THE EFFECT OF COLD SHOCK ON SPERMATOZOA

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Summary

The effect of cold shock on bull and ram spermatozoa was studied by rapidly cooling semen suspensions to 0°C over 3 min. Bull spermatozoa that survived this treatment were less susceptible to subsequent rapid cooling.

Dilute suspensions of bull spermatozoa were more susceptible to cold shock than more concentrated samples.

Reconstituted skim milk powder and proteins partially protected spermatozoa from cold shock. The protein content of milk probably explains this beneficial effect during rapid cooling.

I. Introduction

The motility of ram and bull spermatozoa is irreversibly reduced by sudden cooling to 0°C, the phenomenon known as cold or temperature shock. It is accompanied by loss of ions and other substances from the cell (Mann and Lutwak-Mann 1955; Blackshaw and Salisbury 1957); there is disruption of the cell surface (Walton 1957) and an increase in the proportion of cells staining with dyes such as eosin or congo red (Easley, Mayer, and Bogart 1942; Lasley, Easley, and McKenzie 1942; Hancock 1951; Wales and White 1959).

Various diluents have been used to prevent the irreversible loss of motility of spermatozoa caused by the rapid fall in temperature. Walton (1947), Kampschmidt, Mayer, and Herman (1953), and Blackshaw (1954*a*, 1958) reported that the lipid constituents of egg yolk as well as the yolk itself are of value in preventing cold shock. However, Blackshaw and Salisbury (1957) and Blackshaw (1958) stated that lecithin does not give sufficient protection against severe cold shock to enable the spermatozoa to maintain their full metabolic activities.

Milk has been used successfully as a diluent for bull spermatozoa. Erickson, Graham, and Frederick (1954) and Mixner and Saroff (1954) successfully preserved bull spermatozoa in a milk–glycerol diluent. O'Dell and Almquist (1957) stated that heated and cysteine-treated homogenized milk and skim milk diluents gave survival rates after freezing equal to those obtained with egg yolk–citrate diluents. They also reported that spermatozoa frozen in milk diluents maintained a higher percentage motility after thawing and storage at 5°C than spermatozoa frozen in egg yolk–citrate. Thus, it is of interest to study the effect of milk and milk constituents on the reaction of spermatozoa to cold shock.

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II. Materials and Methods

(*a*) *Semen Collection*

An artificial vagina was used for the collection of bull semen; ram semen was collected after electrical stimulation with the bipolar electrode of Blackshaw (1954*b*). During collection semen samples were carefully protected from sudden temperature changes and only apparently normal ejaculates with high initial motility were used for the motility studies.

Table 1

PERCENTAGE OF MOTILE BULL SPERMATOZOA AFTER REPEATED COOLING TO 0°C

Mean values for six ejaculates are given

No. of Times Shocked	Mean No. of Motile Spermatozoa (%)	No. of Times Shocked	Mean No. of Motile Spermatozoa (%)
0 (control)	78·6	3	26·8
1	37·8	4	25·0
2	28·6		

Summary of the Analysis of Variance after Logarithmic Transformation

Source of Variation	Degrees of Freedom	Variance Ratio
Between ejaculates	5	12·2**
Between treatments		
Linear	1	24·0**
Quadratic	1	9·0**
Cubic	1	0·1
Quartic	1	0·1
Residual	20	0·05

** $P<0·01$.

(*b*) *Diluents*

Isotonic synthetic diluent (pH 7) consisted of 0·0136M $Na_2HPO_4.12H_2O$, 0·0064M $NaH_2PO_4.2H_2O$, 0·005M KCl, 0·0015M $MgCl_2$, 0·0641M NaCl, and 0·1111M glucose. In some experiments bovine albumin, bovine globulin, egg albumin, casein, acacia, alanine, and serine were added to the diluent, and the sodium chloride content adjusted to keep the diluent isotonic.

