Evaluation by the heterospermic insemination technique of the differential effect of freezing at -196°C on fertility of individual bull semen

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This paper gives evidence that deep freezing depresses the fertility of the semen of different bulls unequally, that the period of time spent in the frozen state has no additional effect, and that replicate experiments with mixed (heterospermic) insemination give concordant results. The paper marks a further stage in an experiment with heterospermic insemination replicated over 6 successive weeks in December 1969–January 1970. In each replicate one semen mixture was made containing equal numbers of spermatozoa newly collected from the same four bulls. Some of each mixture was inseminated unfrozen (fresh semen) and another part after standard freezing at -196°C in liquid nitrogen and use within 2–3 days (now termed Series A). The paternity of calves was established by blood-typing; their relative numbers defined the ‘relative heterospermic fertility’ of the bulls. The results at that stage (Stewart, Spooner, Bennett, Beatty & Hancock, 1974) showed that the ‘relative heterospermic fertility’ of the bulls was close to 1:1:1:1 after use of fresh semen but there was a marked departure from that ratio with the frozen Series A semen. Evidently the semen of individual bulls responded unequally to deep freezing. Another part of each mixture had been set aside at -196°C for a later repeatability test (Series B) carried out in the period June–August 1973 and reported in the present paper. If repeatability could be established, the data of Series A and B were to be combined to give an improved estimate of relative heterospermic fertility after deep freezing. Lack of repeatability would have suggested an effect of the period of storage. Materials and methods remain as described by Stewart *et al.* (1974), and a *P* value of 0.05 continues to be our criterion of statistical significance. The earlier progeny ratios are tabulated again here so that the experiment may be visualized and analysed as a whole.

In Table 1(a) the numbers of progeny sired per bull are shown for each of the 6 replicates. The numbers are analysed in Table 1(b) in terms of deviance, *d*. Deviance analysis is preferable to χ^2 analysis in this problem. Deviance, however, is a close relative of χ^2 and for purposes of visualization the deviance values may be regarded simply as χ^2 values. The GLIM programme (Nelder, 1975) based on that of Nelder & Wedderburn (1972) was used. The vital point of homogeneity of data between replicates could reasonably be assumed, since the bull $\times$ replicate interaction and the bull $\times$ replicate $\times$ treatments interaction were both non-significant, as was a ‘pooled bull $\times$ replicate interaction within treatments’ obtained by adding the deviances of the two interactions ($d_{[45]} = 55.09$, $P = 0.2-0.1$). Item (ii) in the analysis (Table 1b) was non-significant, thus showing no evidence of a real difference in progeny ratios between the two Series of frozen semen, and justifying combination of the two Series by adding their data. Item (i) showed that the progeny ratios of the 4 bulls differed between fresh and (combined) frozen semen with a high degree of significance. Item (iii) is uncomfortably close to our criterion of significance and was used as an alternative basis for judging the significance of Item (i). The resulting *F* test was: $F_{[3, 15]} = (17.22/3)/(24.09/15) = 3.57$; $P = 0.05-0.01$. This probably over-stringent test still left Item (i) formally significant.

The preceding analysis permitted the main results to be summarized as follows. The numbers sired by the four bulls (DCP: TG: NN: FJP) in Series A (50:13:12:36) did not differ significantly

Table 1. Progeny numbers and their analysis after heterospermic insemination of fresh (from Stewart *et al.*, 1974) or frozen (Series A from Stewart *et al.*, 1974; Series B, present results) bull semen mixtures containing equal numbers of spermatozoa from four sires (DCP, TG, NN, FJP)

(a) Results

Replicate semen mixtures (6 successive weeks)	Treatments											
	Fresh				Frozen, Series A				Frozen, Series B			
	DCP	TG	NN	FJP	DCP	TG	NN	FJP	DCP	TG	NN	FJP
1	8	4	6	6	8	2	1	2	7	6	2	4
2	1	5	6	4	13	2	3	3	8	2	12	3
3	5	2	3	12	6	4	2	5	8	4	4	6
4	5	6	4	5	8	3	4	9	8	3	1	6
5	4	3	5	6	8	1	1	9	9	2	2	9
6	5	6	6	7	7	1	1	8	12	2	2	3
Σ over replicates	28	26	30	40	50	13	12	36	52	19	23	31

(b) Analysis of deviance (d) for bull interactions

Item	d.f.	Deviance	P
(i) Bulls $\times$ (fresh versus Series A + Series B)	3	17.22	<0.001
(ii) Bulls $\times$ (Series A versus Series B)	3	4.23	0.3-0.2
(iii) Bulls $\times$ replicates	15	24.09	0.1-0.05
(iv) Bulls $\times$ replicates $\times$ treatments	30	31.00	0.5-0.3

from the numbers in Series B (52:19:23:31). The pooled data from Series A and B (102:32:35:67) differed from those obtained with fresh semen (28:26:30:40) with a high degree of significance. It was also found that the numbers of offspring after use of fresh semen did not differ significantly from an assumed 1:1:1:1 ratio, but Series A and B, separately or after pooling their data, differed significantly from that ratio. The effect of the deep freezing on relative heterospermic fertility was due mainly to a change in the performance of bull DCP as compared with the other 3 bulls, which showed little change relative to one another. Bull DCP sired 23 ($\pm$ S.E. 4)% of offspring in the fresh semen Series, but 43 ($\pm$ S.E. 3)% in the combined results of the two frozen semen Series.

The present results, in conjunction with the part of the experiment already reported by Stewart *et al.* (1974), show that progeny ratios after heterospermic insemination were repeatable between semen mixtures made from ejaculates collected up to 6 weeks apart and that two experimental Series employing the same frozen semen mixtures were concordant. The heterospermic fertility of individual bulls was affected unequally by the deep freezing process, but not by the period of storage, the changes being traceable to an alteration in the heterospermic performance of one bull relative to the other three. The reasons for inequality might repay further study. The efficiency of the heterospermic method of comparing fertility (see Beatty *et al.*, 1969) is illustrated by the fact that these various conclusions were well established from only 360 conceptions.

It has been shown from ordinary conception rate tests that semen stored for 4 years between -68°C and -79°C does not change its fertility in that time (Reading Cattle Breeding Centre, 1960—see Maule, 1962). Our results show that maintenance at -196°C for $3\frac{1}{2}$ years does not in itself affect relative heterospermic fertility. Virtually no biological or biochemical change would be expected. Spermatozoa are not, however, necessarily immune from potential change while deep-frozen for a long time. They may experience a cumulative dosage of natural radiation greater than they would receive during their relatively short life in an intact animal, and any repair mechanism that spermatozoa might possess would certainly be inactivated by the low temperature. Further, physical stresses set up by freezing might slowly release themselves at -196°C and damage spermatozoa mechanically. Deep-frozen stocks of our semen mixtures still exist and testing in the years ahead remains practicable.

Acknowledgments remain as in our previous paper (Stewart *et al.*, 1974) and in addition we thank Mr R. Thompson of the A.R.C. Unit of Statistics (Edinburgh) for the deviance calculations, and Dr F. B. Leech for suggesting use of the method.

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