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Indirect Assessment of Sperm Capacitation Using Zona-free Hamster Eggs in the Goat

I. Penetration into Zona-free Hamster Eggs by Goat Spermatozoa Preincubated in the Uteri Isolated from Hamsters and Rats

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Summary

When goat spermatozoa were preincubated for 4-6 h and 6 h in the uteri isolated from hamster and rat, and for 6 h in the hamster uterus *in situ*, they developed the ability to penetrate zona-free hamster eggs *in vitro*.

Zona-free hamster eggs were not penetrated after insemination with goat spermatozoa preincubated in the hamster uterus *in situ* and isolated hamster uterus 15 h before the expected time of ovulation and that of rat, but 12 and 6% of eggs were penetrated by spermatozoa preincubated in the isolated hamster uterus 4 h before and 2 h after the expected time of ovulation, respectively.

Zona-free hamster eggs were not penetrated after insemination with goat spermatozoa preincubated for 4 h in the isolated hamster uterus, but 10 and 18% of eggs were penetrated by spermatozoa preincubated for 5 and 6 h in the isolated uterus.

Introduction

In most *in vitro* fertilization procedures, it is necessary to use sperm previously capacitated in the female genital tract or in physiologically defined conditions. Based on studies of penetration of zona-free hamster eggs by spermatozoa from guinea pig (Yanagimachi, 1972), mouse (Hanada and Chang, 1972), rat (Hanada and Chang, 1976) and human (Yanagimachi et al., 1976), it has been suggested that the penetration of zona-free hamster eggs by foreign sperm may required capacitation.

In domestic animals, studies on sperm penetration into zona-free hamster eggs have been conducted to examine capacitation of bull (Hanada and Nagase, 1981; Sirard et al., 1984) and boar (Imai et al., 1977, 1979, 1980) spermatozoa preincubated in the uterus of heterologous species. Capacitation of goat spermatozoa using a hamster test has been performed using spermatozoa preincubated in the uterus of heterologous species (Kim et al., 1980; Kim, 1981; Kato et al., 1984), but has not been performed on spermatozoa preincubated in the uteri isolated from estrous hamsters and rats.

The present study was conducted to investigate the possibility of capacitation of goat spermatozoa in the uteri isolated from estrous hamsters and rats. Since freshly ovulated eggs are not easily available in goats, another purpose of this study was to examine the possibility of using zona-free hamster eggs for indirect assessment of the fertilizing capacity of goat spermatozoa.

Materials and Methods

The medium used for the manipulation of gametes was a modified Krebs-Ringer bicarbonate (m-KRB) solution (Toyoda and Chang, 1974). The composition of m-KRB solution is shown in Table 1.

Semen (0.4-1.0 ml) was collected from two goats of the Saanen breed using an artificial vagina and the percentage of motile spermatozoa after collection was 80-90%. The semen was diluted to 10 ml m-KRB solution (BSA 1 mg/ml) and washed once by centrifugation at 500g for 10 min. The sedimented spermpack was resuspended in about 2 ml m-KRB solution. About 0.03-0.05 ml of the sperm suspension at the concentration of 15-20 x 10^8/ml was introduced into uterine horn isolated from hamsters superovulated by PMSG and hCG or exposed by laparotomy 11 h after hCG injection, or into the uteri of estrous rats, and then the uterotubal junction and the cervical end of the uterus were ligated. The isolated uteri were dipped in 0.9% NaCl solution and kept in a CO_2 incubator (5% CO_2 in air at 37°C) for 5-5.5 h (Table 2), or for 4, 5 and 6 h (Table 3). The laparotomized hamsters were killed 5-5.5 h later and then the uteri were isolated.

The spermatozoa were recovered by flushing the reproductive tracts after incubation. Contaminating blood cells and cellular debris were removed by centrifugation at 150g for 10 min. All sperm samples were washed again by centrifugation at 500g for 10 min. For insemination, spermatozoa (5 μl of the concentrated suspension from the tract flushings) were introduced into each plastic dish (35 x11 mm) containing zona-free hamster eggs to give a concentration of 0.02-2.0 x 10^6 spermatozoa/ml. The motility of spermatozoa slightly decreased 4 h after incubation in the reproductive tracts, but was lost or very poor after 5 or 6 h insemination. These spermatozoa, however, always became motile after washing although the motility was not better than that of spermatozoa incubated for 4 h.