Reconstituted skim milk diluent consisted of 10% (w/v) skim milk powder in glass-distilled water. Such a diluent contains the equivalent of 3% (w/v) casein. To

construct diluents containing less milk, appropriate amounts of the synthetic diluent were added.

(c) *Techniques*

To study the effect of sudden temperature change, four drops of diluent were kept at 0°C for 5 min before adding two drops of semen which had been held at 30°C. The semen–diluent mixture was left for 3 min in an ice-bath in order to reach 0°C

TABLE 2

EFFECT OF DILUTION ON THE MOTILITY OF BULL SPERMATOZOA AFTER COLD SHOCK

Mean values for six ejaculates are given

Ratio of Semen to Seminal Plasma	Shock Temperature	
	30°C (control)	0°C
1 : 0	4·0	2·3
1 : 1	3·9	2·3
1 : 3	3·8	1·9
1 : 7	3·6	1·2

Summary of the Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
Effect of dilution	3	16·3**
Effect of shock	1	489·3**
Ejaculate differences	5	17·7**
Interactions:		
Dilution × shock	3	3·8*
Dilution × ejaculate	15	0·5
Shock × ejaculate	5	12·7**
Residual	15	0·086

* $P<0·05$. ** $P<0·01$.

before transferring to a water-bath at 30°C. For motility studies drops of spermatozoal suspension were examined under a low-power microscope at 0, 1, and 2 hr for bull semen and 0, $\frac{1}{2}$, and 1 hr for ram semen. Motility was scored by the system of Emmens (1947); full motility was rated as 4 and complete immobility as zero. In some experiments visual estimates of the percentage of motile spermatozoa were made at the same time.

(d) *Preparation of Extracts*

(i) *Ethanol Extracts.*—One volume of reconstituted skim milk powder (10% w/v) was precipitated with 6 volumes of redistilled ethanol and centrifuged.

(ii) *Dialysed Extracts.*—A 10-ml sample of reconstituted skim milk powder was dialysed against 100 ml of glass-distilled water for 24 hr, the dialysate replaced with 100 ml of glass-distilled water, and dialysis continued for another 24 hr. "Cellophane" was used as a membrane.

The ethanol-soluble and dialysable fractions were evaporated to dryness *in vacuo* at 40°C and each made up to the original volume with glass-distilled water. The ethanol-insoluble and non-dialysable portions were lyophylised and made up to the original volume with synthetic isotonic diluent.

TABLE 3

EFFECT OF VARIOUS SUBSTANCES ON THE REACTION OF BULL SPERMATOZOA TO COLD SHOCK

Values represent the mean for six ejaculates in each experiment

Substance Added	Experiment A		Experiment B	
	Concentration (mg/100 ml)	Mean Motility	Concentration (g/100 ml)	Mean Motility
Nil (shocked control)	—	0·9	—	1·3
Bovine albumin	250	1·1	1	1·7**
Bovine globulin	250	1·1	1	1·3
Egg albumin	250	1·0	1	1·7**
Casein	250	1·3*	1	1·9**
Acacia	250	1·0	—	—
Alanine	1000	0·6	—	—
Serine	1000	0·7	—	—
Nil (unshocked control)	—	3·5**	—	—

* Significantly different from shocked control at $P<0·05$.

** Significantly different from shocked control at $P<0·01$.

(e) *Statistical Analysis*

The significance of the results has been assessed by analyses of variance which are presented in summary form giving only degrees of freedom and variance ratios for each source of variation.

Where numbers of independent treatments have been compared with controls, the standard error of the difference between each treatment and the control mean has been calculated from the interaction mean square of the analysis of variance. The significance of the difference between means has then been assessed by the *t*-test using the degrees of freedom associated with the interaction mean square from the analysis of variance.

III. RESULTS

In preliminary tests of the effect of cold shock on bull spermatozoa, samples of six ejaculates were repeatedly cooled from 30 to 0°C in synthetic diluent. The results (Table 1) were transformed to logarithms for the analysis of variance, which

is presented in summary form in Table 1. There was a significant deviation from linearity in the analysis, indicating that the cells which survive the initial treatment are less susceptible to subsequent cold shock.

