THE EFFECTS OF INCUBATION TEMPERATURE AND COLD SHOCK ON THE METABOLISM OF RAM SPERMATOZOA

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Summary

The glycolysis of ram semen was measured at 37, 21, and 5°C. Measurements of Q_{10} over this range gave values between about 2 and 3 for changes in lactic acid and total reducing substances. Cold shock greatly reduced glycolysis and dehydrogenase activity which were only partially maintained by egg yolk and lecithin.

I. Introduction

The motility of spermatozoa may be reduced by decreasing the environmental temperature and this has been used as a means of storing semen for artificial insemination. The metabolic activity of semen may be expected to show a similar decline to motility and a few reports have shown that this does occur (Gladcinova 1936; Moore and Mayer 1941; Mann 1946; Blackshaw, Salisbury, and VanDemark 1957).

Linked with the effects of low temperature on metabolic functions is the phenomenon of cold or temperature shock produced by a rapid fall in temperature, and characterized by irreversible loss of motility. It has been shown by Walton (1947), Kampschmidt, Mayer, and Herman (1953), and Blackshaw (1954*a*) that various lipid constituents of egg yolk as well as the yolk itself are of value in preventing cold shock.

Metabolic studies of the consequences of cold shock are few, but indicate that metabolism is severely affected and the normal permeability of the cells is destroyed (Walton 1942; Mayer 1955; Mann and Lutwak-Mann 1955; Blackshaw and Salisbury 1957). This paper describes experiments in which the glycolysis of ram spermatozoa was measured at 5, 21, and 37°C. The effects of cold shock on the metabolic rate and the protective action of egg yolk and lecithin were also studied.

II. Materials and Methods

Ram semen was collected by the electrical stimulation of ejaculation (Blackshaw 1954*b*) and was used for the metabolic studies within 3 hr.

The diluents used in all tests were modifications of that described by White (1953). At pH 7·0 the diluent contained 0·048M $Na_2HPO_4.12H_2O$, 0·032M $NaH_2PO_4.2H_2O$, and 0·040M NaCl and 200 mg per cent. fructose. At pH 7·5 the

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amounts of $Na_2HPO_4.12H_2O$ and $NaH_2PO_4.2H_2O$ were 0·058M and 0·018M respectively and at pH 6·5, 0·022M and 0·066M. In some experiments egg yolk was added to the diluent to give a concentration of 20 or 50 per cent. (v/v).

TABLE 1

EFFECT OF INCUBATION TEMPERATURES AND THE PRESENCE OF EGG YOLK ON THE METABOLISM AND MOTILITY OF RAM SPERMATOZOA

Each result is the mean of 9 ejaculates

Temperature (°C)	Egg Yolk (%)	Final Motility	pH	(*A*) Decrease in Total Reducing Substances ($\mu g/10^8$ cells/hr)	(*B*) Increase in Lactic Acid ($\mu g/10^8$ cells/hr)	Ratio *B*/*A*
37	0	2·6	6·60	121	97	0·80
	20	1·7	6·40	140	100	0·71
21	0	2·3	6·75	25	21	0·84
	20	2·1	6·65	52	30	0·58
5	0	2·4	6·85	−8	4	—
	20	2·6	6·70	1	14	14·00

Analyses of Variance

Source of Variation	Degrees of Freedom	Total Reducing Substances		Lactic Acid	
		Mean Square	Variance Ratio	Mean Square	Variance Ratio
Ejaculates (1)	8	2,376	12·2**	6,796	60·7**
Yolk (2)	1	4,630	23·8**	690	6·2*
Temperature (3)	2	84,444	436·2**	40,631	363·0**
Interactions					
1×2	8	1,540	7·9*	670	6·0**
1×3	16	1,497	7·7*	1,957	17·5**
2×3	2	2,855	14·6**	58	0·5
Residual	16	194·5		111·9	

*$P<0·05$. **$P<0·01$.

A partially purified preparation of lecithin was made by extracting the acetone-insoluble fraction of egg yolk in a blendor with 95 per cent alcohol. This was vacuum-dried, dissolved in ether, and precipitated by acetone in which it was stored. A 1 per cent. (w/v) suspension of the extract dried under vacuum was used in the tests.

