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FERTILITY OF DEEP FROZEN BOAR SPERMATOZOA AT VARIOUS INTERVALS BETWEEN INSEMINATION AND INDUCED OVULATION

INFLUENCE OF BOARS AND THAWING DILUENTS*

By

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LARSSON, K.: *Fertility of deep frozen boar spermatozoa at various intervals between insemination and induced ovulation. Influence of boars and thawing diluents.* Acta vet. scand. 1976, *17*, 63—73. — This study was performed to investigate the influence of boars and thawing diluents on the fertilizing capacity of deep frozen spermatozoa at various intervals between inseminations and ovulation.

Forty-four Swedish crossbred gilts were inseminated following injection of HCG late in the prooestrus. Inseminations were performed 22, 28, 34 and 38 hrs. after injection of HCG. Ovulation was expected to occur 40 hrs. after injection of HCG. Two boars, previously tested for fertility with frozen semen, supplied the spermatozoa. Boar seminal plasma and OLEP were utilized as thawing diluents. The gilts were slaughtered 32—48 hrs. after estimated ovulation. The genital tracts were removed immediately after stunning and bleeding and the numbers of recent ovulations, recovered ova and fertilized ova were recorded. Additionally recovered ova were classified according to estimated numbers of spermatozoa attached to the zona pellucida.

Similar fertilization rates were obtained when inseminations were performed 2 and 6 hrs. before estimated ovulation. A clear decline in fertility appeared when inseminations were performed earlier than 6 hrs. before expected ovulation. The results were influenced by the boars as well as by the thawing diluents. Seminal plasma yielded a higher fertilization rate than OLEP in inseminations performed 2 hrs. before estimated ovulation. The boars yielded similar fertility in inseminations performed 2 hrs. before estimated ovulation. With increasing intervals between inseminations and ovulation the difference between the boars increased. The single gilt in which fertilized ova were found after insemination 18 hrs. before ovulation was inseminated with spermatozoa from the superior boar, thawed in seminal plasma. The present results indicate that spermatozoa with low resistance to freezing-thawing have a short fertile life in the female genital tract after insemination.

deep frozen boar spermatozoa; fertility; in vivo survival; boars; thawing diluents.

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Under conditions of natural service boar spermatozoa remain fertile for 24—48 hrs. after mating (cf. *Austin* 1975). The information on the fertile life in the female genital tract of boar spermatozoa prepared for artificial insemination is so far limited. *Alanko* (1974) reported a higher fertility rate in gilts inseminated with fresh semen (< 2 hrs. old) than with older semen (> 24 hrs. old). With the older semen the best fertility results were obtained if inseminations were performed close to the time of ovulation. The number of live spermatozoa in the oviducts at the time of ovulation was in that study considered a determining factor for the fertility results.

With frozen boar spermatozoa *Pursel & Johnson* (1975) obtained similar fertility results in gilts inseminated twice with 5 hrs.' interval, the first inseminations being performed 20, 15, 10 or 5 hrs. prior to or at the time of induced ovulation. Spermatozoa from three boars were pooled in this study. Differences in fertility among the boars were thus not considered.

The possibilities of precise control of the time of ovulation in pigs by means of chorionic gonadotrophin (HCG) injection late in the prooestrus (*Dziuk & Polge* 1962, *Polge & Dziuk* 1965, *Hunter* 1967 a, b, 1972, 1974) have been utilized in a number of investigations concerning fertilization in pigs. The accuracy of the method was evaluated by *Hunter* (1967 a) who concluded that ovulation appears at a predictable interval after injection of HCG. The duration of the ovulation under such conditions was approx. 1 hr. Depending on the breed of the gilts the interval from injection to ovulation has been estimated to be 39—42 hrs. (*Hunter* 1967 a, 1974, *Hunter & Dziuk* 1968, *Alanko, Pursel & Johnson* 1975).

The aim of the present investigation was to compare the fertilizing capacity of deep frozen spermatozoa from two boars thawed in seminal plasma or in OLEP and inseminated at various intervals before induced ovulation.