Mature female golden hamsters were superovulated with an intraperitoneal injection of 25 IU PMSG, followed by an intraperitoneal injection of 25 IU hCG 48-52 h later (Yanagimachi and Chang, 1964). They were killed 15-17 h after injection of hCG.

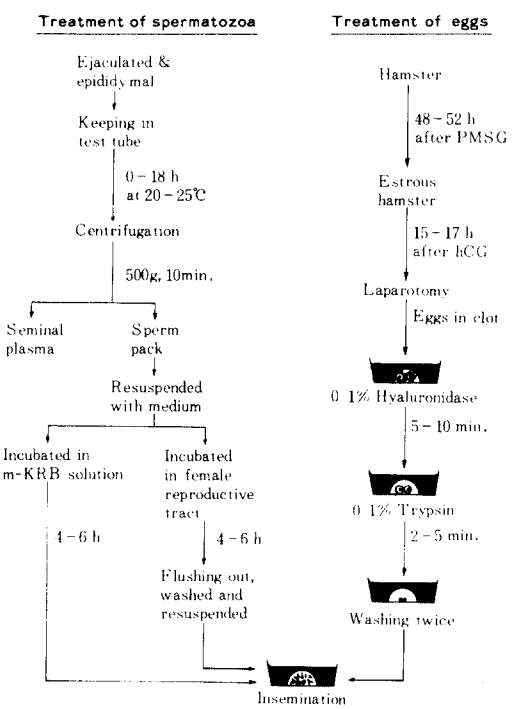
Eggs recovered from the ampulla of the oviducts were treated for 5-10 min. with m-KRB solution (without BSA) containing 0.1% hyaluronidase (Type I: Sigma Chemical Co.) for dispersion of cumulus cells and for 2-5 min. with the medium containing 0.1%

Table 1. Composition of modified Krebs-Ringer bicarbonate solution

Componten	mM
NaCl	94.60
KCl	4.78
$CaCl_2 \cdot 2H_2O$	1.71
$MgSO_4 \cdot 7H_2O$	1.19
KH_2PO_4	1.19
$NaHCO_3$	25.07
Glucose	5.56
Na-pyruvate	0.50
Na-lactate	21.58
Streptomycin	50 ug/ml
Penicillin	75 ug/ml
BSA	*
Phenol red	2 ug/ml

* Crystalline bovine serum albumin (BSA) was supplemented 1mg/ml for sperm washing and preincubation, and 4 mg/ml for oocyte culture and fertilization

Figure 1. Experimental procedures for treatment of goat spermatozoa and zona-free hamster eggs, and insemination *in vitro*.



bovine pancreatice trypsin (Type III: Sigma Chemical Co.) for removal of the zona pellucida, according to the procedures reported elsewhere (Imai et al., 1979). The zona-free eggs were washed twice with m-KRB solution (BSA 4 mg/ml), and about 20 eggs were then introduced into 0.4 ml medium covered with paraffin oil in a culture dish (Figure 1).

After the incubation of the zona-free hamster eggs with goat spermatozoa for 5 h, all the spermatozoa were removed from the egg surfaces by pipetting, and the eggs were mounted *in toto* on a slide, fixed with formalin, stained with lacmoid (Chang, 1952), and examined for evidence of sperm penetration using a phase-contrast microscope. Eggs which had an enlarged sperm head(s) or male pronucleus(ei) with the corresponding sperm tail in the vitellus were considered to be penetrated. The number of eggs which had resumed the second maturation division at various stages after sperm penetration was also recorded.

Table 2. *In vitro* penetration of zona-free hamster eggs by ejaculated goat spermatozoa preincubated in the uteri isolated from hamsters and rats

preincubation		Time of sperm introduction (h before (−) or after (+) the expected time of ovulation)*	No. of eggs examined	No. of eggs penetrated (%)
Hamster uterus	Isolated	− 15	50	0 (0)
		− 4	104	12 (12)
		+ 2	102	6 (6)
	In situ	− 4	50	0 (0)
Rat uterus	Isolated	− 16	20	0 (0)
		+ 9	36	0 (0)

* The isolated uterus was immersed in physiological saline and kept in a CO_2 incubator, and spermatozoa were recovered 5−6 h later.