For the experiments on temperature effects the neat semen was slowly cooled to 5°C and diluted 1 to 4 with the test diluent. Samples were taken for the deter-

mination of initial lactic acid and reducing sugar levels. The cold, diluted semen was then rewarmed to the required incubation temperature. The period of incubation was 4 hr at 37°C and 8 hr at 21 and 5°C.

To produce cold shock, 1·5 ml of semen, diluted at room temperature, was chilled by plunging into an ice-bath for 10 min. Samples were taken from the chilled and control tubes for chemical analysis immediately before incubation at 37°C and at the end of 3 hr.

Lactic acid was estimated by the method of Barker and Summerson (1941) and total reducing substances by a method using the chromogenic mixture of Nelson (1944) and the reducing agent of Somogyi (1952).

TABLE 2

Q_{10} FOR LACTIC ACID ACCUMULATION AND THE CHANGE IN TOTAL REDUCING SUBSTANCES

Results from means of 9 ejaculates

Temperature Range (°C)	Egg Yolk (%)	Lactic Acid Accumulation	Change in Total Reducing Substances
37–21	0	2·6	2·7
	20	2·1	1·9
37–5	0	2·7	—
	20	1·9	4·8

The effect of cold shock on the dehydrogenase activity of semen was estimated by the reduction of 2,3,5-triphenyltetrazolium chloride (Kun and Abood 1949). The diluted semen (1 : 4) in 1·5-ml amounts was mixed with sufficient dye to give a concentration of 0·25 mg/ml. A layer of liquid paraffin (2 ml) was added and the tubes incubated for 2 hr at 37°C in the dark. One-ml aliquots of semen were removed, mixed with 7 ml of acetone, and centrifuged to obtain a clear supernatant. The optical density was measured at 495 μ in a Coleman model 14 spectrophotometer.

The motility of spermatozoal suspensions was scored by the method of Emmens (1947) in which maximum motility is scored as 4 and complete immotility as 0.

The Q_{10} values were calculated from the following equation:

$$\log Q_{10} = \frac{10\ (\log k_1 - \log k_2)}{(t_1 - t_2)},$$

where k_1 and k_2 are the velocity constants at the temperatures t_1 and t_2.

III. RESULTS

The effects on metabolism of incubation temperatures of 37, 21, and 5°C are given in summary form (Table 1). Analysis of variance of both the lactic acid and reducing sugar changes (Table 1) showed that egg yolk increased the metabolic rate

but the effect was small. The mean values from Table 1 were used to calculate Q_{10} for the temperature ranges of 37–21°C and 37–5°C (Table 2).

Further tests were made to observe the effects of cold shock on the motility and metabolism of ram spermatozoa. The influence of 20 per cent. (v/v) egg yolk on glycolysis following shock was measured at 37°C (Table 3). In this series the yolk

TABLE 3

EFFECT OF COLD SHOCK ON THE METABOLISM AND MOTILITY OF RAM SPERMATOZOA

Results are means of 9 ejaculates

Treatment	Egg Yolk (%)	Final Motility	(*A*) Decrease in Total Reducing Substances (μg/10^8 cells/hr)	(*B*) Increase in Lactic Acid (μg/10^8 cells/hr)	Ratio *B*/*A*
Control	0	3·0	137	115	0·87
	20	2·7	129	96	0·74
Shock	0	0·0	−9	9	—
	20	1·5	34	47	1·40

Analyses of Variance

Source of Variation	Degrees of Freedom	Total Reducing Substances		Lactic Acid	
		Mean Square	Variance Ratio	Mean Square	Variance Ratio
Ejaculates (1)	8	3,686	2·5	3,504	3·3*
Shock (2)	1	132,860	89·3**	53,592	50·2**
Yolk (3)	1	2,584	1·7	702	0·7
Interactions					
1×2	8	2,574	1·7	1,685	1·6
1×3	8	383	0·3	923	0·9
2×3	1	5,256	3·5	7,253	6·8*
Residual	8	1,488		1,067	—

*$P<0·05$. **$P<0·01$.

did not affect the metabolism of the control semen and only the lactic acid production of shocked semen was significantly improved (Table 3) by egg yolk. Similar results were obtained when 1 per cent. (w/v) lecithin was used in place of yolk; the pH of the medium did not affect the results with shocked semen but at pH 6·5 lactic acid accumulation in the controls was reduced (Table 4).

