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Human Spermatozoa Attach to Trypsin-treated Hamster Zonae Pellucidae but do not Undergo Acrosome Reactions

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ABSTRACT

The acrosome reaction by spermatozoa is an indispensable prerequisite for fertilization, and zonae pellucidae of human oocytes induce this reaction in the human spermatozoa attached to them. Human spermatozoa can attach to the zona pellucida of hamster oocytes when the oocytes have been treated with trypsin. We examined whether or not trypsin-treated hamster zona pellucida induces the acrosome reaction of human spermatozoa.

Ten semen samples from 7 donors of proven fertility were examined in the present study. Highly motile spermatozoa were obtained by a swim-up method, and pre-incubated for 1 or 6 hours in modified Biggers, Whitten and Whittingham's (mBWW) medium supplemented with human serum albumin. The spermatozoa were then co-incubated for 1 hour with trypsin-treated hamster oocytes to allow sperm attachment to the zona. The spermatozoa on the zona were incubated for 3 additional hours in the mBWW medium. The percentage of acrosome reacted spermatozoa (%AR) was determined before and after the 3-hours of incubation. The %AR in the sperm suspension was also determined.

There was no significant difference in the %AR between the spermatozoa attached to the zona pellucida and those in suspension during the incubation for 3 hours. These results indicate that the trypsin-treated hamster zona pellucida does not induce the acrosome reaction of human spermatozoa.

Key words: *Spermatozoa, Acrosome reaction, Zona pellucida*

Mammalian spermatozoa must attach to the zona pellucida (ZP), penetrate it and then fuse with the vitellus to complete normal fertilization of the oocyte. To achieve these tasks, spermatozoa must first undergo capacitation and then complete the acrosome reaction[14]. Several tests have been developed to evaluate the fertilizing ability of human spermatozoa. However, the sperm-zona interaction remains one of the most difficult aspects to investigate because human spermatozoa can only attach to homologous human ZP[10], and the availability of human oocytes is limited.

In 1979, zona-free oocytes of the golden hamster were found to allow human spermatozoa to penetrate into the vitellus. This finding has led to the development of the human sperm penetration assay using zona-free hamster oocytes[12] instead of human oocytes. Recently, it was found that human spermatozoa can attach to the ZP of hamster oocytes when the oocytes have been pre-treated with trypsin[6]. It is therefore convenient to use trypsin-treated hamster ZP in the study of the human sperm-zona interaction.

In the present study, we examined whether or not trypsin-treated hamster ZP has the ability to induce the acrosome reaction in human spermatozoa.

MATERIALS AND METHODS

Sperm preparation and pre-incubation:

Ten semen samples from 7 donors of proven fertility were obtained by masturbation after 3 days' abstinence. All the semen samples exhibited normal semen parameters (World Health Organization, 1987[11]).

The media used in the present study were modified Biggers, Whitten and Whittingham's (mBWW) medium, and mBWW medium supplemented with 0.5% (w/v) human serum albumin (HSA; Fr.V, Sigma) (HSA-mBWW). The spermatozoa were washed twice in HSA-mBWW medium (centrifugation at 300g for 5 minutes), and then the sperm pellet was gently placed under 1 ml of HSA-mBWW medium in a test tube to allow motile spermatozoa to swim up into the medium. After incubation at 37°C 5% CO_2 in air (the same conditions employed throughout the experiments) for 1 hour, motile spermatozoa in the medium were transferred to a conical tube, and the sperm

