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The effect of cooling rate before freezing and the temperature of the semen upon addition of DMSO on the fertilizing capacity of chicken semen stored at — 196 °C

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Summary. Semen was collected from Single Comb White Leghorn roosters, diluted 1:4 with Beltsville Poultry Semen Extender at 35 °C and cooled at various rates to 5 °C. DMSO was added to the semen between 10 and 120 min after ejaculation at temperatures between 15 and 5 °C, respectively. Following the addition of DMSO, the semen was allowed to equilibrate for 2 h in a 5 °C environment. The semen was then frozen at 1 °C per min from 5 °C to — 20 °C, transferred into liquid nitrogen vapour for 4 to 10 min and then immersed in liquid nitrogen for 4 to 60 days. The thawed semen was inseminated on two consecutive days and fertility was calculated during 5 and 7 days commencing on the second day after the last insemination. In general, fertility was unaffected by the rate of cooling and the temperature at which the DMSO was added. In one trial, however, the fertilizing capacity was significantly greater if the DMSO was added within 45 min when the temperature of the semen was 15 °C. Approximately 42 to 71 p. 100 of eggs laid by hens during the 5 day period after the second insemination were fertile regardless of the method of cooling or the temperature of the semen when the DMSO was added. The motility of the ejaculates after thawing and before insemination varied between 15 and 55 p. 100 and this measure of physiological quality was a poor indicator of fertilizing capacity. Embryonic mortality was unaffected by the cooling and freezing procedure and hatchability of fertile eggs was not significantly different for the hens inseminated with either fresh or frozen semen.

Introduction.

In recent years, two techniques for obtaining at least 50 p. 100 fertility in hens inseminated with semen stored at — 196 °C have been reported (Sexton, 1976 ; Lake and Stewart, 1978). In preliminary experiments, we were unable to obtain satisfactory fertility in hens inseminated with semen which had been frozen in buffers containing glycerol using several variations of the technique reported by Lake and Stewart (1978) (see Etches *et al.*, 1980). In the experiments described in this paper, we investigated the use of DMSO as the cryoprotectant using the method described by Sexton (1976) and incorporating some modifications to ascertain the effects on motility and fertilizing capacity of the time and temperature at which DMSO was added to the semen.

Materials and methods.

Semen collection and estimation of sperm concentration. — Semen from 12 Single Comb White Leghorn roosters was collected every second or third day by abdominal massage (Burrows and Quinn, 1937) into a glass tube with a funnelled opening. The ejaculates were combined, immediately mixed and an aliquot was removed for the determination of sperm concentration using a haemocytometer. To minimize contamination of the semen with clear fluids and feces, drinking water was removed 2 h prior to ejaculation and only the first part of the ejaculate was collected. All of the diluted semen was extended immediately by adding 1 part of semen to 4 parts of Beltsville Poultry Semen Extender (BPSE) (Sexton, 1977) at 35 °C.

Semen processing. — In the first trial, the control hens were inseminated with either (*a*) undiluted semen, (*b*) semen diluted 1:4 with BPSE or (*c*) semen diluted 1:4 with BPSE and containing 4 p. 100 (v/v) DMSO (Reagent Grade, J. T. Baker Chemical Co., Philipsburg, N. J.). The DMSO was added after dilution and within 15 min after collection when the temperature of the semen was 24 °C. Immediately after the DMSO was added, all of the fresh semen was placed in an ice chest at 5 °C until the time of insemination. Hens were inseminated with fresh semen within 45 min of collection and within 30 min after the addition of DMSO.

Before freezing, the temperature of the semen was reduced to 5 °C by one of eight different methods. In the first two methods, 5 ml aliquots of semen diluted 1:4 in BPSE at 35 °C were exposed to the 5 °C ambient temperature in 15 × 110 mm test tubes for 10 and 120 min at which time DMSO was added to a final concentration of 4 p. 100 (v/v). The temperatures of the semen at the time of addition of DMSO were 15 and 5 °C respectively. The third and fourth methods were identical to the first two methods except that the test tubes were insulated in 80 ml of water at 35 °C before they were exposed to the 5 °C ambient temperature in the cold room. The DMSO was added either 45 or 120 min after placing the water bath with the semen into the cold room at which time the temperatures of the semen were 15 and 7 °C respectively. The fifth and sixth methods were similar to the third and fourth except that the water bath contained 150 ml of water at 35 °C and the semen was exposed to the 5 °C ambient temperature for 60 and 120 min before the DMSO was added to a final concentration of 4 p. 100 (v/v). The temperatures of the semen at the time of addition of DMSO were 15 and 9 °C respectively. For the last two methods, a 250 ml water bath with an initial temperature of 35 °C was used. The DMSO was added to a final concentration of 4 p. 100 (v/v) when the temperatures of the semen were 15 and 10 °C after 70 and 120 min of cooling.

