Zona-free Hamster Eggs for the Study of Sperm Capacitation in Farm Animals

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Studies on *in vitro* fertilization, which have been performed in many mammalian species by various research workers, contributed to our better understandings on the mechanism of mammalian fertilization. In recent years, the birth of "test tube babies" of human was given much publicity from mass communication. In domestic animal species it is more advantageous to develop a solid technique of *in vitro* fertilization, because their eggs are more easily available from living and slaughtered animals than human eggs. However, the study of *in vitro* fertilization in domestic animal species has been handicapped owing to two major problems; one from spermatozoa and the other from eggs. For the achievement of normal fertilization sperm cells and eggs have to be matured. Final maturation processes of sperm cells, i.e. sperm capacitation and the acrosome reaction, are known to be induced during their sojourn in female reproductive tracts. In small laboratory animals and human these processes are known to be able to be induced *in vitro* by incubating sperm suspension in various kinds of media. Until now, the mechanism of these processes are not well understood with spermatozoa of domestic animals. Lots of matured eggs are necessary for the study of *in vitro* fertilization but their preparation requires labour-consuming works.

An idea to break this bottleneck in the study is to utilize the newly ovulated eggs from small laboratory animals which can be obtained easily in place of the eggs from domestic animals. At least as far as the purpose of examining the fertilizing capacity of spermatozoa is concerned, it is not always necessary to use the eggs of the same species if the eggs of certain species react to the penetration by foreign species spermatozoa in a similar manner as in the reaction to the penetration by homologous spermatozoa.

To furnish a firm basis for this idea a series of interspecific fertilization experiments was performed between eggs from mice, rats, golden hamsters and rabbits and spermatozoa from eleven different species (mouse, rat, hamster, guinea pig, mongolian gerbil, white-footed mouse, prairie deer mouse, rabbit, bull, boar and goat.[4,5,6,7,8] After ovulation the cytoplasm of mammalian eggs is typically surrounded by the vitelline membrane and the zona pellucida, a noncellular mucoprotein structure. Therefore, the points of these studies were to compare the attitudes of animal eggs, especially of their zonae pellucida and vitelline membranes, and to investigate the requirement of sperm capacitation (including the sperm acrosome reaction) for the penetration into foreign species eggs. Effective methods for *in vitro* induction of sperm capacitation were also investigated with spermatozoa of domestic animal species.[7,8,15]

Species specificity of the zona pellucida and the vitelline membrane

From the results of cross fertilization experiments *in vitro* the possibility of penetration of intact and zona-free eggs by foreign spermatozoa is summalized in Table 1. In these studies special care was paid for providing adequate incubation conditions to expect

Table 1. Penetration of intact and zona-free eggs by foreign spermatozoa *in vitro*

Spermatozoa of	Intact eggs of			Zona-free eggs of			
	Mouse	Rat	Hamster	Mouse	Rat	Rabbit	Hamster
Mouse		Yes**	No		Yes	Yes	Yes
Rat	No*		No	No		Yes	Yes
Hamster	No	No		No	No	No	
Guinea pig	No*	No	No	No	No	No	Yes
Mongolian gerbil	No	No	No	No	No		Yes
White-footed mouse	No	No*	No	No	No		Yes
Prairie deer mouse	No*	No	No	No	No		Yes
Rabbit							Yes
Bull							Yes
Boar							Yes
Human[1)]							Yes
Dolphin[2)]							Yes

* Penetration only into the perivitelline space was observed on very rare occasions.
** Penetration into the vitellus was observed in 3.2% of eggs examined.

the achievement of sperm capacitation except for spermatozoa of mongolian gerbils and white-footed mice. However, none of the eggs were penetrated by foreign spermatozoa in most of the combination experiments with intact eggs. Thus it appears that the zona pellucida has a function to prevent the penetration of foreign spermatozoa and sperm capacitation is not directly related to the failure of interspecific fertilization. But this function of the zona pellucida is not so rigid, because in particular combination experiments it was possible to observe the firm attachment of spermatozoa on the surface of the zonae (e.g., mouse eggs by deer mouse sperm), or the penetration of spermatozoa into the perivitelline space but not into the cytoplasm (e.g., rat eggs by white-footed mouse sperm), or the complete penetration of the vitellus by spermatozoa and the formation of male pronuclei (e.g., rat eggs by mouse sperm).

