THE INFLUENCE OF THE AGEING OF RABBIT SPERMATOZOA *IN VITRO* ON FERTILIZING CAPACITY AND EMBRYONIC MORTALITY

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Summary. Female rabbits were inseminated with spermatozoa which had either been freshly ejaculated or stored for 24 or 48 hr at 5° C. One oviduct of each female was flushed 26 to 28 hr after insemination and HCG injection. The ova were examined and returned to the same oviduct if cleavage had occurred. This procedure did not affect the subsequent development of the ova, as compared with those in the undisturbed oviduct. The number of implantations was counted at 8 days and the number of live fetuses at 20 days of pregnancy. Compared with fresh spermatozoa, the stored samples showed significantly lower fertilizing capacity and the fertilized ova showed significantly higher pre- and postimplantation losses.

INTRODUCTION

Ageing of spermatozoa *in vitro* causes a decrease in their fertilizing capacity and an increase in embryonic mortality. This has been amply demonstrated in relation to artificial insemination in cattle by Salisbury & Flerchinger (1961, 1967). Dziuk & Hanshaw (1958) and First, Stratman & Casida (1963) found increased embryonic mortality after insemination with boar spermatozoa which had been stored for 72 hr.

Spermatozoa also gradually lose their fertility in the isolated epididymis *in vivo*, though after a much longer time interval (Hammond & Asdell, 1926; Tesh & Glover, 1966). Functional changes evoked by ageing occur rapidly in spermatozoa in the female reproductive tract (rabbits—Hammond & Asdell, 1926; Maurer, Whitener & Foote, 1969; Tesh, 1969; guinea-pigs—Soderwall & Young, 1940).

The aim of the present work was to investigate the fertilizing capacity of rabbit spermatozoa aged *in vitro* by direct evaluation of ova, and the pre- and postimplantation losses by examination of the uterine contents.

MATERIALS AND METHODS

Sexually mature females were isolated from males for at least 3 weeks before

insemination. Semen from six males of known fertility was collected by means of an artificial vagina and investigated microscopically. Ejaculates of good quality were diluted with a tris-egg yolk solution (Koefoed-Johnsen, 1964) to a concentration of 75 to 100×10^6 spermatozoa/ml. An aliquot of the diluted semen was used immediately, and the remainder was cooled to $+5^\circ \text{C}$ and stored for 24 or 48 hr. The females were inseminated intravaginally with 20 to 40×10^6 spermatozoa. An injection of HCG (Physex-Leo, Copenhagen-10 i.u./kg body weight) was given immediately after insemination. In most cases, two to three females of each group were inseminated with fresh semen, and the same number with semen which had been stored 24 and 48 hr, respectively. However, if ejaculates were of inferior quality, the last group of females (semen stored for 48 hr) was reduced or the ejaculate was discarded.

The females were subjected to laparotomy under ether anaesthesia 26 to 28 hr after insemination, the abdomen was opened by a flank incision on the left side, or by an incision in the linea alba. After checking the ovaries and determining ovulation points on both ovaries, a polyethylene catheter fitted with a glass tube was inserted from the infundibular end into one oviduct to a depth of 1 to 2 cm. With a syringe and a thin needle (No. 20) introduced near the utero-tubal junction, the contents of the oviduct were flushed into a watch glass. Two to three ml of medium TC 199, pH adjusted to 7.5 to 7.8 with sodium carbonate, were used for flushing.

The contents of the oviduct were investigated under a stereomicroscope at $\times 20$ magnification. All cleaved ova were returned to the oviducts by means of a thin glass capillary. The whole procedure was carried out under constant temperature conditions. Uncleaved ova were evaluated under the microscope after fixation with acetic acid: alcohol and staining with lacmoid (Chang, 1952).

Only one oviduct from each female was flushed, the other served as a direct control of the experimental procedure. The females were again subjected to laparotomy 8 days after insemination in order to check the number of implanted embryos and corpora lutea. Twenty days after insemination, the females were killed and their uterine contents investigated.

RESULTS

The data summarized in Table 1 show the progressive decrease in the fertilizing capacity of stored spermatozoa and the increase in preimplantation losses. The decrease in fertilizing capacity after storage for 24 hr reaches a statistically significant value. A further decrease, though not statistically significant, was found between 24 and 48 hr. Embryonic mortality in the preimplantation period also increased with the time of storage *in vitro*.

The experimental procedures, i.e. flushing of oviducts and return of ova, appeared to have no adverse effect on the further development of the embryos, since the number of implantations expected in the control uterine horns did not significantly exceed the actual implantations in the treated ones.