In a second trial, hens were inseminated with fresh semen diluted 1:4 in BPSE after 60 or 120 min of equilibration in a 5 °C environment with or without 4 p. 100 DMSO. The DMSO was added within 15 min after collection when the temperature of the semen was 24 °C. At the time of addition of DMSO, all of the semen was placed in an ice chest at 5 °C until the time of insemination.

In the second trial, the temperature of the semen to be frozen was reduced to 5 °C by four different methods. In the first two methods, 5 ml aliquots of semen diluted 1:4 in BPSE were exposed to 5 °C in 15 × 110 mm test tubes for 10 and 18 min at which time

DMSO was added to a final concentration of 4 p. 100 (v/v). The temperatures of the semen at the time of addition of DMSO were 15 and 10 °C respectively. In the third and fourth methods, the aliquots of semen were insulated in a 250 ml water bath and the DMSO was added 70 and 120 min later when the temperatures of the semen were 15 and 10 °C respectively.

The rate of cooling to 5 °C for semen exposed to all of the above methods was non-linear. Therefore, an average rate of cooling was not applicable and the changes in semen temperature throughout the cooling period are illustrated in figure 1.

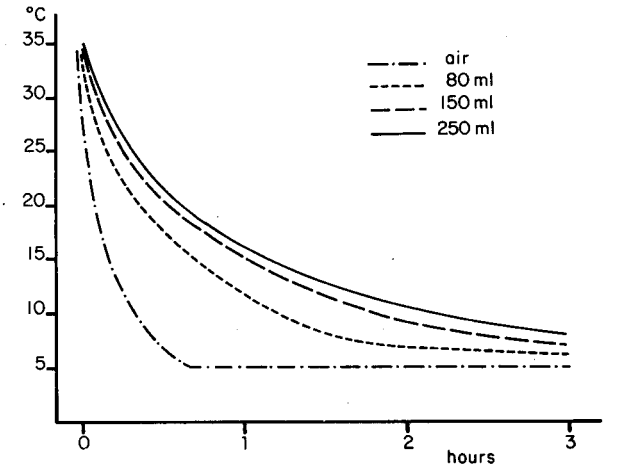

FIG. 1. — *The rate at which the temperature of diluted semen declined when exposed to an ambient temperature of* 5 °C *or when insulated in* 80, 150 *or* 250 *ml of water before exposure to* 5 °C.

After a 2 h equilibration period with DMSO, the semen was packaged in 0.5 ml plastic straws (United Breeders, Guelph, Ontario) and placed in 3.5 l of ethanol in a Dewar flask at 5 °C. The semen was frozen by reducing the temperature of the ethanol by 1 °C per min between 5 °C and — 20 °C with a Cryocool CC60 freezing unit (Neslab Instruments, Portsmouth N. H.). The straws were then rapidly transferred into liquid nitrogen vapour where they remained for 4 to 10 min at which time the temperature of the semen was between — 90 °C and — 135 °C. The straws were then immersed in liquid nitrogen and stored for 4 to 60 days.

Assessment of motility. — The motility of the spermatozoa was subjectively assessed as a percentage for each ejaculate at the time of collection, immediately after the addition of DMSO, immediately before the semen was frozen, *i.e.*, after equilibration with DMSO for 2 h and immediately after the semen was thawed. Motility was assessed in aliquots which were diluted 1:4 on a slide maintained at 30 °C.