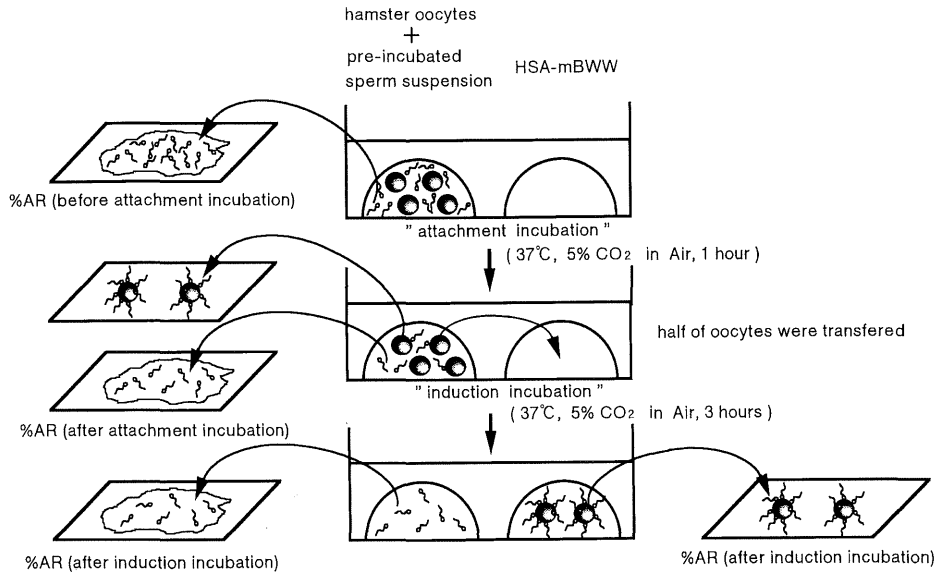

Fig. 1. Experimental design

concentration was adjusted to 3 to 5 × 10^6/ml in the HSA-mBWW medium. The sperm suspension was then incubated either for 1 hour (5 semen samples) or 6 hours (5 semen samples).

Preparation of trypsin-treated hamster zonae pellucidae:

Mature unfertilized oocytes were obtained from adult golden hamsters. The animals were superovulated by an intraperitoneal injection of pregnant mare serum gonadotropin (40 IU) followed by an injection of human chorionic gonadotropin (hCG) (40 IU) 55 to 60 hours later. The animals were sacrified 18 hours after the hCG injection. Cumulus masses containing oocytes were removed from the oviducts and collected in a Petri dish. The cumulus cells were completely dispersed by treatment with 0.1% (w/v) hyaluronidase in mBWW medium, then the cumulus-free oocytes were collected and rinsed thoroughly in HSA-mBWW medium. The oocytes were introduced into mBWW medium containing 0.01% (w/v) trypsin (1:250, Difco) and pipetted gently until each ZP swelled to approximately 1.5 times its original diameter (approximately 5 minutes). The oocytes were then washed in HSA-mBWW medium 3 times. After washing, the oocytes were carefully examined under a microscope and those with inadequately swollen ZP were discarded.

Insemination and incubation:

An aliquot (0.2 ml) of the pre-incubated (1 or 6 hours) sperm suspension and an equal aliquot of HSA-mBWW medium were placed in separate drops under mineral oil in a plastic Petri dish. The oocytes with swollen ZP (20 to 30) were introduced into the sperm suspension and co-incubated for 1 hour to ensure sperm attachment (Fig. 1). Hereafter, this 1 hour of co-incubation is referred to as "attachment incubation". After the attachment incubation, all the oocytes were removed from the sperm suspension and washed by gently pipetting them in fresh HSA-mBWW medium in a Petri dish to remove the loosely attached spermatozoa (Fig. 2). The sperm suspension (without oocytes) was kept in the plastic Petri dish and incubated for 3 hours.

Half of the washed oocytes were placed on glass slides and flattened by air-drying to allow examination of the acrosomal status of the spermatozoa attached to the ZP. The remaining oocytes were introduced into the aliquot of HSA-mBWW medium (without spermatozoa) under mineral oil and incubated for 3 hours. Hereafter, this period of incubation is referred to as the "induction in-

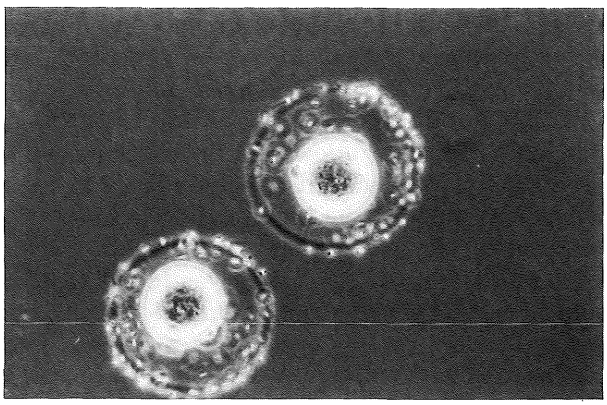

Fig. 2. Human spermatozoa attached to trypsin-treated zonae pellucidae of hamster oocytes