Results and Discussion

When ejaculated goat spermatozoa were preincubated for 4 h in the uterus isolated from hamster and/or for 6 h in the hamster uterus *in situ*, the spermatozoa showed good (70%) motility and quickly attached themselves to the surface of the vitelline membrane after insemination. Conversely, spermatozoa preincubated for 5 and/or 6 h in the uterus isolated from hamster or rat exhibited only 40% motility, but this motility was well maintaining during incubation with zona-free hamster eggs. These phenomena agree with the results for the goat by Kim et al. (1980) and Kato et al. (1984). Miyamoto and Chang (1973) mentioned that good motility did not necessarily ensure the possibility of fertilization. Observations in the present study also suggested that factors other than motility may be involved in penetration *in vitro* of zona-free hamster eggs.

As shown in Table 2, zona-free hamster eggs were not penetrated after insemination of goat spermatozoa preincubated in the isolated hamster uterus 15 h before the expected time of ovulation, but about 10% of eggs were penetrated by spermatozoa preincubated in the isolated hamster uterus 4 h before and 2 h after the expected time of ovulation. On the other hand, eggs were not penetrated with spermatozoa preincubated in the hamster uterus *in situ* 4 h before the expected time of ovulation. Preincubation in the rat uterus isolated 16 h before and 9 h after the expected time of ovulation could not capacitate spermatozoa. These results suggested that the isolated hamster uterus might be a better

Table 3. Effect of preincubation period of ejaculated goat spermatozoa in isolated hamster uteri on penetration *in vitro* of zona-free hamster eggs

Duration of preincubation (h)	No. of eggs examined	No. of eggs penetrated* (%)	No. of eggs activated (%)
4	63	0 +0/63 (0)	0 / 0 (0)
5	51	2 +3/51 (10)	2 / 5 (40)
6	56	5 +5/56 (18)	3 /10 (30)

* The first figure denotes the number of eggs with enlarged sperm head and the second denotes the number of eggs with male pronucleus.

environment for capacitation of goat spermatozoa than the uterus *in situ*.

Effects of preincubation time of goat spermatozoa in the uteri isolated from hamsters were examined. As shown in Table 3, eggs were not penetrated by spermatozoa preincubated for 4 h, but 10% and 18% of the eggs were penetrated by spermatozoa preincubated for 5 and 6 h, respectively. In the penetrated eggs, various stages of enlarged sperm heads and transformation into the male pronucleus containing many nucleoli were observed. A high proportion (10/15=67%) of penetrated eggs had only an enlarged sperm head 5 h after insemination. The male pronucleus derived from goat spermatozoa in hamster eggs was morphologically similar to that from hamster spermatozoa (Plate a-b).

Kim et. al. (1980) reported that 53-63% and 38-76% of zona-free hamster eggs were penetrated 2-5 h after insemination with goat spermatozoa preincubated for 5-5.5 h in the uterus and oviduct of the maturing gilt, respectively. Kato et al. (1984) also reported that 60-100% of zona-free hamster eggs were penetrated 4-6 h after insemination with goat spermatozoa preincubated in the isolated hamster uterus. In the present study, overall penetration rates were comparatively low (10 and 18%) when eggs were inseminated with goat spermatozoa preincubated for 5 and 6 h in the isolated hamster uterus and examined 5 h after insemination. These figures were lower than those by Kim et al. (1980) who used gilt uteri for sperm preincubation and Kato et al. (1984) who used Tyrode's solution.

The present results suggested that some physiological changes occurred in goat spermatozoa during preincubation in the isolated hamster uterus and these changes might be concomitant with capacitation and/or the acrosome reaction. It was furhter inferred that the time requirement for inducing these changes was 5-6 h in the present experimental conditions. These results also indicate that zona-free hamster eggs can be used for the assessment of the fertilizing capacity of goat spermatozoa *in vitro*.

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Explanation of Plates

Zona-free hamster eggs penetrated by goat spermatozoa preincubated in the isolated hamster uterus and photographed under a phase-contrast microscope after being stained with aceto-lacmoid.

a) An egg 5 h after insemination with spermatozoa preincubated for 6 h in the isolated hamster uterus, showing one female pronculeus (FP) and one male pronucleus (MP) with their corresponding sperm tails (arrows). x 480.

b) An egg 5 h after insemination with spermatozoa preincubated for 6 h in the isolated hamster uterus, showing four enlarged sperm heads (large arrows) and tails (small arrows), but egg chromosomes(Ch) are still unactivated at second meiotic division. x 480.