Insemination of hens. — The fertilizing capacity of semen which had been cooled or cooled and frozen by the methods described below was tested by inseminating Single Comb White Leghorn hens using the intravaginal method described by Burrows and Quinn (1937). The semen was thawed by immersing the straws in ice water for 3 to

6 min. The straws were then transferred to an ice chest at approximately 5 °C and the semen was recombined according to the method of freezing in 15 × 110 mm test tubes. Individual inseminations were prepared in the ice chest by filling turkey insemination pipettes (Arnold Nasco, Guelph, Ontario) with 0.17 ± 0.04 (mean ± s.d.) ml of semen containing 200 million spermatozoa. Hens were inseminated on two successive days with frozen-thawed semen whereas only one insemination was given with fresh semen. All inseminations were performed within 20 min of thawing the semen and within 2 min after removal from the ice chest.

Calculation of fertility. — The hens were maintained in individual cages and all eggs laid during the experiments were identified with the cage number of the hen and the date of oviposition. Only hens which laid more than 2 eggs in 5 days and 3 eggs in 7 days commencing on the second day after the last insemination were used to calculate fertility. All eggs were candled after 7 days of incubation and eggs which were apparently infertile were broken open and examined macroscopically for evidence of fertilization. Fertility was calculated for each hen individually and expressed in six different ways ; (i) as the percentage of fertile eggs within the 5-day period beginning on the second day after the last insemination (p. 100 fertility in 5 days) ; (ii) as the percentage of fertile eggs within the 7-day period commencing on the second day after the last insemination (p. 100 fertility in 7 days) ; (iii) as the percentage of embryos in eggs laid during the 5-day period which died during the first 7 days of incubation (p. 100 early dead in 5 days) ; (iv) as the percentage of embryos in eggs laid during the 7-day period which died during the first 7 days of incubation (p. 100 early dead in 7 days) ; (v) as the percentage of embryos alive at 7 days of incubation in eggs laid during the 5-day period which hatched (p. 100 hatchability in 5 days), and (vi) as the percentage of embryos alive at 7 days of incubation in eggs laid during the 7-day period which hatched (p. 100 hatchability in 7 days). The latter two measures of fertility were calculated in the second trial only.

Statistical analysis. — For both trials, the data were analyzed as a one-way analysis of variance and individual means were compared using Duncan's Multiple Range Test as modified by the General Linear Models procedure of the Statistical Analysis System (Helwig and Council, 1979) to accommodate unequal subclass numbers. Although the data were not normally distributed and the variances were not homogeneous, the usual transformations did not rectify these violations of the assumptions for an analysis of variance and, therefore, were not done.

Results.

The concentration of spermatozoa in a random selection of 28 pooled ejaculates of semen which were used in these experiments was 5.9 billion ± 1.2 billion cells per ml. The motility of the spermatozoa in each pool of semen at the time of collection was 85 p. 100 or greater. In all cases, the addition of DMSO reduced the motility to between 70 and 80 p. 100. At the end of the 2 h equilibration period the motility was further reduced to between 60 and 70 p. 100. The lowest levels of motility were observed after freezing and before insemination, at which time the estimates of motility ranged between 15 and 55 p. 100.

TABLE 1

The percentage of fertile eggs laid by hens inseminated with fresh and frozen semen (Trial 1)

Vol. (ml) of water bath	Addition of DMSO		Mean p. 100 fertility		Number of hens inseminated
	Time (min)	Temp. (°C)	in 5 days after insemination	in 7 days after insemination	
0 ([1, 2])	—	—	95 ([a])	90 ([a])	11
0 ([2])	—	—	96 ([a])	95 ([a])	10
0 ([2])	15	24	94 ([a])	93 ([a])	10
0 ([3])	10	15	71 ([ab])	71 ([ab])	9
0 ([3])	120	5	47 ([b])	34 ([d])	9
80 ([3])	45	15	71 ([ab])	61 ([bc])	8
80 ([3])	120	7	62 ([b])	60 ([bcd])	7 ([4])
150 ([3])	60	15	52 ([b])	43 ([cd])	10
150 ([3])	120	9	62 ([b])	56 ([bcd])	8
250 ([3])	70	15	60 ([b])	57 ([bcd])	10
250 ([3])	120	10	66 ([b])	57 ([bcd])	9
s.d.			27	25	

([1]) Undiluted semen. ([2]) Inseminated within 45 min after ejaculation. ([3]) Semen was stored at −196 °C before insemination. ([4]) Eight hens were used to calculate « p. 100 fertility in 5 days after insemination ».

