THE VIABILITY OF FOWL SPERMATOZOA IN DILUTE SUSPENSION

By R. G. WALES* and I. G. WHITE*

[*Manuscript received May*, 3, 1961]

Summary

The motility of fowl spermatozoa is depressed at relatively high dilutions and the use of diluents with a high tonicity is important in helping to offset the detrimental effect.

Protection by as little as 2·5% of fowl seminal plasma is due to a heat-stable substance which is not present in dialysed plasma or in the supernatant after ethanol precipitation.

Supernatants obtained from washed fowl spermatozoa depress motility.

Bovine albumin, bovine globulin, casein, and acacia gum give some protection against the harmful effect of dilution but none of these substances is as beneficial as seminal plasma.

I. INTRODUCTION

The inactivation of mammalian spermatozoa on high dilution has been extensively studied since Milovanov (1934) noted that 1% sodium chloride had this effect. Emmens and Swyer (1948), investigating the motility of rabbit spermatozoa in dilute suspension, demonstrated that the harmful effect of dilution was not due, as Milovanov thought, to the toxicity of sodium chloride, but occurred as readily in sulphate and tartrate diluents. Other investigators have reported similar findings for bull, ram, and human spermatozoa (Salisbury *et al.*1943; Kennedy 1947; Cheng, Casida, and Barrett 1949; Blackshaw 1953; White 1954).

The viability of dilute rabbit spermatozoa is improved by adding accessory secretion. Supernatants from spermatozoa left overnight, however, are even more effective (Emmens and Swyer 1948). Thus it would seem that the detrimental effect of dilution, in the rabbit at least, is due to both dilution of plasma material and the loss of substances from the spermatozoa.

The nature of the substance or substances lost from spermatozoa at low cell concentration, however, is unknown. White (1953*a*, 1953*b*, 1953*c*) demonstrated that potassium increased the motility and glycolysis of repeatedly washed or moderately diluted ram and bull spermatozoa. At lower cell concentration, however, some other factor must be limiting as potassium is ineffective.

Munro (1938*a*), Bonnier and Trulsson (1939), and Wilcox (1958) have reported lowered fertility with diluted fowl semen, which is partly offset by adding seminal plasma (Munro 1938*b*). Except for the works of Grodzinski and Marchlewski (1935) and Lorenz and Tyler (1951) there is little information on the effects of high dilution on avian spermatozoa *in vitro*, and the studies reported in this paper were, therefore, undertaken.

* Department of Veterinary Physiology, University of Sydney.

II. Materials and Methods

(a) *Semen*

Semen was collected by abdominal massage (Burrows and Quinn 1939). Duplicate spermatozoal counts were made with the neat semen which was then diluted to the required cell concentrations. The spermatozoa were kept at 20–25°C and motility scored at hourly intervals for 4 hr by the system of Emmens (1947). Full motility was rated as four and complete immotility as zero. As quarter grades were frequently used, the actual scores have been multiplied by four, and the total score multiplied by four over the experimental period, the motility index, has been used as unit observation in the analyses of variance.

(b) *Diluents*

A.R. chemicals and glass-distilled water were used. Relative tonicity (100% ≡ 0·9% NaCl) was calculated on the assumption that there was complete primary dissociation of all electrolytes and no secondary dissociation. The diluent (relative tonicity 140%, pH 7) used in most fowl experiments had the following composition: 0·0136M $Na_2HPO_4.12H_2O$, 0·0064M $NaH_2PO_4.2H_2O$, 0·005M KCl, 0·0015M $MgCl_2.6H_2O$, 0·0641M NaCl, 0·25M glucose.

(c) *Preparation of Seminal Plasma Extracts*

The following extracts were prepared from pooled ejaculates:

(i) *Heated extract.*—Plasma was heated in a boiling water-bath for 10 min, cooled, centrifuged, and the supernatant removed.

(ii) *Ethanol extract.*—One volume of plasma was precipitated with six volumes of redistilled ethanol. The supernatant after centrifuging was evaporated to dryness in an incubator at 37°C and made up to the original volume with glass-distilled water.

(iii) *Dialysed extract.*—The plasma sample was dialysed against glass-distilled water for 2 days at 5°C using "Cellophane" as a membrane.

(d) *Preparation of Supernatants*

Spermatozoa were separated from accessory secretion by centrifuging at 1500 r.p.m. (approx. 300 *g*) for 15 min, washed once, and made up to the original volume with diluent. Half of the resulting washed suspension was incubated at 37°C for 6 hr and the other half repeatedly frozen and thawed during the same period. The samples were then centrifuged and the supernatants removed.