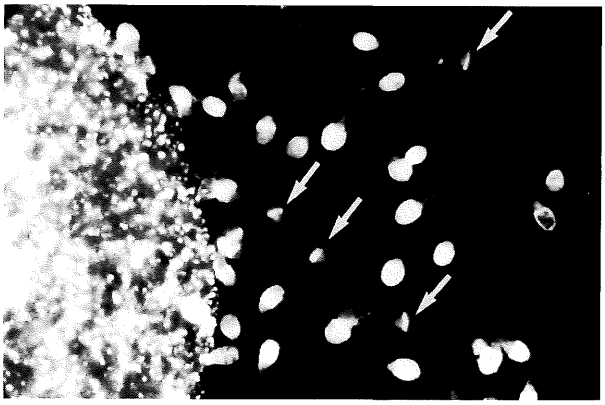

Fig. 3. Human spermatozoa stained with FITC-PSA on a trypsin-treated hamster zona pellucida. Arrows indicate the acrosome reacted spermatozoa.

cubation". After the induction incubation, all the oocytes were removed for examination of the acrosomal status of the spermatozoa.

Acrosomal status of the spermatozoa:

A small portion of each sperm suspension was smeared on a glass slide at 3 different times: before and after the attachment incubation, and after the induction incubation (after removal of oocytes). Simultaneously, sperm motility was examined using a Makler counting chamber. As stated above, the spermatozoa attached to the ZP were also examined for their acrosomal status after the attachment incubation and the induction incubation.

The slides were fixed by immersing them in 100% ethanol and storing them at 4°C overnight. The next day, the slides were stained with *Pisium sativum* agglutinin conjugated to fluorescein isothiocynate (FITC-PSA; Vector laboratories, CA) (100 μg/ml in distilled water) for 15 minutes, washed gently in distilled water and examined using an epifluorescence microscope ($\times$100, B2 filter)[1].

At least 300 spermatozoa per sample were examined to determine the percentage that were acrosome-reacted (%AR). Spermatozoa with bright staining at the equatorial segment and without staining at the acrosomal cap were considered to be acrosome-reacted. The spermatozoa overlying the vitellus were not examined because the vitellus was also brightly stained by FITC-PSA (Fig. 3).

Statistical analysis:

The %AR in the sperm suspension and in the spermatozoa attached to the ZP were compared by Student's t-test. A P-value of 5% or less was considered to be significant.

RESULTS

Sperm motility was greater than 90% in all sperm suspensions.

After pre-incubation for 1 hour in the HSA-mBWW medium, the %AR in the sperm suspension was low, ranging from 0.7 to 3.7% (mean, 2.2%) (Table 1). After the attachment incubation and the induction incubation (after removal of oocytes), the %AR in the sperm suspension remained low, with means of 2.5% and 3.1%, respectively. There was no significant difference in %AR among the 3 sperm suspensions.

The %AR of spermatozoa attached to the ZP was higher than that of spermatozoa in suspension before or after the induction incubation, but the difference in %AR was not significant. In addition, the percentage of spermatozoa that un-

Table 1. Percentage of Acrosome Reacted Spermatozoa (%AR) and Reaction Rate during Incubation for 3 hours*

Preincubation time	%AR			Reaction Rate (%)
	Before attachment incubation	After attachment incubation	After induction incubation	
1 hour (n=5)				
Sperm Suspension	2.2 ± 1.1 (0.7~3.7)	2.5 ± 0.9 (1.2~3.7)	3.1 ± 1.3 (1.7~4.9)	0.6 ± 0.5 (-0.2~4.5) N.S.
Spermatozoa on the zonae		3.4 ± 2.4 (1.5~6.9)	5.6 ± 3.2 (1.4~10.4)	2.3 ± 2.0 (-0.1~4.5)
6 hours (n=5)				
Sperm Suspension	3.8 ± 1.5 (2.0~5.7)	5.8 ± 1.7 (4.2~8.6)	7.0 ± 3.6 (3.0~11.8)	1.4 ± 2.2 (-1.3~3.5) N.S.
Spermatozoa on the zonae		7.3 ± 3.0 (3.8~10.5)	7.7 ± 3.6 (3.8~13.5)	0.4±3.4 (-4.1~5.0)

* All results are presented as the mean ± SD and the range.

$$\text{Reaction Rate} = \frac{\%\text{AR after induction incubation} - \%\text{AR after attachment incubation}}{100 - \%\text{AR after attachment incubation}} \times 100$$

derwent acrosome reactions during the induction incubation (Reaction Rate) was not significantly different between the spermatozoa in suspension and those attached to the ZP.