([abcd]) Means in columns with the same letter are not significantly different ($P \geqslant 0.05$).

TABLE 2

The percentage of fertile eggs laid by hens inseminated with fresh and frozen semen (Trial 2)

Vol. (ml) of water bath	Addition of DMSO		Mean p. 100 fertility in 5 days after insemination	Number of hens	Mean p. 100 fertility in 7 days after insemination	Number of hens
	Time (min)	Temp. (°C)				
0 ([1])	—	—	100 ([a])	8	100 ([a])	8
0 ([2])	—	—	86 ([a])	17	83 ([a])	17
0 ([1])	15	24	79 ([a])	10	71 ([a])	10
0 ([2])	15	24	48 ([b])	18	47 ([b])	18
0 ([3])	10	15	52 ([b])	25	45 ([b])	30
0 ([3])	18	10	39 ([b])	30	42 ([b])	31
250 ([3])	70	15	54 ([b])	31	44 ([b])	32
250 ([3])	120	10	42 ([b])	32	37 ([b])	33
s.d.			34		31	

([1]) Inseminated 75 min after ejaculation. ([2]) Inseminated 135 min after ejaculation. ([3]) Semen was stored at −196 °C before insemination.

([ab]) Means in columns with the same letter are not significantly different ($P \geqslant 0.05$).

The mean percentage of fertile eggs laid by the hens inseminated with fresh or frozen-thawed semen are given in tables 1 and 2. The fertilizing capacity of undiluted semen was not significantly different from that of semen diluted 1 :4 in BPSE or semen to which DMSO had been added to a final concentration of 4 p. 100 (v/v) for up to 60 min.

When spermatozoa were exposed to the DMSO for 120 min, however, their fertilizing capacity was significantly reduced (table 2). Freezing and thawing of the ejaculate caused no further reduction in the fertilizing capacity of the spermatozoa (table 2).

In trial 1, the number of fertile eggs produced in the 5-day period by hens inseminated with frozen-thawed semen to which DMSO had been added at 15 °C within 45 min was lower, but not significantly different from the number of fertile eggs produced in the 5-day period by hens inseminated with undiluted or fresh semen (table 1). The fertilizing capacity of semen prepared for freezing by any other method was significantly lower than that of the control groups. When fertilizing capacity was expressed as « p. 100 fertility in 7 days », however, this relationship failed and only the fertilizing capacity of semen to which DMSO had been added at 15 °C in 10 min was not significantly different from the fertilizing capacity of fresh semen. The fertilizing capacity of the semen prepared for freezing by the other methods was separated into 3 subsets by Duncan's Multiple Range Test when the production of fertile eggs was expressed as « p. 100 fertility in 7 days ».

In trial 2, the differences between the fertilizing capacity of fresh and frozen semen were the same when expressed as either « p. 100 fertility in 5 days » or « p. 100 fertility in 7 days » (table 2). There were no significant differences between the fertilizing capacity of frozen-thawed semen which was cooled and equilibrated with DMSO by any of the methods. The fertilizing capacity of semen to which DMSO had been added for 120 min but not frozen was not significantly different from the fertilizing capacity of semen which had been frozen and thawed by any methods.

Although there was an apparent increase in the number of embryos which died during the first 7 days of incubation when hens were inseminated with frozen-thawed

TABLE 3

The percentage of embryos which died within the first 7 days of incubation in fertile eggs laid within 5 and 7 days after insemination by hens inseminated with fresh and frozen semen (Trial 1). None of the differences between the mean percentages of embryos which died were statistically significant ($P \geqslant 0.05$).