(e) *Chemicals*

The vitamins and other fine chemicals were obtained from the following sources: folic acid—Roche Products Ltd.; *p*-aminobenzoic acid, riboflavin, pyridoxine, and niacin—Andrews Laboratories; calcium pantothenate and thiamine hydrochloride—General Biochemicals Inc.; vitamin B_{12}—Merck and Co. Inc.; biotin—Delta Chemical Works; inositol—Difco Laboratories; ascorbic acid, serine, and alanine—British Drug Houses Ltd.; glycine—May and Baker Ltd.; creatine—Light and Co.

TABLE 1

EFFECT OF 0·005M POTASSIUM AND 0·0015M MAGNESIUM ON THE MOTILITY OF FOWL SPERMATOZOA

Mean motility indices for six ejaculates over 4 hr are given

Sperm Density (millions/ml)	Ion Added	Relative Tonicity (0·9% NaCl = 100)		
		100	125	150
200·0	Nil	49·5	55·2	52·3
	K^+	47·7	52·2	55·5
	Mg^{++}	48·7	53·2	54·7
	$K^+ + Mg^{++}$	44·0	53·3	56·0
20·0	Nil	6·5	19·8	23·5
	K^+	12·2	30·7	38·3
	Mg^{++}	7·2	16·8	28·8
	$K^+ + Mg^{++}$	13·5	34·3	40·3
2·0	Nil	0·7	2·8	5·8
	K^+	0·3	5·2	8·3
	Mg^{++}	1·5	3·7	8·0
	$K^+ + Mg^{++}$	0·0	2·2	11·0
0·2	Nil	0·0	0·0	0·7
	K^+	0·0	0·8	0·7
	Mg^{++}	0·0	0·2	0·2
	$K^+ + Mg^{++}$	0·0	0·2	0·7

Summary of the Analysis of Variance†

Source of Variation	Degrees of Freedom	Variance Ratio	Source of Variation	Degrees of Freedom	Variance Ratio
Effect of dilution	2	2748·7**	First-order interactions		
			Dilution × tonicity	4	35·3**
Effect of tonicity	2	193·4**	Dilution × potassium	2	49·8**
			Dilution × magnesium	2	1·3
Effect of potassium	1	48·4**	Tonicity × potassium	2	9·2**
			Tonicity × magnesium	2	3·2*
Effect of magnesium	1	1·3	Potassium × magnesium	1	0·0
			Ejaculate interactions	30	4·9**
Ejaculate differences	5	26·8**	Residual	161	15·2

* $P<0·05$.

** $P<0·01$.

† The results at a spermatozoal concentration of 0·2 million/ml have been omitted from the analysis.

Ltd.; cytochrome *c* and flavine adenine dinucleotide—Nutritional Biochemicals Corp; crystalline bovine albumin and crystalline bovine γ-globulin—Armour Laboratories; crystalline egg albumin—prepared by the method of Cole (1933).

(*f*) *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 analyses of variance,

TABLE 2

EFFECT OF SEMINAL PLASMA ON THE MOTILITY INDICES OF DILUTED FOWL SPERMATOZOA

Mean values for six ejaculates are given

Sperm Density (millions/ml)	Seminal Plasma Concn. (%)			
	0	2·5	5·0	10·0
20	42	46	43	42
2	11	37	38	37

and the significance of the difference has then been assessed in a *t*-test using the degrees of freedom associated with the interaction mean square.

In cases where motility falls rapidly, variances are probably not completely independent of the level of response. In such instances, however, there is rarely any doubt about the significance of the effects observed.

III. RESULTS

(*a*) *Effect of Composition of Diluent on Diluted Fowl Spermatozoa*

Potassium (0·005M) and magnesium (0·0015M) chlorides were added alone and in combination to diluents composed of 0·0136M $Na_2HPO_4.12H_2O$, 0·0064M $NaH_2PO_4.2H_2O$, 0·0641M NaCl, and enough glucose to bring the relative tonicity to 100, 125, and 150 respectively. Fowl semen was diluted to give final cell concentrations of 200, 20, 2, and 0·2 millions/ml in these diluents and the mean motility scores for six ejaculates are given in Table 1. Motility indices were very low at a cell concentration of 0·2 million/ml and to avoid heterogeneous variances, these results were omitted from the analysis of variance which is summarized in Table 1. Motility at low spermatozoal concentration was maintained best in potassium-containing diluents of relative tonicity 150. Magnesium, on the other hand, had no

effect on diluted fowl spermatozoa. In all subsequent tests optimal conditions were maintained by using the hypertonic diluent containing potassium and magnesium ions.

