8	2·8	1·5	3·0	3·0	2·8	2·0	2·8	2·8
9					2·5	1·5	2·8	2·8
10					3·0	1·5	3·0	2·8
Totals	22·6	9·8	23·1	21·9	30·9	18·3	31·0	27·8
Mean score	2·8	1·2	2·9	2·7	3·1	1·8	3·1	2·8

In order to find out the nature of any active substance in egg yolk, several extracts were made. Egg yolks were extracted several times in a blender with acetone, alcohol, and ether in succession. Exhaustive extraction was not attempted and the use of heat was avoided, all extractions being made at room temperature. The various extracts and residues were retained and dried *in vacuo* to remove all traces of the solvents.

The solvent-free products were suspended in the glucose-sodium chloride diluent and tested for capacity to prevent cold shock. The quantity of material obtained from the ether extraction was extremely small and no tests were made

with it. The results are given in Table 2 (bull and ram). Analyses of variance of the results showed significant diluent and ejaculate effects with both species. Comparisons were made between the glucose-sodium controls (1) and the corresponding cold-shocked group (2) and gave $t_{(21)} = 6{\cdot}5$, $P < 0{\cdot}001$ (bull) and $t_{(35)} = 8{\cdot}6$, $P < 0{\cdot}001$ (ram). Further comparisons were made between the control group (1) and the acetone-soluble group (5), $t_{(21)} = 6{\cdot}9$, $P < 0{\cdot}001$ (bull) and $t_{(35)} = 6{\cdot}8$, $P < 0{\cdot}001$ (ram) and also between (1) and the insoluble residue (8), $t_{(21)} = 4{\cdot}6$, $P < 0{\cdot}001$ (bull) and $t_{(35)} = 7{\cdot}7$, $P < 0{\cdot}001$ (ram). Other comparisons were made but the differences were not significant. These results indicated that the activity of egg yolk in preventing cold shock, was to be found in the alcohol-soluble fractions.

TABLE 4

EFFECT OF 0·5 PER CENT. PURE LECITHIN ON THE DEGREE OF COLD SHOCK

½ Hr motility scores

Ejaculates	Bull				Ram			
	Buffered Glucose Sodium Chloride		0·5 Per Cent. Pure Lecithin		Buffered Glucose Sodium Chloride		0·5 Per Cent Pure Lecithin	
	Control	Shocked	Control	Shocked	Control	Shocked	Control	Shocked
1	3·0	2·0	3·0	3·0	2·8	2·0	2·5	2·3
2	2·8	0·0	2·8	2·3	2·8	0·0	2·8	2·3
3	3·0	1·5	3·0	2·8	3·3	2·0	2·8	2·8
4	2·8	1·8	2·5	2·3	2·8	2·3	3·0	2·8
5	2·8	2·3	2·8	3·0	4·0	0·5	4·0	1·0
6	3·0	2·3	3·0	3·0	4·0	1·0	4·0	2·3
7	3·3	2·3	3·3	2·8	3·5	0·5	3·0	1·0
8	2·5	1·5	2·5	2·5	4·0	1·0	4·0	2·8
9	2·3	1·0	2·8	2·5	2·8	1·3	2·8	2·0
10	2·8	0·0	2·5	2·5	3·5	2·0	4·0	2·8
11	2·5	1·3	3·3	3·0	3·3	1·3	3·3	2·8
12	2·5	2·0	3·0	3·0	3·5	1·0	3·0	2·5
13	2·5	1·8	3·0	3·0	3·5	1·0	3·5	2·3
14					3·5	2·0	3·8	2·8
Total	35·8	19·8	37·5	35·7	47·3	17·9	46·5	32·5
Mean score	2·7	1·5	2·9	2·7	3·4	1·3	3·3	2·3

Further tests were made using the acetone precipitate of the alcohol-soluble extract (Table 3), and *t*-tests were made comparing the two cold-shocked groups which gave $t_{(7)} = 4{\cdot}8$, $P < 0{\cdot}01$ (bull), and $t_{(9)} = 10{\cdot}5$, $P < 0{\cdot}001$ (ram), clearly indicating the superiority of the yolk extract.

