FACTORS AFFECTING STORAGE OF SEMEN

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SUMMARY

The effect of ampulla and seminal plasma on sperm quality and livability is discussed. Amino acid oxidase activity was absent in epididymal sperm, but present in ejaculated sperm. Passage of sperm through the ampulla accounts for the presence of, and difference between bulls in the percentage of dead H_2O_2-producing sperm. Seminal plasma reduces livability of ejaculated sperm. Egg yolk protects sperm against the adverse effects of seminal plasma but enhances the effect of dead sperm. A number of field trials are reported. The main features of these trials were (1) Fertility of diluted semen was higher when stored at ambient compared with 5° C; (2) Fertility increased, at ambient temperatures, when egg yolk was reduced from 20 to 5%; (3) Catalase improved fertility of diluted semen when stored at ambient temperatures, but not when stored at 5° C.

Ejaculated semen consists of a mixture of sperm derived from the ampulla and vas deferens and secretions of the accessory glands. Ejaculated sperm differs from epididymal sperm, having a shorter survival time *in vitro* and reduced resistance to such stress conditions as rapid cooling or centrifuging.

Apart from these generalizations, the quality of ejaculates from different bulls, and to a lesser extent different batches from the same bulls, vary both in the above qualities and in the percentage of live sperm and motility.

It is of some interest to determine in which area of the reproductive tract these differences arise. One useful parameter is the percentage of live sperm. We have lately demonstrated the presence of a hydrogen peroxide-producing aromatic amino acid oxidase (Shannon and Curson, 1972) which is completely inactive in live sperm but becomes active on the death of sperm (Table 1). This appears to be a useful biochemical test to determine the presence of dead sperm.

TABLE 1: OXYGEN CONSUMED (μl) BY 50×10^6 SPERM IN DEGRADATION OF PHENYLALANINE

*Source of Sperm**			
Untreated		*Killed*	
Vas	*Ejaculated*	*Vas*	*Ejaculated*
0	2.4	10.1	9.2

*$n = 6$

The results show nil activity for sperm derived from the vas but appreciable activity from ejaculated sperm. The activity of killed sperm derived from the vas was as high as killed ejaculated sperm demonstrating that the enzyme is as potentially active in epididymal sperm as ejaculated sperm.

In ejaculated semen there is a very good relationship between the percentage of dead sperm measured by the eosin staining technique and oxidase activity. The activity of ejaculated dead sperm is between 80 and 90% of freshly-killed sperm indicating (based on studies of the stability of the enzyme at 37 °C) that sperm have been dead, on average, only one to two days before ejaculation.

The evidence indicates that differences between bulls in the percentage of dead sperm ejaculated arise as a consequence of conditions in the upper part of the tract. Further, the consequence of differences in the percentage of live sperm is not simply that there is a difference between ejaculates in the percentage of sperm available for fertilization, but that there are considerable differences between ejaculates in their ability to produce hydrogen peroxide.

On ejaculation epididymal sperm are mixed with seminal plasma. This reduces the survival time of epididymal sperm and renders it more susceptible to stress conditions such as centrifuging, an effect illustrated in the following experiment.

Pooled epididymal sperm derived from three bulls was suspended either in 14 g diluent or 14 g + 20% seminal plasma. After centrifuging the estimated percentage of motile sperm was 60% for epididymal sperm centrifuged in the absence and 10% in the presence of seminal plasma. Supernatants depressed livability of semen diluted in caprogen + 20% egg yolk by 20 hours for epididymal sperm centrifuged in the presence and 11 hours in the absence of seminal plasma. The increased toxicity was due to greater amounts of amino acid oxidase. One is tempted to conclude that seminal plasma increases the fragility of the cell membrane resulting in distintegration and death in certain stress conditions.

While seminal plasma significantly increases the susceptibility of epididymal sperm to centrifuging, evidence for its effect on cold shock is less convincing (Table 2). Although there was

TABLE 2: EFFECT OF COLD SHOCK ON LIVABILITY IN HOURS OF SPERM AT 37° C*

Source of Sperm		
Ejaculated	*Vas*	*Vas + Seminal Plasma*
3	28	19

*$n = 5$

a significant reduction in livability of epididymal sperm after cold shock in the presence of seminal plasma, epididymal sperm were, nevertheless, much more resistant to cold shock even in the presence of seminal plasma than ejaculated sperm.