TABLE 4

EFFECT OF DIFFERENT CONCENTRATIONS OF CASEIN ON THE MOTILITY OF RAM AND BULL SPERMATOZOA SUDDENLY COOLED TO 0°C

Mean values for six ejaculates are given

Treatment of Spermatozoa	Concentration of Casein in Synthetic Diluent (%)	Motility after Shock	
		Ram Spermatozoa	Bull Spermatozoa
Unshocked	Nil	3·9	3·4
Shocked	Nil (control)	1·2	1·4
,,	0·04	2·0	1·9
,,	0·20	2·1	2·1
,,	1·00	2·0	2·4
,,	4·00	2·4	2·5

Summary of the Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		Ram Spermatozoa	Bull Spermatozoa
Between treatments			
Overall effect of shock	1	80·7**	74·9**
Shocked control *v.* casein diluents	1	17·6**	25·4**
Within casein diluents			
Linear	1	1·9	12·4**
Quadratic	1	0·6	0·1
Cubic	1	1·1	1·7
Ejaculate differences	5	15·7**	9·7**
Interaction (error)	25	0·253	0·128

** $P<0·01$.

As dilution is known to decrease the viability of spermatozoa, the effect of cold shock on bull spermatozoa at various dilutions was studied. Aliquots of six ejaculates were mixed with varying proportions of pooled seminal plasma obtained by centrifuging fresh bull ejaculates. Samples of undiluted and diluted semen were rapidly cooled to 0°C and subsequent motility compared with that of diluted but unshocked controls. Even at the low dilution rates used there was a decrease in the motility of diluted controls (Table 2). The effect of dilution, however, was even more marked in the shocked suspensions than in the unshocked specimens.

Bovine albumin, bovine globulin, egg albumin, casein, and acacia (all at 250 mg/100 ml), and alanine and serine (both at 1 g/100 ml) were added to aliquots of the synthetic diluent and tested for their ability to prevent the adverse effect of cold shock. The results with six ejaculates are shown in Table 3. The standard error calculated from the interaction mean square of the analysis of variance was 0·17

TABLE 5

COMPARISON OF THE EFFECT OF MILK AND CASEIN ON THE MOTILITY OF BULL SPERMATOZOA SUDDENLY COOLED TO 0°C

Mean values for six ejaculates are given

Concn. of Casein in Diluent (%)	Motility after Shock	
	Synthetic Diluent	Milk Diluent
0	0·5	0·7
1	1·3	1·5
3	1·7	1·9

Summary of the Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
Ejaculate differences	5	3·3*
Milk *v.* synthetic diluent	1	3·2
Effect of casein concentration		
Linear	1	81·1**
Quadratic	1	2·9
Interactions		
Concentration × diluent	2	0
Diluent × ejaculate	5	1·6
Concentration × ejaculate	10	1·1
Residual	10	0·106

* $P<0·05$. ** $P<0·01$.

(d.f. = 40) and has been used for the estimation of t. Casein was the only substance which significantly decreased the effect of cold shock. In further tests the concentration of the proteins in the diluent was raised to 1000 mg/100 ml. A standard error of 0·18 (d.f. = 25) was calculated from the results with six ejaculates (Table 3). At this concentration bovine albumin and egg albumin, as well as casein, gave partial protection against the detrimental effect of cold shock.

The ability of varying concentrations of casein to prevent cold shock of bull and ram spermatozoa was tested. Ram spermatozoa were used in this experiment

because they are even more severely affected by rapid change in temperature than are bull spermatozoa. The results of tests with six ejaculates for each species are shown in Table 4. The beneficial effect of casein on bull spermatozoa during rapid cooling increased as the concentration of casein increased. Casein also proved beneficial to ram spermatozoa during cold shock, but a concentration of 0·04% was about as effective as the higher concentrations tested.