The fatty nature of the active extract, insolubility in acetone, and solubility in alcohol suggested that an active substance in egg yolk might be lecithin,

which is the principle phospholipid present (Romanoff and Romanoff 1949). Pure lecithin was prepared from egg yolks by the method of Levene and Rolf (1927) and later by that of Pangborn (1951), the latter being preferred because of the relative ease of preparation. The lecithin was stored under acetone in the refrigerator and before use was dried *in vacuo* for at least ½ hr before suspension in buffered glucose-sodium chloride.

TABLE 5

LECITHIN CONCENTRATION AND THE DEGREE OF COLD SHOCK

½ Hr motility scores

Bull Ejaculates	Control	Cold Shocked				
		Glucose Sodium Chloride	Lecithin (% w/v)			
			0·50	0·25	0·12	0·06
1	2·3	1·3	2·5	2·5	2·8	1·3
2	3·5	2·8	3·5	3·8	3·5	3·8
3	2·8	2·0	2·5	2·5	2·5	2·3
4	2·5	2·0	2·5	2·5	2·5	2·5
5	2·8	1·0	3·3	3·3	2·8	2·8
6	2·8	1·5	3·0	3·0	2·8	2·3
Totals	16·7	10·6	17·3	17·6	16·9	16·2
Ram Ejaculates						
1	4·0	1·0	1·0	2·0	2·0	2·5
2	3·5	1·0	1·5	2·5	2·5	2·0
3	4·0	2·3	3·3	3·3	3·5	3·0
4	4·0	2·8	3·5	3·5	3·3	3·5
5	3·5	1·0	2·8	3·3	3·3	3·3
6	3·5	1·0	2·8	3·0	2·8	2·5
Totals	22·5	9·1	14·9	17·6	17·4	16·8

Tests of the pure lecithin were made (Table 4), the results of a *t*-test between the two cold-shocked groups indicating ability of lecithin to prevent cold shock, $t_{(12)} = 5{\cdot}5$, $P < 0{\cdot}001$ (bull), and $t_{(13)} = 6{\cdot}5$, $P < 0{\cdot}001$ (ram). It may be noted, however, that protection with ram spermatozoa was far from complete and comparison of the two lecithin-treated groups gave $t_{(13)} = 2{\cdot}8$, $P < 0{\cdot}02$.

The effects of various levels of lecithin were observed (Table 5) and analysis showed no significant differences between levels in both species. All levels of lecithin afforded complete protection from cold shock with bull spermatozoa but in the case of the ram, as shown by comparison of the 0·25 per cent.

level with the glucose-sodium chloride control ($t_{(25)} = 3{\cdot}3$, $P < 0{\cdot}01$) only partial protection occurred.

All the previous experiments were carried out at a pH of 7·0 to 7·2, but subsequently it was found that pH played an important part in the action of lecithin in preventing cold shock with ram spermatozoa, although this does not appear to be so with bull spermatozoa.

TABLE 6

EFFECT OF pH ON THE ACTION OF 0·25 PER CENT. LECITHIN IN PREVENTING COLD SHOCK IN RAMS

½ Hr. motility scores

	0·25 Per Cent. Lecithin			Buffered Glucose Sodium Chloride
Nominal pH Range	6·5	7·2	8·0	7·2
	6·2-6·6	7·0-7·2	7·4-7·7	6·9-7·2
Ejaculates				
1	3·5	2·5	2·3	0·5
2	3·3	2·3	2·0	1·0
3	3·0	1·5	1·0	1·3
4	2·5	2·0	1·0	0·5
5	3·3	2·3	2·0	0·5
6	3·3	2·0	2·0	1·5
7	2·8	1·5	2·0	0·0
8	2·3	2·3	2·0	0·0
9	2·8	1·3	1·0	0·0
10	4·0	3·0	2·5	2·0
11	3·0	2·0	1·0	1·5
12	3·5	2·5	1·5	1·5
13	3·3	2·5	1·5	1·5
14	3·3	2·8	1·0	1·0
15	2·8	2·0	2·0	1·5
Totals	46·7	32·5	24·8	14·3
Mean score	3·1	2·2	1·7	1·0

Lecithin at 0·25 per cent. concentration was suspended in a diluent containing 3 per cent. glucose, 0.26 per cent. sodium chloride, and buffered with 0·1M sodium phosphates, which were added at the rate of 20 ml buffer per 100 ml total diluent. The nominal pH levels used were 6·5, 7·2, and 8·0, variations are recorded in Table 6, which contains the results from 15 ram ejaculates. Analysis of variance of the results is given in Table 7 and clearly shows that pH 6·5 gave best results, protection from cold shock being greatly decreased above pH 7·2.