Vol. (ml) of water bath	Addition of DMSO		p. 100 Early dead in 5 days after insemination	Number of hens	p. 100 Early dead in 7 days after insemination	Number of hens
	Time (min)	Temp. (°C)				
0 ([1, 2])	—	—	5	11	5	11
0 ([2])	—	—	8	10	8	10
0 ([2])	15	24	5	10	7	10
0 ([3])	10	15	23	8	20	8
0 ([3])	120	5	23	7	21	7
80 ([3])	45	15	13	7	15	7
80 ([3])	120	7	13	8	0	7
150 ([3])	60	15	18	9	17	9
150 ([3])	120	9	17	7	17	8
250 ([3])	70	15	17	9	11	9
250 ([3])	120	10	9	9	12	9
s.d.			27		23	

([1]) Undiluted semen. ([2]) Inseminated within 45 min after ejaculation. ([3]) Semen was stored at −196 °C before insemination.

semen in trial 1 (table 3), there were no significant differences between any of the methods in either the number of embryos which died during the first seven days of incubation (tables 3 and 4) or the number of live embryos which continued development and hatched (table 5).

TABLE 4

The percentage of embryos which died within the first 7 days of incubation in fertile eggs laid within 5 and 7 days after insemination by hens inseminated with fresh and frozen semen (Trial 2). None of the differences between the mean percentages of embryos which died were statistically significant ($P \geqslant 0.05$).

Vol. (ml) of water bath	Addition of DMSO		p. 100 Early dead in 5 days after insemination	Number of hens	p. 100 Early dead in 7 days after insemination	Number of hens
	Time (min)	Temp. (°C)				
0 (1)	—	—	0	8	0	8
0 (2)	—	—	9	16	9	16
0 (1)	15	24	12	10	10	10
0 (2)	15	24	5	12	8	12
0 (3)	10	15	3	21	3	26
0 (3)	18	10	11	22	10	25
250 (3)	70	15	9	26	11	27
250 (3)	120	10	9	24	14	26
s.d.			21		21	

(1) Inseminated 75 min after ejaculation. (2) Inseminated 135 min after ejaculation. (3) Semen was stored at −196 °C before insemination.

TABLE 5

The percentage of embryos alive at 7 days of incubation which hatched from eggs laid by hens inseminated with fresh and frozen semen (Trial 2). None of the differences between the mean percentages of embryos which hatched were statistically significant ($P \geqslant 0.05$).

Vol. (ml) of water bath	Addition of DMSO		Mean p. 100 hatchability in 5 days after insemination	Number of hens	Mean p. 100 hatchability in 7 days after insemination	Number of hens
	Time (min)	Temp. (°C)				
0 (1)	—	—	100	8	100	8
0 (2)	—	—	96	15	96	16
0 (1)	15	24	100	10	98	10
0 (2)	15	24	92	12	96	12
0 (3)	10	15	90	21	93	26
0 (3)	18	10	90	21	89	24
250 (3)	70	15	97	25	95	26
250 (3)	120	10	87	23	90	24
s.d.			22		19	

(1) Inseminated 75 min after ejaculation. (2) Inseminated 135 min after ejaculation. (3) Semen was stored at −196 °C before insemination.

Discussion.

The concentrations of spermatozoa in the ejaculates which were used in these experiments were greater than those normally reported (Lake, 1962 ; Smyth, 1973 ; Nalbandov, 1976). This may be due to the removal of drinking water 2 h before ejaculation and collection of only the first part of the ejaculate. The collection of good quality semen is generally considered to be a prerequisite for successful artificial insemination with either fresh or frozen-thawed semen in any species and was probably a major factor contributing to the relatively good fertility of hens inseminated with frozen-thawed semen (tables 1 and 2).

The correlation between motility and fertilizing capacity of poultry semen has generally been reported to be poor (Lake, 1962 ; Wilson *et al.*, 1979) and this generalization was confirmed in the present study. For example, the addition of DMSO reduced motility immediately by 5 to 15 p. 100 but had no effect on fertility (tables 1 and 2). In contrast, the freezing and thawing procedure reduced motility of the semen by approximately 50 p. 100 although its fertilizing capacity (table 2) was equal to that of fresh semen equilibrated for 2 h with DMSO.