Preliminary experiments showed that reconstituted skim milk diluent also protected bull spermatozoa from the effects of cold shock. To test if this beneficial effect was due to the casein content of the milk, synthetic diluents containing 1 or 3% casein were compared with skim milk diluents containing an equivalent amount of casein. Samples of six bull ejaculates were rapidly cooled to 0°C in the diluents

TABLE 6

EFFECT OF MILK EXTRACTS ON THE MOTILITY OF BULL SPERMATOZOA SHOCKED TO 0°C

Mean values for six ejaculates are given

Treatment of Spermatozoa	Diluent	Casein Content (%)	Motility after Shock
Unshocked	Synthetic control	0	3·7**
Shocked	Synthetic control	0	1·4
,,	Reconstituted skim milk powder	1	2·1**
,,	Ethanol-soluble milk extract	0	1·4
,,	Ethanol-insoluble milk extract	1	2·2**
,,	Dialysable milk extract	0	1·2
,,	Non-dialysable milk extract	1	2·2**
,,	Casein in synthetic diluent	1	2·1**
,,	Heated whole milk diluent	1	2·0**

** Significantly different from shocked control at $P<0·01$.

and the motility during the subsequent 2 hr is shown in Table 5. The casein-containing diluents were equally as effective as skim milk diluents in preventing the effects of cold shock.

Further proof that milk proteins prevent the effects of cold shock was obtained by using various milk extracts. Samples of the extracts were diluted with two volumes of synthetic diluent and their ability to prevent the effect of cold shock compared with that of similarly diluted skim milk diluents. The results for six bull ejaculates are shown in Table 6. A standard error of 0·16 (d.f. = 40) was calculated from the analysis of variance and used for the estimation of t. Both the ethanol-insoluble and the non-dialysable fractions were as effective as either casein or milk in preventing the loss of motility subsequent to cold shock.

IV. Discussion

The work of Wales and White (1959) suggests that resistance to cold shock is mainly a property of the cell and is little affected by accessory secretions. In the present experiments, it was found that the spermatozoa surviving rapid cooling are less susceptible to subsequent cold shock. This observation also indicates that resistance or susceptibility to cold shock is an inherent property of the cell. It would seem, however, that accessory secretions do play some part in the susceptibility to cold shock since dilution with seminal plasma increases the detrimental effect of rapid cooling.

The effect of cold shock is associated with changes in the cell membrane (Walton 1957) and is accompanied by loss of ions and other substances from the cell (Mann and Lutwak-Mann 1955; Blackshaw and Salisbury 1957). Similarly, the rapid immobilization occurring at high dilution (Kennedy 1947; Emmens and Swyer 1948; Blackshaw 1953; Wales and White 1961) is thought to be due to surface changes associated with the loss of intracellular substances. Thus, it is not surprising to find that even moderate dilution, while having little or no effect on the viability of unshocked spermatozoa, increases the susceptibility of these cells to cold shock. The various unrelated proteins that partially protect spermatozoa against the detrimental effects of cold shock probably help to maintain the cell membrane and prevent loss of intracellular substances.

Although milk has been used extensively as a diluent for spermatozoa, there is apparently no mention in the literature that it reduces the effect of cold shock on ram and bull spermatozoa. The present studies indicate that the beneficial effect of milk is due to its protein content. Casein-containing diluents give results similar to milk and both the non-dialysable and ethanol-insoluble fractions of milk protect the spermatozoa. The fact that albumin and globulin, as well as casein, will help to maintain viability under these conditions suggests that the proteins have a physical rather than a chemical effect. Beljkevic *et al.* (1959), however, suggest that the action of protective substances is due to their effects on calcium ions lost from spermatozoa during rapid cooling. Whatever the mechanism of action, the addition of 1–2% protein to the diluent, as well as lecithin, seems advisable if spermatozoa are liable to be subjected to fluctuations in temperature.

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VI. References

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