Ejaculated sperm are therefore equipped with two major sources of self destruction, seminal plasma and hydrogen peroxide-producing dead sperm.

Some protection against these deleterious factors is clearly necessary. Egg yolk has long been used as a protective agent against the effects of rapid cooling (Philips, 1939; Philips and Lardy, 1940). In experiments to determine the reasons for dilution rate effects in sperm, seminal plasma was added to egg yolk diluents. Additional seminal plasma depressed the livability of diluted semen and the effect was modified by the level of egg yolk used (Table 3).

TABLE 3: LIVABILITY OF SPERM IN HOURS AT 37° C* AT TWO EGG YOLK LEVELS

Seminal Plasma	*Egg Yolk Level*	
	5%	*20%*
0	64	63
10%	33	49

*$n = 18$

Semen diluted in diluents containing 20% egg yolk showed less effect from the addition of seminal plasma than diluents containing 5% egg yolk. Egg yolk is at least partly protective against seminal plasma and there is a definite dose response relationship. Of course one must ask whether in fact the effect of seminal plasma is just reflected in reduced livability at 37° C or whether continued exposure to seminal plasma results in permanent damage.

To test this, sperm were diluted in diluents with either 5 or 30% egg yolk with or without 30% seminal plasma. After 30 min the seminal plasma and egg yolk levels were adjusted to contain the same levels and incubated at 37° C. There was a reduction of 7 hours in incubated life in semen which had been exposed to 30% seminal plasma in the presence of 5%

TABLE 4: LIVABILITY OF SPERM IN HOURS AT 37° C*

Seminal Plasma	*Egg Yolk Level for First 30 min*	
	5%	*30%*
0	35	47
30%	28	46

*$n = 6$

egg yolk but no depression in the presence of 30% egg yolk (Table 4). Seminal plasma therefore depresses livability and it appears that the damage is permanent.

The actual toxic action of seminal plasma is unknown. It contains several enzymes, lipases (Shannon, 1965), proteases (weak) NAD-ases, ATP-ases, etc. (Mann, 1964), any of which or all could possibly be involved in the toxic effect.

Nevertheless, some observations are in order: (1) The toxic substance is non-dialysable so that its molecular weight is greater than 10 000. (2) Its toxicity is only slightly reduced by heat treatment, even at temperatures of 100° C. (3) The toxicity of the factor is not reduced by catalase, so its effect is not due to peroxide formation. (4) The solution may not necessarily be a simple one. At least two toxic fractions have been detected following sequential precipitation by ammonium sulphate.

These negative results at least eliminate certain possible toxic effects, but give little indication of what is taking place. Recent experiments have given some indication of the nature of the beast.

Although seminal plasma does not make epididymal sperm as susceptible to cold shock as ejaculated sperm, there are, nevertheless, some similarities between the response of epididymal sperm plus seminal plasma and ejaculated sperm. Both are more susceptible to centrifuging, and egg yolk will protect both from its deleterious effects. One can postulate that the factor in seminal plasma is similar to the cold shock factor but less drastic in its effect. Therefore that fraction of egg yolk that protects against cold shock will also protect against the seminal plasma. If this is the case then the addition of seminal plasma should block the protective action of egg yolk against cold shock.

This in fact it does. The recovery of ejaculated sperm following rapid cooling to 1° C was 20% in the absence of egg yolk, and 70% in the presence of 1% egg yolk. Inclusion of 5, 10, and 20% seminal plasma to the egg yolk diluent reduced recoveries to 60, 37, and 23%, respectively. So at least seminal plasma blocks the protective action of egg yolk.

This still does not explain the point at which the seminal plasma toxin is working. A large number of substances will protect sperm against cold shock — milk, tomato juice, apple juice, cocoa to name but a few — but one of the most interesting is yeast cell walls. These are, of course, insoluble and when mixed with seminal plasma and centrifuged lose their ability to protect sperm against cold shock. A similar loss of activity is obtained when yeast cell walls are suspended in egg yolk and centrifuged.

The observed facts would fit the theory that a substance is attached to the sperm, derived either from the ampulla or seminal plasma, which makes them susceptible to stress conditions. Protection is obtained from egg yolk by the replacement of the factor. In the case of yeast cell walls, the factor is transferred to the yeast cell wall. Loss of activity by exposure of yeast cell walls to either seminal plasma or egg yolk could be due to the blocking of protective sites on yeast cell walls by either seminal plasma or egg yolk.