The fertilizing capacity of the semen reported in tables 1 and 2 was similar to that reported by Sexton (1976) and Bakst and Sexton (1979). The methods used in these experiments were modifications of these original methods aimed at identifying the effects of rates of cooling and the temperature of addition of DMSO on fertilizing capacity. In general, the results in tables 1 and 2 indicate that cooling the semen as rapidly as possible by exposure to a 5 °C environment and adding the DMSO within 10 min when the temperature of the semen was 15 °C produced no deleterious effects. From a pragmatic viewpoint, this is the most convenient technique and, therefore, the method of choice for most applications. It should also be noted, however, that this cooling rate was the most rapid in the experiment and 15 °C was the highest temperature at which DMSO was added. It is possible, therefore, that more rapid rates of cooling and/or adding the DMSO at higher temperatures may be equally or more successful techniques.

Bakst and Sexton (1979) reported that the fertilizing capacity of chicken semen was reduced after 2 h of equilibration with DMSO and further reduced by the actual procedure of freezing and thawing. In the present study, a reduction in the fertilizing capacity of semen incubated in the presence of DMSO was also observed (table 2), but no further reduction in fertilizing capacity was observed after freezing and thawing. An explanation of this difference is not apparent but may be related to differences in the procedures for handling semen after thawing and before insemination.

Hens inseminated with semen which was equilibrated with DMSO for only 60 min produced significantly more fertile eggs than hens inseminated with semen equilibrated with DMSO for 120 min (table 2). This observation may suggest that the preparation of the ejaculates for freezing might be further improved by reducing the equilibration period with DMSO. Similar modifications have been used with limited success in preserving semen from Sandhill cranes (Sexton and Gee, 1978).

The estimates of fertilizing capacity of semen prepared for insemination by any method was always lower when calculated during a 7-day period rather than a 5-day

period. The reduction in « p. 100 fertility in 7 days » was similar for both frozen and fresh semen indicating that fertilizing capacity did not decline more rapidly when hens were inseminated with frozen semen. In all cases, optimum fertility could only be maintained by inseminating hens more frequently than once per week.

Inseminating hens with frozen-thawed semen did not appear to cause any malformation of the embryos. The data in tables 3 to 5 show that neither embryonic mortality during the first 7 days of incubation nor embryonic mortality during the last 14 days of incubation was significantly affected by the processing method. The techniques used to determine embryonic mortality, however, could not detect embryonic development which ceased during the first 18 to 24 h of incubation (Kosin *et al.*, 1951) and, therefore, the possiblity of an effect of using frozen-thawed semen on very early embryonic mortality cannot be excluded at this time.

In conclusion, we have shown that the fertilizing capacity of fowl semen is best maintained by adding the DMSO at 15 °C and by cooling the semen rapidly to 5 °C prior to freezing. This method not only preserves the fertilizing capacity of a relatively high proportion of spermatozoa but offers technical simplicity for practical applications in the poultry industry.

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Résumé. Du sperme de coqs Leghorn Blanc à crête simple a été récolté, dilué 1 :4 avec du dilueur de Beltsville à 35 °C et refroidi à différentes vitesses jusqu'à 5 °C. Du DMSO a été ajouté au sperme dilué de 10 à 120 min après éjaculation, à la température de 15 ou 5 °C. Après l'addition de DMSO, le sperme a été laissé à équilibrer pendant 2 h à 5 °C, puis congelé à — 1 °C/min de + 5 °C à — 20 °C, transféré dans les vapeurs d'azote pendant 4 à 10 min, puis immergé dans l'azote liquide pendant 4 à 60 jours. La semence décongelée a été inséminée 2 jours de suite et la fertilité calculée à partir des œufs du 2e au 7e jour ou du 2e au 9e jour post-insémination. En général, la fertilité n'a été affectée ni par la vitesse de refroidissement, ni par la température à laquelle le DMSO a été ajouté. Dans un essai, cependant, la fécondance a été significativement plus élevée dans le cas où le DMSO a été ajouté à 45 min à 15 °C. 42 à 71 p. 100 des œufs pondus dans les 5 jours suivant la seconde insémination ont été fécondés, quelles que soient la méthode de refroidissement ou la température d'addition du DMSO. La moitié des éjaculats après décongélation et avant insémination a varié de 15 à 55 p. 100, et n'a constitué qu'un mauvais indicateur du pouvoir fécondant. La mortalité embryonnaire n'a pas été affectée par les conditions de refroidissement et de congélation et l'éclosivité des œufs fécondés était la même que le sperme ait été utilisé à l'état frais ou après congélation-décongélation.

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