The effect of different temperatures on the development of cold shock was studied using 50 per cent. egg yolk and 1 per cent. lecithin as protective agents.

The diluted semen was cooled from 30·0 to 22·5, 15·0, 7·5, and 0°C and held at these temperatures for 10 min and then incubated at 37°C for 3 hr. The mean lactic acid changes for seven ejaculates and summary analyses of variance for each diluent are given in Table 5.

TABLE 4

EFFECT OF LECITHIN ON THE ACCUMULATION OF LACTIC ACID BY RAM SPERMATOZOA AFTER COLD SHOCK

Lecithin (% w/v)	Control pH	Lactic Acid Accumulation (μg/10^8 cells/hr)	Shock pH	Lactic Acid Accumulation (μg/10^8 cells/hr)
0	6·5	66	6·5	11
	7·5	99	7·5	15
1	6·5	78	6·5	39
	7·5	95	7·5	34

Analyses of Variance

Source of Variation	Degrees of Freedom	Control		Shock	
		Mean Square	Variance Ratio	Mean Square	Variance Ratio
Ejaculates (1)	5	4641	18·8**	564	0·9
Lecithin (2)	1	85	0·3	3197	5·0*
pH (3)	1	3798	15·4**	0	0·0
Interactions					
1×2	5	161	0·6	242	0·4
1×3	5	1040	4·2	614	0·9
2×3	1	369	1·5	126	0·2
Residual	5	246		640	

*$P<0·05$. **$P<0·01$.

The damage produced by cold shock on the dehydrogenase systems of ram spermatozoa was demonstrated by the reduction of 2,3,5-triphenyltetrazolium chloride. Preliminary tests with normal ram semen showed that the reduction was slow aerobically but under a layer of liquid paraffin reduction proceeded rapidly. This technique was used in the experimental procedure.

After cold shock the semen was incubated at 37°C for 2 hr with the dye and the colour extracted with acetone. The mean values for the optical density readings are given in Table 6 for both egg yolk and lecithin. The analyses of variance (Table 6) indicate that both substances partially prevented the decline in dehydrogenase activity produced by cold shock.

IV. Discussion

The metabolic activity of ram sperm is greatly decreased by a reduction in incubation temperature and the Q_{10} obtained show that the effect is similar to that found in other tissues in which a Q_{10} of between 2 and 3 is common. It is clear that ram spermatozoa are very susceptible to rapid temperature changes and that egg

Table 5

Effect of diluent and the degree of cold shock on the accumulation of lactic acid by ram spermatozoa

Results are means of 7 ejaculates

Shock Temperature (°C)	Lactic Acid Accumulated (μg/10^8 cells/hr)		
	Control	50% Egg Yolk	1% Lecithin
30·0	98	114	106
22·5	53	73	116
15·0	67	72	106
7·5	26	69	75
0·0	8	39	5

Analyses of Variance

Source of Variation	Degrees of Freedom	Variance Ratios		
		Control	Egg Yolk	Lecithin
Ejaculates	6	13·7**	4·7*	42·3**
Temperature	(4)			
Linear	1	25·8**	9·8**	17·0**
Quadratic	1	0·0	0·1	6·8*
Cubic	1	0·8	1·8	0·1
Quartic	1	3·2	0·0	0·0
Residual	24	1180†	1671†	2430†

*$P<0·05$. **$P<0·01$.

†Residual mean squares.

yolk and lecithin do not give sufficient protection against severe cold shock to enable the spermatozoa to maintain their full metabolic activities during a subsequent period of incubation at 37°C.