MATERIAL AND METHODS

Forty-four Swedish crossbred gilts were inseminated in this trial. The gilts were approx. seven to 11 months of age and their live weights averaged 100—150 kg. One Swedish Landrace boar (172) and one Swedish Yorkshire boar (388) supplied the semen. The boars were previously tested for fertility with frozen semen and the fertility of boar 388 was known to be superior to that of

boar 172 (*Larsson & Einarsson* 1976). Seven ejaculates from each boar were utilized in the trial.

Experimental design

All gilts were inseminated once with 6×10^9 frozen-thawed spermatozoa in 70 ml of thawing diluent. Inseminations were performed 22, 28, 34 and 38 hrs. following HCG injection late in the prooestrus. According to the literature previously cited, ovulation could be expected to occur 40 hrs. after injection. Therefore the intervals between insemination and ovulation were estimated at 18, 12, 6 and 2 hrs. The utilization of boars and gilts as related to the interval from HCG injection to insemination and to the use of thawing diluents is presented in Table 1.

Table 1. Utilization of boars and gilts as related to time of insemination and thawing diluents (A = boar seminal plasma, C = OLEP).

Boar	Thawing diluent	Interval from injection of HCG to insemination (number of gilts inseminated)			
		22	28	34	38
172	A	2	3	3	3
172	C	2	3	3	3
388	A	2	3	3	3
388	C	2	3	3	3

Collection and processing of semen

Semen was collected by the gloved hand technique and frozen once a week during the experimental period. Freezing was performed according to the method of *Crabo & Einarsson* (1971) as described by *Larsson & Einarsson*. Thawing was performed as described by *Larsson & Einarsson*. Only seminal plasma deriving from the sperm-poor fractions of the boars supplying the spermatozoa was utilized. OLEP was prepared and stored as described by *Larsson & Einarsson*.

Heat detection, induction of ovulation and inseminations

The gilts were checked for oestrus once daily by an experienced stockman with the aid of vasectomized boars. After a minimum of two recorded normal oestrus periods ovulation was induced by one injection of 500 i.u. HCG (Prolan ad us vet®,

Bayer) late in the prooestrus. The prooestrus was defined by the swelling and reddening of the vulva and by the length of the preceding oestrus interval(s). During the prooestrus period heat was controlled at least twice daily and all gilts were confronted with a vasectomized boar immediately before HCG injection to check that they were still in prooestrus. The inseminations were performed with rubber spiral tip catheters according to *Melrose & O'Hagan* (1961).

Postmortem examination

The gilts were slaughtered 32—46 hrs. after estimated ovulation (in one case 22 hrs.). Immediately after stunning and bleeding the genital tracts were removed. Within 45 min. after slaughter the genital tracts were examined and the numbers of fresh corpora lutea (c. l.) in the ovaries were recorded. The oviducts were cut into an isthmic and an ampullary part. Each oviductal part as well as the proximal 10 cm of the uterine horns were flushed with 5 ml of physiological saline solution. The flushing fluids were collected in separate tubes and examined for presence of ova under a dissection microscope at 15 $\times$ magnification. Wet preparations of recovered ova were examined for cleavage and the numbers of spermatozoa attached to the zona pellucida were estimated under a phase-contrast microscope at a maximum of 400 $\times$ magnification. Ova were classified according to the estimated number of spermatozoa attached to the zona pellucida as follows: 0, 1—4, 5—8, > 8. Only normally cleaved ova were recorded as fertilized, uncleaved and asymetrically cleaved ova were recorded as unfertilized.

STATISTICAL ANALYSES

Differences in percentages of fertilized ova out of all recovered ova as related to time of insemination, boars and thawing diluents were tested by chi-square analysis (*Snedecor* 1966). The degree of significance is expressed as follows:

$0.05 > P > 0.01$ almost significant*
$0.01 > P > 0.001$ significant**
$P < 0.001$ highly significant***

RESULTS

The results are summarized in Tables 2—5. The mean recovery rate of ova was 84 %, ranging from 64 to 100 % in single groups of gilts.