We have not as yet isolated the toxic factors involved. However, in the ammonium sulphate fractions which exhibit toxicity it is associated with a fraction that precipitates at pH 4.3. This fraction has a considerable solubilizing effect on egg yolk proteins. This solubilizing effect is also demonstrable in seminal plasma and it is perhaps significant that complete solubilization of egg yolk proteins occurs at ratios of 10 seminal plasma to 1 egg yolk, a ratio at which seminal plasma markedly reduces the protective action of egg yolk.

Protection against H_2O_2 produced by dead sperm can be obtained by four methods: (1) By using chilled semen at 5° C at which temperature the production of H_2O_2 is very low; (2) By using simple sulphhydryl compounds like cysteine which act by reducing hydrogen peroxide; (3) By EDTA which slows down the oxidizing effect of hydrogen peroxide; or (4) By catalase which degrades hydrogen peroxide to water and oxygen.

The effect of cysteine on the livability of diluted semen is curious. In samples with few dead sperm, cysteine depresses livability, but in cases where the percentage of dead sperm is high, it enhances livability. It appears that in its reduced form cysteine is harmful to live sperm.

TABLE 5: EFFECT OF EDTA AND CATALASE ON LIVABILITY OF SEMEN IN HOURS AT 37° C*

Diluent Additions	*Additional Killed Sperm*	
	Nil	*5 × 10⁶*
Nil	64	36
+ Catalase	96	92
Nil	57	87
+ EDTA	87	74

*$n = 8$

Both EDTA and catalase have similar effects on the livability of diluted semen. Both increase livability very markedly

and both protect diluted sperm from the effects of additional killed sperm.

The first decision to be made is at what temperature semen should be stored. For liquid semen the obvious choices are between 5° C or at ambient temperatures. The advantages of storage at 5° C are that the metabolic rate of live sperm and production of H_2O_2 by dead sperm is reduced; against storage at 5° C is the potential damage caused by cooling, even when the cooling rate is slow.

Cooling depressed livability by 6 hours (15 split ejaculates) even though it was carried out at the slow rate of 0.3° C/min. For storage periods of up to one week incubation life was always greater for material stored at ambient temperatures.

The effect of temperature of storage was also shown in conception rate results (Table 6). Material held at ambient temperatures gave better results than material stored at 5° C and this difference increased with increasing age of semen. In other trials it has been shown that this advantage holds, at least for semen used for up to 3 days after collection.

TABLE 6: EFFECT OF TEMPERATURE OF STORAGE ON CONCEPTION RATES

Semen Used on	*Temperature of Storage*	
	5° C	*Ambient*
Day of collection	65.3[1]	66.2[2]
Day after collection	66.6[3]	68.9[4]

[1] $n = 268\,201$; [2] $n = 44\,354$; [3] $n = 172\,663$; [4] $n = 29\,772$

It is not possible to give an entirely satisfactory explanation for the advantages of ambient temperature storage but one reason must be that it avoids the damage caused by the cooling process, against which damage egg yolk is not a complete protection. The second decision that has to be made is the level of egg yolk to be used. While egg yolk protects sperm against the adverse effects of seminal plasma, it enhances the

TABLE 7: EFFECT OF EGG YOLK LEVEL AND DEAD SPERM ON LIVABILITY OF SPERM IN HOURS AT 37° C*

No. Dead Sperm ($\times 10^6$)	*Egg Yolk Level*	
	5%	*20%*
0	64	63
5	45	34

$n = 18$

effect of dead sperm by providing a substrate for the amino acid oxidase. Thus 5 million dead sperm depressed livability by 28 hours in the presence of 20% egg yolk and 19 hours in the presence of 5% egg yolk (Table 7).

The livability of semen stored for 7 days at ambient temperatures in 5% egg yolk is superior to that stored in 20% egg yolk. Two large-scale trials on the effect of egg yolk level have been conducted. In the first, there was no difference in the conception rate obtained at the two egg yolk levels but variation between bulls was much greater when 5% egg yolk was used.