Over a temperature shock range from 30 to 0°C the decline in metabolic activity was linear for both the control semen and that diluted with the yolk medium but at 7·5°C the semen in the lecithin diluent showed an abrupt decline in lactic acid accumulation not seen in the semen in egg yolk.

These observations are in marked constrast to those described for bull semen by Blackshaw and Salisbury (1957). In this species egg yolk and lecithin were both highly effective in preventing the occurrence of cold shock and also in maintaining the metabolism of the semen during subsequent incubation at 37°C. In addition, yolk stimulated the glycolysis of unshocked semen.

TABLE 6

EFFECT OF EGG YOLK AND LECITHIN ON THE OCCURRENCE OF COLD SHOCK AS SHOWN BY THE REDUCTION OF 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE

Results given as mean optical densities

	Egg Yolk (% v/v) (mean of 6 ejaculates)	Optical Density	Lecithin (% w/v) (mean of 9 ejaculates)	Optical Density
Control	0	0·396	0	0·256
	20	0·262	1	0·250
Shock	0	0·202	0	0·106
	20	0·231	1	0·188

Analyses of Variance

Source of Variation	Degrees of Freedom	Variance Ratio	Degrees of Freedom	Variance Ratio
		Egg Yolk		Lecithin
Ejaculates	5	9·00**	8	15·35**
Shock	1	20·83**	1	34·8**
Agent	1	4·54	1	4·56*
Shock × agent	1	10·98**	1	6·20*
Error	15	0·0036†	24	0·0028†

*$P < 0·05$. **$P < 0·01$.

†Error mean squares.

The usual temperature for the storage of semen for artificial insemination has been 5°C, but it has been indicated (Emmens and Blackshaw 1956) that the fertility of stored ram semen is low. Recently it has been shown that at 20°C, in the presence of CO_2, the fertile life of bull semen could be extended to 1 week (VanDemark and Sharma 1957). In view of the high sensitivity of ram spermatozoa to cold shock and the relative inefficiency of protective agents, the use of this method of storage for ram semen at 20°C may be preferable.

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VI. References

Barker, S. B., and Summerson, W. H. (1941).—*J. Biol. Chem.* **138**: 535.
Blackshaw, A. W. (1954*a*).—*Aust. J. Biol. Sci.* **7**: 573.
Blackshaw, A. W. (1954*b*).—*Aust. Vet. J.* **30**: 249.
Blackshaw, A. W., and Salisbury, G. W. (1957).—*J. Dairy Sci.* **40**: 1093.
Blackshaw, A. W., Salisbury, G. W., and VanDemark, N. L. (1957).—*J. Dairy Sci.* **40**: 1099.
Emmens, C. W. (1947).—*J. Physiol.* **106**: 471.
Emmens, C. W., and Blackshaw, A. W. (1956).—*Physiol. Rev.* **36**: 277.
Gladcinova, E. F. (1937).—*Usp. Zootek. Nauk* **4**: 56. (Anim. Breed. Abstr. **7**: 373 (1939).)
Kampschmidt, R. F., Mayer, D. T., and Herman, H. A. (1953).—*J. Dairy Sci.* **36**: 733.
Kun, E., and Abood, L. G. (1949).—*Science* **109**: 144.
Mann, T. (1946).—*Biochem. J.* **40**: 481.
Mann, T., and Lutwak-Mann, C. (1955).—*Arch. Sci. Biol.* **39**: 578.
Mayer, D. T. (1955).—Mich. St. Univ. Centenn. Sym. p. 45.
Moore, B. H., and Mayer, D. T. (1941).—Res. Bull. Mo. Agric. Exp. Sta. No. 338.
Nelson, N. A. (1944).—*J. Biol. Chem.* **153**: 375.
Somogyi, M. (1952).—*J. Biol. Chem.* **195**: 19.
Walton, A. (1947).—"The Family Planning Association Conference on Infertility, London." p. 36. (Oxford Univ. Press.)
White, I. G. (1953).—*J. Expt. Biol.* **30**: 200.
VanDemark, N. L., and Sharma, U. D. (1957).—*J. Dairy Sci.* **40**: 438.