Table 2. Influence of estimated interval between insemination and ovulation on the fertility of deep frozen boar spermatozoa.

Interval between insemination and ovulation (hrs.)	Number of gilts with fertilized ova out of all gilts	Total and mean numbers in all gilts inseminated of			Percentages of fertilized ova in	
		corpora lutea (c.l.)	recovered ova (r.o.)	fertilized ova (f.o.)	all gilts	gilts with f.o.
2	Total 10/12	159	121	64		
2	Mean (83 %)	13.3	10.1	5.3	53	62
6	Total 10/12	152	121	59		
6	Mean (83 %)	12.7	10.1	4.9	49[a]	61
12	Total 8/12	149	132	44		
12	Mean (75 %)	12.4	11.0	3.7	33[ab]	44
18	Total 1/8	118	95	5		
18	Mean (13 %)	14.8	11.9	0.1	4[b]	7

a: Difference between intervals 6 and 12 hrs. is almost significant ($P < 0.05$)*
b: Difference between intervals 12 and 18 hrs. is highly significant ($P < 0.001$)***

Table 3. Influence of thawing diluents on the fertility of deep frozen boar spermatozoa as related to estimated interval between insemination and ovulation.

Interval between insemination and ovulation (hrs.)	Thawing diluent	Number of gilts with fertilized ova out of all gilts	Mean numbers in all gilts inseminated of			Percentages of fertilized ova in	
			c.l.	r.o.	f.o.	all gilts	gilts with f.o.
2	A	6/6	13.5	10.5	6.8	65[a]	66
2	C	4/6	13.0	9.7	3.8	40[a]	58
6	A	5/6	14.3	10.3	5.0	48	62
6	C	5/6	11.0	9.8	4.8	49	60
12	A	4/6	12.0	11.2	4.0	36	31
12	C	4/6	12.8	10.8	3.3	31	37
18	A	1/4	14.5	12.8	1.3	10	15
18	C	0/4	15.0	11.0	0	0	0
Total	A	16/22	13.6	11.2	4.5	41[b]	49
	C	13/22	12.8	10.3	3.3	32[b]	39

a: Difference between thawing diluents is significant ($P < 0.01$)**
b: Difference between thawing diluents is almost significant ($P < 0.05$)*

Table 4. Influence of boars on the fertility of deep frozen spermatozoa as related to estimated interval between insemination and ovulation.

Interval between insemination and ovulation (hrs.)	Boar	Number of gilts with fertilized ova out of all gilts	Mean numbers in all gilts inseminated of			Percentages of fertilized ova in	
			c.l.	r.o.	f.o.	all gilts	gilts with f.o.
2	172	5/6	14.0	9.8	5.3	54	60
2	388	5/6	12.5	10.3	5.3	52	63
6	172	5/6	13.3	11.7	4.5	39[a]	51
6	388	5/6	12.0	8.5	5.3	63[a]	71
12	172	3/6	11.8	11.8	1.8	15[b]	28
12	388	5/6	13.0	10.2	5.5	54[b]	61
18	172	0/4	12.5	10.5	0	0	0
18	388	1/4	17.0	13.3	1.3	9	15
Total	172	13/22	13.0	11.0	3.2	29[c]	35
	388	16/22	13.3	10.3	4.6	45[c]	53

a: Difference between boars is almost significant ($P < 0.05$)*
b: Difference between boars is highly significant ($P < 0.001$)***
c: Difference between boars is highly significant ($P < 0.001$)***

Table 2 shows the general influence of the interval from insemination to ovulation. A clear decline in fertility as reflected by the number of gilts with fertilized ova and by numbers and percentages of fertilized ova appeared when inseminations were performed earlier than 6 hrs. before estimated ovulation. Very few spermatozoa remained fertile as long as 18 hrs. after insemination. The decline in percentage of fertilized ova was almost significant ($P < 0.05$) between 6 and 12 hrs. and highly significant ($P < 0.001$) between 12 and 18 hrs.