(*b*) *Effect of Seminal Plasma*

As little as 2·5% seminal plasma almost completely protected fowl spermatozoa against the harmful effect of dilution and was as effective as higher concentrations of plasma (Table 2). The results of further tests on the effect of seminal plasma are

TABLE 3

EFFECT OF SEMINAL PLASMA ON THE MOTILITY OF FOWL SPERMATOZOA AT 15 AND 25°C

Mean motility indices for duplicate observations on four ejaculates are given

Cell Concentration (millions/ml)	Control		5% Seminal Plasma	
	15°C	25°C	15°C	25°C
200·0	56·3	57·4	54·9	57·9
20·0	55·2	53·7	54·5	56·1
2·0	51·4	46·0	54·4	54·6
0·2	34·4	19·5	51·9	52·8

Summary of the Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
Between temperatures	1	6·1*
Effect of plasma	1	103·2**
Effect of dilution	3	98·4**
Ejaculate differences	3	3·6*
First-order interactions		
Temperature × plasma	1	17·6**
Temperature × dilution	3	6·7**
Plasma × dilution	3	58·4**
Ejaculate interactions	15	3·3**
Higher-order interactions	64	1·2
Between duplicates	64	19

* $P<0·05$. ** $P<0·01$.

shown in Table 3. Fowl spermatozoa diluted to concentrations of 200, 20, 2, and 0·2 millions/ml were incubated at 15 and 25°C with and without the addition of 5% seminal plasma. Duplicate motility observations on four ejaculates showed that seminal plasma completely protected the spermatozoa against the dilution effect at

both temperatures. Without added plasma, however, the effects of dilution were more marked at 25°C.

In view of the beneficial action of seminal plasma, extracts of four samples were tested. The effect of 10% (v/v) plasma, heated plasma, ethanol-extracted plasma, and dialysed plasma on fowl spermatozoa at a concentration of 0·2 million/ml is shown in Table 4. From the analysis of variance, the standard error of the difference between the treatment and control means was found to be 2·1 (d.f. = 45) and has been used for the estimation of t. Ethanol extracts and dialysed samples depressed motility, but heated plasma was as effective as the untreated material in preventing the effects of dilution.

TABLE 4

EFFECT OF EXTRACTS OF FOUR SEMINAL PLASMA SAMPLES ON THE MOTILITY OF DILUTED FOWL SPERMATOZOA

Mean values for four ejaculates tested on each of four samples are given

Plasma Extract	Sperm Concentration (million/ml)	Mean	Plasma Extract	Sperm Concentration (million/ml)	Mean
Nil (control)	0·2	23·9	Ethanol extract	0·2	18·1**
Whole plasma	0·2	44·8**	Dialysed plasma	0·2	16·2**
Heated plasma	0·2	44·8**	Nil (control)	20·0	54·5**

** Significantly different from control (0·2 million/ml) at $P<0·01$.

(c) *Effect of Supernatants from Washed Spermatozoa*

To check on the possible occurrence of a protective substance in the spermatozoa which might diffuse out, supernatants from three samples of washed fowl spermatozoa were tested. The motility scores of four fowl ejaculates diluted to cell concentrations of 20 and 2 millions/ml are given in Table 5. Neither of the supernatants had any beneficial effect, in fact those prepared by freezing consistently depressed motility at the lower cell concentration.

(d) *Effects of Various Substances on Diluted Spermatozoa*

Table 6 shows the effect of a number of seminal constituents and other possible beneficial substances on the motility of fowl spermatozoa at a cell concentration of 0·2 million/ml. No analysis was considered necessary in experiment A as motility was usually zero. In experiment B, the treatments giving zero or near zero scores were omitted in the analysis of variance from which a standard error of 4·8 with 77 degrees of freedom was calculated. Bovine albumin, bovine globulin, casein, and gum acacia gave some protection against the dilution effect although none of these substances were as beneficial as seminal plasma.

IV. Discussion

Fowl spermatozoa rapidly become immotile at high dilutions. The range of cell concentrations over which the phenomenon appears may vary from ejaculate to ejaculate but is of the same order as reported for other species (Kennedy 1947; Emmens and Swyer 1948; Blackshaw 1953; White 1954) and suggests it is of general occurrence with vertebrate spermatozoa. There seems to be no apparent reason why ejaculates collected under similar conditions should vary in their reaction

Table 5

Motility of fowl spermatozoa in diluents containing spermatozoal supernatants

Three sets of supernatant samples were obtained after (*a*) incubation and (*b*) alternate freezing and thawing of washed fowl spermatozoa and tested on four ejaculates. Mean motility indices are given

Cell Concentration (millions/ml)	Preparation of Supernatant	% of Supernatant		
		0	3	9
20	Incubation	54·8	51·4	47·3
	Freezing	55·1	52·4	41·7
2	Incubation	47·1	45·5	40·8
	Freezing	50·5	37·8	23·0