Table 2 shows the postimplantation losses, including the number of live embryos after 20 days of pregnancy. The dependence on the age of the spermatozoa was also evident here. The differences of postimplantation losses between

TABLE 1
FERTILIZATION AND IMPLANTATION RATES AFTER INSEMINATION WITH FRESH AND STORED SPERMATOZOA

Age of sperm. (hr)	Treatment of oviducts	No. of oviducts	No. of ovulation points	No. of ova			% of ova fertilized	No. of implanted embryos at 8 days	% of implanted embryos	
				Flushed	Fertilized	Redeposited			From ova redeposited	From ovulation points
0	Flushed	28	157	151	140	140	92.7*	123	87.8**	78.3
24		29	153	152	119†	114†	78.3*	89	78.9**	58.2
48		19	100	97	71	71	73.2*	46	64.8**	46.0
0	Control	28	128					105		82.0
24		29	131					84		64.1
48		19	96					47		48.9

* Difference 0 to 24 hr: $\chi^2 = 11.570$ ($P < 0.005$)
0 to 48 hr: $\chi^2 = 16.288$ ($P < 0.005$)

† Five fertilized ova uncleaved at the time of examination.

** Difference

0 to 24 hr: $\chi^2 = 3.681$ ($0.1 > P > 0.05$)
0 to 48 hr: $\chi^2 = 14.319$ ($P < 0.005$)
24 to 48 hr: $\chi^2 = 3.268$ ($0.1 > P > 0.05$)

TABLE 2
EMBRYO SURVIVAL AFTER IMPLANTATION

Age of sperm. (hr)	No. of females	Treatment of oviducts	No. of implanted embryos at 8 days	No. of live embryos at 20 days	No. of live embryos/female		% postimplantation mortality
					At 8 days after insemination	At 20 days after insemination	
0	24	Flushed Control Total	103 86 189	91 78 169			
					7.87 $\pm$ 0.72	7.04 $\pm$ 0.72	10.5
24	25	Flushed Control Total	75 74 149	62 61 123			
					5.96 $\pm$ 0.58	4.92 $\pm$ 0.53	17.4
48	17	Flushed Control Total	39 35 74	29 29 58			
					4.35 $\pm$ 0.81	3.41 $\pm$ 0.76	21.6

The differences between the number of females and implanted embryos at 8 days in Tables 1 and 2 are due to the elimination of animals with postoperative complications which may have affected the normal development of the foetus. Difference 0 to 24 hr: $\chi^2 = 2.782$ ($0.1 > P > 0.05$)
0 to 48 hr: $\chi^2 = 4.590$ ($0.05 > P > 0.025$)

the groups inseminated with fresh spermatozoa and spermatozoa stored for 48 hr were statistically significant, but they did not reach the level of the pre-implantation losses.

DISCUSSION

The changes which occur during the storage of spermatozoa which are responsible for their functional inadequacy are as yet insufficiently known. The original findings of Salisbury, Birge, de la Torre & Lodge (1961) and more recently of other authors concerning the loss of spermatozoal DNA during storage *in vitro* have not been fully confirmed (Koefoed-Johnsen, Fulka & Kopečný, 1968; Miller & Blackshaw, 1968; Kopečný & Trebichavský, 1969). Further detailed studies allowed Salisbury (1968) to assume that the changes evoked by ageing affect the spermatozoal DNA or DNP molecule qualitatively rather than quantitatively and may cause damage to the genetic information transfer system.

Spermatozoa stored *in vitro* can lose their fertilizing capacity to different degrees depending on the storage method used. In our experiments, in spite of the relatively high initial fertility, a decline was observed, which was more rapid than that reported for rabbit spermatozoa by Norman (1964) who used NJ 2 medium, or Miller & Blackshaw (1968) who diluted ejaculates in IVT medium.

Besides decreased fertility, high preimplantation losses, depending on the period of storage, were observed in the present study. These results are in accordance with those of Maurer *et al.* (1969), who found that spermatozoa ageing in the female reproductive tract were associated with reduced fertility and cleavage rate, and a lower proportion of embryos developing into blastocysts. In contrast, Tesh (1969) found, for the same type of spermatozoa, reduced fertility only and to a lesser extent an increase of postembryonic losses. He did not observe any effect on preimplantation development. Thibault (1967) described an increased incidence of digyny after fertilization with ageing spermatozoa.

In females with a considerably reduced fertilization rate, especially those which were inseminated with spermatozoa which had been stored for 48 hr, the 'undercrowding' effect (Hafez, 1968) may have been one of the causes of higher embryonic mortality.

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