In Table 3 is shown the influence of the thawing diluents on the fertility at the various intervals. Spermatozoa thawed in seminal plasma seemed to maintain their fertilizing capacity a little longer than did spermatozoa thawed in OLEP. Sixty-five % of the recovered ova were fertilized when the inseminations were made 2 hrs. before the estimated time of ovulation with spermatozoa thawed in seminal plasma. The corresponding fertilization rate with spermatozoa thawed in OLEP was 40 %. This difference is significant ($P < 0.01$). Smaller and insignificant differences occurred between the thawing diluents at the intervals 12 and

Table 5. Numbers of recovered ova as related to estimated interval between insemination and ovulation to boars and to thawing diluents. Percentages of ova in classes according to estimated numbers of spermatozoa attached to zona pellucida.

Interval between insemination and ovulation (hrs.)	Boar	Thawing diluent	Total number of recovered ova	Number of spermatozoa attached to zona pellucida			
				0	1—4	5—8	> 8
				(% of ova in classes)			
2	172	A	31	32	61	7	0
	388	A	32	25	75	0	0
	172	C	28	39	54	7	0
	388	C	30	53	47	0	0
6	172	A	35	48	29	9	14
	388	A	27	30	52	18	0
	172	C	35	63	31	6	0
	388	C	24	29	54	17	0
12	172	A	38	76	24	0	0
	388	A	29	55	41	4	0
	172	C	33	97	3	0	0
	388	C	32	25	72	3	0
18	172	A	22	100	0	0	0
	388	A	29	72	28	0	0
	172	C	20	100	0	0	0
	388	C	24	100	0	0	0

18 hrs. The total difference between the thawing diluents was almost significant ($P < 0.05$).

The effect of the boars was more marked than the effect of the thawing diluents (Table 4). In gilts inseminated 2 hrs. before estimated ovulation the fertility results were very similar for the two boars. The fertility of boar 172 then declined gradually with prolonged intervals from insemination to ovulation. In boar 388 no similar decline appeared until the interval reached 18 hrs. In gilts inseminated 6 and 12 hrs. before the estimated time of ovulation the percentages of fertilized ova out of all recovered ova differed almost significantly ($P < 0.05$) and highly significantly ($P < 0.001$) respectively, between the boars. The single gilt with fertilized ova following insemination 18 hrs. before estimated ovulation was inseminated with spermatozoa from boar 388 thawed in seminal plasma. In total, inseminations with spermatozoa from boar 388 yielded 45 % fertilized ova in con-

trast to 29 % for boar 172. This difference is highly significant ($P < 0.001$).

In Table 5 the recovered ova have been classified according to estimated numbers of spermatozoa attached to the zona pellucida. It appears that ova recovered from gilts inseminated with spermatozoa thawed in seminal plasma at 2 hrs. before estimated ovulation tended to have more spermatozoa in the zona pellucida than did ova recovered from gilts inseminated with spermatozoa thawed in OLEP at the corresponding time. In gilts inseminated closer to the time of HCG injection this tendency was less marked. On the other hand there seems to be increasing influence of the boars on the percentages of recovered ova with spermatozoa attached to the zona pellucida when the time from HCG injection to insemination decreased. In general, spermatozoa from boar 388 attached to the zona pellucida at much higher rates than did spermatozoa from boar 172.

DISCUSSION

In the present study the influence of boars and thawing diluents on the fertilizing capacity of frozen-thawed spermatozoa at various times after insemination was investigated.

The results (Table 2) indicate that good fertility with deep-frozen boar spermatozoa could be expected if inseminations were performed shortly (2 to 6 hrs.) before estimated ovulation. However, under the conditions of this study, the thawing diluents and in particular the boars appeared to have a marked influence on the maintenance of the fertilizing capacity of the frozen-thawed spermatozoa after insemination.