IV. Discussion

The occurrence of cold shock has been demonstrated in the spermatozoa of several species, and means to avoid it by slow cooling or the addition of egg yolk have been suggested. The observations presented here point to the phospholipid lecithin as a factor in egg yolk which will considerably reduce the damage caused by cold shock, as judged by motility observations. The good protection afforded to ram spermatozoa at the rather low pH 6·5 is somewhat less than that for optimal spermatozoal activity (pH 6·9) but this may be related to the isoelectric point of lecithin which Bull and Frampton (1936) give as pH 6·4.

The nature of the change in the cell caused by sudden temperature changes is unknown, but Walton (1947) believes it to be a surface phenomenon, involving damage to the motor mechanism and flagellar surface. The loss of resistance to cold shock which occurs on ejaculation may support this view, especially as Milovanov (1934) states that the accessory secretions remove the lipoid capsule from the spermatozoa. The presence of egg yolk or lecithin may repair the deficiency.

Table 7

SUMMARY ANALYSIS OF VARIANCE OF THE DATA IN TABLE 6

Source of Variation	Degrees of Freedom	Variance Ratio†
Ejaculates	14	3·6*
Diluents		
1. Lecithin *v.* no lecithin	1	122·0*
2. pH 6·5 *v.* 7·2, and 8·0	1	85·2*
3. pH 7·2 *v.* 8·0	1	11·6*
Residual	42	*0·17*

*$P<0·01$.

†The actual value of the residual variance is given in the last column in italics.

The occurrence of phospholipids in human spermatozoa has been demonstrated by Wislocki (1950) and Brown (1952), the midpiece in particular showing their presence. More recently Boguth (1952) has investigated the plasmalogen content of egg yolk and bull semen and has found that the 20 per cent. egg yolk used in semen diluents may be completely replaced by 0·1 per cent. of phosphatides derived from the egg yolk. The total content of plasmalogen in bull semen is given as 0·3 to 0·9 mg/ml, of which one-third is found in the seminal plasma.

The observations recorded in this report are in accord with the brief account of Walton (1947) but differ in all respects from those of Mayer and Lasley (1944, 1945). The active substance isolated by them was a non-lipoidal white crystal-like material, while the acetone-insoluble, alcohol-soluble fraction of egg yolk, described as toxic, contains principally lecithin and cephalin which

have been amply shown as very beneficial to spermatozoa. No explanation of this apparent contradiction can be given. However, recently Kampschmidt, Mayer, and Herman (1953) state that lipoprotein and the phospholipids which occur in egg yolk will protect bull spermatozoa from cold shock, but the evidence presented for the action of the phospholipids is not clear. Also the lipoprotein will replace egg yolk for the storage of spermatozoa.

The part played by phospholipids in the physiology of the cell is not clear, but it has been shown (Lardy and Phillips 1941*a*) that egg lecithin decreases the respiration and greatly prolongs the motility of bull spermatozoa in Ringer phosphate; oxygen consumption is not appreciably increased by lecithin added to a glucose medium, and this Lardy and Phillips (1941*b*) state is an indication that the intracellular reserves of the spermatozoa are phospholipid in nature. Sea urchin spermatozoa also utilize endogenous phospholipid as a source of energy (Rothschild and Cleland 1952).

Milovanov and Selivanova (1932) found that lecithin improves the viability of stored bull semen while Phillips and Spitzer (1946) recommend the use of lecithin in their synthetic pabulum for bull semen; the fertility rates for the latter being equal to those obtained using the normal egg yolk diluent. It would thus seem reasonable to use lecithin for the prevention of cold shock, with the expectation of no undesirable side effects.

V. References

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