Results were similar for spermatozoa pre-incubated for 6 hours and for 1 hour (Table 1).

DISCUSSION

Due to the recent development of techniques[1,8] for evaluating the acrosomal status of human spermatozoa, the normal course of the acrosome reaction has been gradually revealed. Morales et al[7] reported that both acrosome-unreacted and acrosome-reacted human spermatozoa can attach to the ZP of human oocytes. Cross et al[2] reported that the human ZP can induce acrosome reactions in human spermatozoa attached to the ZP. Since spermatozoa of mammals, including the human, must be acrosome-reacted before penetrating the ZP, it is clear that the zona-induced acrosome reaction plays an important role in normal human fertilization.

The acrosome reaction can be induced by Ca ionophore A23187 or follicular fluid, as well as the ZP. Cummins et al[3] and Fénichel et al[4] reported that when capacitated human spermatozoa were treated with Ca ionophore A23187, the percentage of acrosome reacted spermatozoa was significantly lower in infertile men than in fertile men. These results imply that an impaired acrosomal response, as revealed by a poor response to Ca ionophore A23187, may be responsible for failure in fertilization. It is possible that the spermatozoa of infertile men also exhibit an impaired acrosomal response to human ZP, and that acrosomal dysfunction and failed zona penetration may be a cause of infertility in some cases. Up to now, however, such an impaired acrosomal response to the ZP in infertile men has not been confirmed.

Because the human sperm-zona interaction is strongly species-specific[10], the interaction can be examined only by the use of human oocytes. Although a few tests of sperm function using human ZP have been developed[9,13], the practical application of such tests has been restricted. This is mainly due to the poor availability of human oocytes, and also to ethical concerns in using the oocytes.

Recently, Mishima et al[6] reported that human spermatozoa could attach to the ZP of hamster oocytes when the oocytes had been treated briefly with trypsin. According to the report, human spermatozoa, incubated for 90 minutes in human tubal fluid and treated with Ca ionophore A23187, began to attach to the trypsin-treated ZP shortly after insemination, and the number of attaching spermatozoa increased in proportion to the insemination period and the sperm concentration. In addition, staining with FITC-PSA revealed that both acrosome-reacted and acrosome-unreacted spermatozoa were present on the ZP. Although the mechanism by which the trypsin treatment of the ZP allows the attachment of human spermatozoa is unknown, sperm attachment is not likely to be due to simple degradation of the ZP because the trypsin treated ZP of mouse or rat oocytes does not allow attachment of human spermatozoa[5]. If there is a common mechanism for sperm attachment to human ZP and the trypsin-treated ZP of hamster oocytes, it might be possible to use hamster oocytes for examining the human sperm-zona interaction.

In the present study, we examined whether or not the trypsin treated hamster ZP can induce acrosome reactions in attached human spermatozoa. Cross et al[2] reported that approximately 200 capacitated human spermatozoa bound to the human ZP after co-incubation for 1 minute. On the other hand, our preliminary result (unpublished data) revealed that approximately 1 hour of co-incubation (attachment incubation) was necessary to allow at least 100 human spermatozoa to attach to the ZP of hamster oocytes. Thus, the dynamics of attachment vary greatly according to the heterologus system.

In the present study, the %AR in the spermatozoa attached to the trypsin-treated ZP was not significantly different from that seen in the sperm suspension. This indicates that both acrosome-reacted and acrosome-unreacted human spermatozoa have an equal ability to attach to the ZP, and that the acrosome reactions were not induced by trypsin-treated ZP. Cross et al[2] reported that the %AR in the human spermatozoa attached to human ZP increased from 3% to 46% during a 1 hour incubation period. Therefore, we do not think that the incubation period of 3 hours (induction incubation) employed in the present experiments was too short to induce the acrosome reaction.

We have to conclude that the trypsin-treated hamster ZP has little, if any, capacity to induce acrosome reactions in human spermatozoa. However, it may be useful as a model system to evaluate the zona-binding component of the fertilization process.

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