Seminal plasma seemed to maintain the fertilizing capacity of the frozen-thawed spermatozoa a little longer than did OLEP. The differences between the two thawing diluents were, however, limited and amounted in the total material to about 10 % (Table 3). Only in gilts inseminated shortly before induced ovulation was there a significant difference in percentages of fertilized ova. If the percentages of fertilized ova were calculated only in gilts with fertilized ova, the difference was insignificant. The reason for a possible superiority of seminal plasma might be an effect of the seminal plasma on the sperm transport into the oviducts. Such an effect was demonstrated by *Viring et al.* (1974, 1976). Sperm penetration of pig ova appears already 2 to 3 hrs. after

insemination with undiluted fresh semen (*Hunter & Dziuk* 1968). A promoted transport of frozen-thawed spermatozoa into the oviducts might therefore be of importance for fertilization in gilts inseminated close to the ovulation. However, further experiments are necessary to elucidate this question.

The decline in fertility with prolonged interval from insemination to ovulation was not similar for the boars. Apparently the initial fertilizing capacity of the frozen-thawed spermatozoa was equal. However, the fertile life after insemination was markedly shorter for spermatozoa from the "low-fertile" boar. These results are not in agreement with those of *Pursel & Johnson* (1975) who did not find any differences in fertility of frozen-thawed boar spermatozoa inseminated at various intervals before ovulation. However, the two studies are not fully comparable from a methodological point of view since *Pursel & Johnson* repeated the inseminations after 5 hrs. and used pooled semen from three boars.

The numbers of spermatozoa attached to the zona pellucida of the ova corresponded very well to the incidence of fertilization. In general the numbers of spermatozoa attached to the zona pellucida were much lower than those reported after fresh boar semen inseminations (*Alanko* 1974).

To avoid incidental polyspermic fertilization connected with inseminations late in the oestrus period (*Thibault* 1959, *Alanko*) or with inseminations performed late after induced ovulation (*Hunter* 1967b), all inseminations were performed before the time of estimated ovulation. Therefore it is not possible to establish the most suitable time for insemination of frozen-thawed boar semen from the present results alone. However, for boars with spermatozoa having good resistance to freezing-thawing, acceptable results could be obtained after inseminations performed at least 12 hrs. before ovulation. In terms of spontaneous oestrus the best time for insemination would then be 15 to 30 hrs. after onset of oestrus. These recommendations are in general agreement with those earlier stated for fresh boar semen inseminations (*Thibault, Alanko, Swensson* 1975).

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SAMMANFATTNING

Befruktningsförmågan hos djupfrysta-upptinade galtspermier vid olika intervall mellan insemination och ovulation. Effekt av galtar och upptiningsmedier

Fyrtiofyra korsningsgyltor inseminerades efter injektion av HCG sent i förbrunsten. Inseminationerna utfördes 22, 28, 34 och 38 timmar efter HCG-injektion. Ovulationen beräknades ske i genomsnitt 40 timmar efter HCG injektion. Två galtar, vars fertilitet med fryst sperma var känd, utnyttjades för spermasamling. Spermaplasma från galt och OLEP användes som upptiningsmedier. Gyltorna slaktades 32—46 timmar efter ovulation. Könsorganen tillvaratogs omedelbart efter avblodningen och antalen färska ovulationer, återfunna ägg och befruktade ägg registrerades. Dessutom indelades de återfunna äggen i klasser beroende på antalet spermier i zona pellucida.

Likartade befruktningsfrekvenser erhölls när inseminationerna företogs 2 och 6 timmar före förväntad ovulation. En uppenbar nedgång i fertilitet erhölls när inseminationerna företogs tidigare än 6 timmar före förväntad ovulation. Såväl galtarna som tiningsmedierna påverkade resultaten. Spermaplasma gav högre befruktningsfrekvens än OLEP vid insemination 2 timmar före förväntad ovulation. Galtarna gav likvärdiga befruktningsfrekvenser när inseminationerna gjordes 2 timmar före ovulation. Med ökande intervall mellan insemination och ovulation ökade skillnaden mellan galtarna. Den enda gylta hos vilken befruktade ägg återfanns efter insemination 18 timmar före ovulation var inseminerad med spermier från den bästa galten upptinade i spermaplasma. De föreliggande resultaten visar att spermier med låg motståndskraft mot djupfrysning och upptining bibehåller sin befruktningsförmåga i honliga genitalia kort tid efter insemination.

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