In the second trial a highly significant advantage of 3% was obtained from the use of 5% egg yolk. Both trials included bulls with a considerable range in conception rate so that the difference calls for some comment. In the first trial ejaculated semen was initially diluted in 25 ml of 5% egg yolk diluent and approximately 20 min later was diluted to final volume. The percentage of seminal plasma in the original dilution was approximately 20%. Laboratory tests indicated that this method was inferior to that of making the initial dilution in 20% egg yolk and then making up to final volume in 5% egg yolk. The difference was probably due to an inadequate level of egg yolk at initial dilution in the first experiment to protect against seminal plasma.

The reason for the superior performance with lower egg yolk levels is not known, but it appears that the lowest level of egg yolk necessary to provide protection against seminal plasma gives the best results.

Protection against hydrogen peroxide *in vitro* can be obtained by the four methods previously given. The response is likely to vary according to the temperature of storage. A summary of effects of different methods tried is shown in Table 8.

TABLE 8: EFFECT OF VARIOUS PROTECTIVE AGENTS AGAINST H_2O_2 ON CONCEPTION RATE

Protective Agent	*Temperature of Storage* 5° C	*Ambient*
Cysteine	—5.0[1]	
EDTA		6.0[2]
		0[3]
Catalase	—3.0[4]	0.4[4]

[1] Split ejaculates n for each treatment = 5 000
[2] Split ejaculates n = 3 000
[3] Split ejaculates n = 5 000
[4] Split ejaculates n = 2 500

Cysteine has been used only in chilled material where it depressed conception rates by 5%. This result is not surprising in view of the apparent toxic effect of cysteine on sperm. EDTA has been used in two trials at ambient temperatures. In the first, in which a 6% advantage was obtained, it was tested on the semen of four sub-fertile bulls whose semen would normally have been unusable. In the second trial, it was used on bulls in normal service in the AB scheme. Only catalase has been used with semen stored at both temperatures and in this trial it depressed conception rates by 3% at 5° C storage and increased conception rates by 0.4% at ambient temperatures. The interaction was significant. Although the depression in conception rate at 5° C storage is surprising, the interaction is not. Production of H_2O_2 at 5° C is very low whereas appreciable amounts are produced at ambient temperatures. As there was some indication that catalase could be of benefit at ambient temperatures a large trial was conducted.

TABLE 9: EFFECT OF CATALASE ON CONCEPTION RATES

Semen used on	*Diluent* *Caprogen*	*Caprogen + Catalase*
Day of collection	63.9[1]	64.7[2]
Day after collection	68.1[3]	69.7[4]
Day of collection	63.5[5]	64.5[6]
Day after collection	66.2[7]	68.0[8]

$n^1 = 94\,671$; $n^2 = 16\,117$; $n^3 = 148\,286$; $n^4 = 27\,854$
$n^5 = 30\,233$; $n^6 = 146\,337$; $n^7 = 44\,126$; $n^8 = 230\,107$

The results show an increase in conception rate of 0.8 to 1% with semen used on the day of collection and 1.6 to 1.8% for semen used on the day after (Table 9). Catalase then appears to be an effective protector against the toxic effects of hydrogen peroxide formation at ambient temperatures.

While the effect of and protection against the effect of dead sperm has been well defined, the same cannot be said of the effects of passage through the ampulla and admixture with seminal plasma.

The role of the ampulla certainly warrants further investigation. It appears that the quite marked differences between bulls in the percentage of peroxide-producing sperm arises as a consequence of storage or passage through the ampulla. The reasons for this are not known but could arise for several reasons: (1) That sperm from different bulls are more susceptible to conditions in the ampulla; (2) That a greater proportion of the ejaculate in certain bulls is stored in the

ampulla; or (3) That the ampulla varies in its adverse effect in different bulls. In this respect it is interesting to note that Bialy and Smith (1959) found that epididymal sperm acquired their susceptibility to cold shock by passage through the ampulla. Whether this factor predisposes them to early death is worthy of investigation.

The effect of seminal plasma also needs further research. Certainly there are differences in the toxicity of seminal plasma from different bulls and these are not necessarily related to the quality of ejaculated semen. Both effects, it seems, would need to be investigated independently.

Finally, one may ask, why a toxic fraction in semen at all? A suggestion made by Dr J. P. James that the toxic effect could be due to a bacteriostatic substance is being investigated. Preparatory results indicate that seminal plasma is effective against a wide variety of bacteria.

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