Summary of the Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
Effect of dilution	1	53·5**
Difference in preparation	1	11·0**
Concentration of supernatant	2	36·6**
Between supernatant samples	2	3·8**
Between ejaculates	3	9·4**
First-order interactions		
Dilution × preparation	1	5·1*
Dilution × supernatant concentration	2	2·1
Preparation × supernatant concentration		
Linear difference	1	17·6**
Quadratic difference	1	0·3
Sample and ejaculate interactions	26	1·6
Residual	103	63

* $P<0·05$. ** $P<0·01$.

to dilution. Similar variability has been found by Wales and White (unpublished data, 1961) using dilute dog spermatozoa.

To offset the detrimental effect of dilution, diluents for fowl spermatozoa should have a high tonicity similar to the semen itself. The beneficial action of potassium, especially at high dilution, confirms the opinion of White (1953*b*) that potassium loss from spermatozoa occurs during dilution. Seminal plasma is very beneficial in preventing the effects of dilution and the present work indicates that it contains a heat-stable, dialysable, protective substance, possibly a low molecular weight peptide which is precipitated by ethanol.

TABLE 6

EFFECTS OF VARIOUS SUBSTANCES ON THE MOTILITY OF FOWL SPERMATOZOA DILUTED TO A FINAL CELL CONCENTRATION OF 0·2 MILLION/ML

The mean motility indices of spermatozoa at a concentration of 100 millions/ml were 44·0 and 49·8 respectively

Experiment A (6 replications)		Experiment B (8 replications)	
Substance Added	Mean Motility Index	Substance Added	Mean Motility Index
Nil (control)	3·4	Nil (control)	0·3
Seminal plasma (10%)	31·1	Seminal plasma (10%)	37·2**
Thiamine (5 mg/100 ml)	3·2	Bovine albumin (250 mg/100 ml)	19·6**
Riboflavin (5 mg/100 ml)	0·8	Bovine γ-globulin (250 mg/100 ml)	10·6*
Niacin (5 mg/100 ml)	3·2	Casein (250 mg/100 ml)	16·4**
Inositol (5 mg/100 ml)	1·2	Egg albumin (250 mg/100 ml)	7·1
p-Aminobenzoic acid (5 mg/100 ml)	2·8	Starch (250 mg/100 ml)	1·3
Pantothenate (5 mg/100 ml)	4·2	Gelatin (250 mg/100 ml)	0·9
Pyridoxine (5 mg/100 ml)	1·2	Acacia gum (250 mg/100 ml)	11·4*
Folic acid (5 mg/100 ml)	1·5	Glycogen (250 mg/100 ml)	0·0
Biotin (25 μg/100 ml)	2·2	Agar (250 mg/100 ml)	0·0
Vitamin B_{12} (40 μg/100 ml)	2·7	Glycine (1 g/100 ml)	0·5
Ascorbic acid (10 mg/100 ml)	0·0	Serine (1 g/100 ml)	8·4
Cytochrome *c* (2·5 mg/100 ml)	5·0	Alanine 1 g/100 ml)	2·6
Flavine adenine dinucleotide (2·5 mg/100 ml)	1·0	Creatine (50 mg/100 ml)	4·0

* Significantly beneficial at $P<0·05$. ** Significantly beneficial at $P<0·01$.

Since supernatants from rabbit semen left overnight give more protection than fresh seminal plasma, beneficial substances may be lost from spermatozoa during senescence (Emmens and Swyer 1948). Blackshaw (1953), in reviewing work on rabbit, ram, and bull spermatozoa, suggested that during dilution two types of substance are lost: one diffusible and the other non-diffusible. On this basis, one might expect that supernatants prepared by incubating carefully washed spermatozoa would contain at least the diffusible substance. Supernatants prepared from sper-

matozoa damaged by freezing should be even more effective because of their content of non-diffusible substances. In the present studies, however, frozen and thawed cells apparently released a substance which further depressed the motility of diluted fowl spermatozoa.

Lorenz and Tyler (1951) found that diluted fowl spermatozoa remained motile for a longer period in the presence of 0·003–0·133M glycine. In the present studies, 0·131M glycine did not improve the motility score of highly diluted fowl spermatozoa over 4 hr. Tests with other miscellaneous substances gave results similar to those found for mammalian spermatozoa (Emmens and Swyer 1948; Blackshaw 1953; White 1954). The vitamins were inactive and proteins beneficial.

V. Acknowledgments

The authors are indebted to Professor C. W. Emmens for his interest and advice. This work has been aided by a grant from the Rural Credits Development Fund of the Commonwealth Bank of Australia.

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