In the experiments with zona-free eggs the zonae were removed from all the eggs by enzymic digestion before the incubation with foreign spermatozoa. Mouse eggs completely rejected the penetration by rat, hamster, guinea pig, gerbil, white-footed mouse and deer mouse spermatozoa. Rat eggs were penetrated by mouse spermatozoa but not penetrated by hamster, guinea pig, gerbil, white-footed mouse and deer mouse spermatozoa. Rabbit eggs accepted the penetration of mouse and rat spermatozoa but rejected the penetration of guinea pig and hamster spermatozoa. It seems that the vitelline membranes of these eggs sometimes exert the species specificity against foreign spermatozoa from particular species and physiological affinity is required for the penetration of these eggs by foreign spermatozoa.

On the contrary, zona-free hamster eggs accepted the penetration of all the foreign spermatozoa including bull, boar and goat. It was reported that human[20)] and dolphin[3)] spermatozoa are also able to penetrate into zona-free hamster eggs *in vitro*. Thus the vitelline membrane of hamster egg appears to have no species specificity for foreign sperm penetration.

Sperm capacitation as a prerequisite for the penetration of zona-free hamster eggs by foreign spermatozoa

Sperm capacitation is known as an absolute prerequisite for successive acrosome reaction of sperm cells and only the reacted spermatozoa can penetrate the zona pellucida and

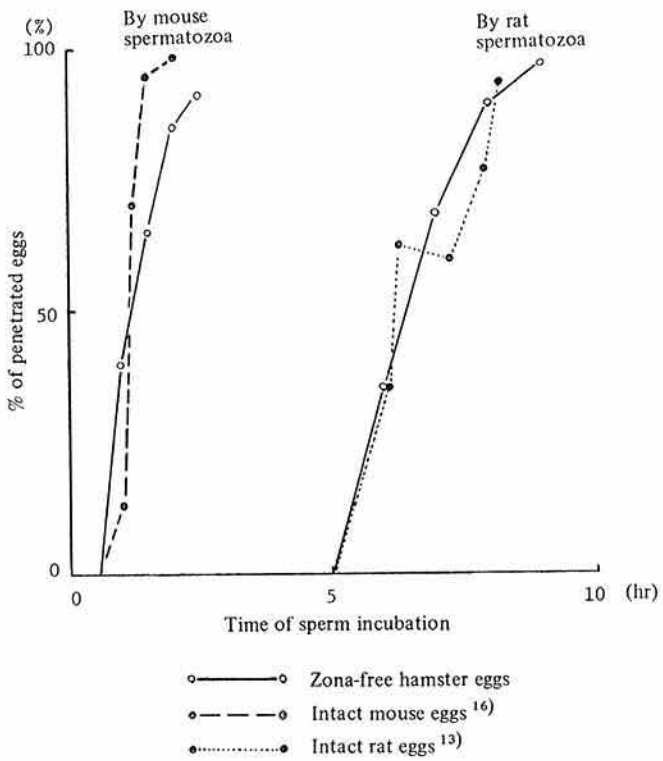

Fig. 1. Time-related changes of the proportions of penetrated eggs by mouse and rat spermatozoa during incubation

fuse with the vitelline membrane of mammalian egg. In the fertilization studies with homologous eggs *in vitro* it is common to use the time required for the first appearance and successive time-related changes of the proportions of penetrated eggs as indicators for determining the efficiency of inducing sperm capacitation and acrosome reaction in various experimental conditions. Thereupon, the changes of the proportions of penetrated eggs during various incubation hours were investigated to know whether or not sperm capacitation is required for the penetration of zona-free hamster eggs by foreign spermatozoa.

Fig. 1 shows that the incubation hours for the first appearance of penetrated eggs and for the attainment to the peak of the penetration by mouse and rat spermatozoa exactly correspond to the values reported in the fertilization studies of these animal eggs *in vitro*.[13,16] These data strongly suggest that the vitelline membrane of hamster eggs has a function to distinguish the achievement of capacitation of foreign spermatozoa. In other words, basic study on sperm capacitation of various species including domestic animals can be proceeded by utilizing the penetration of zona-free hamster eggs in place of homologous eggs *in vitro*.

Induction of sperm capacitation of domestic animals *in vitro*

The capacitation of bull and boar spermatozoa was reported to be induced by incubation of washed sperm suspension in homologous reproductive tracts isolated from oestrous

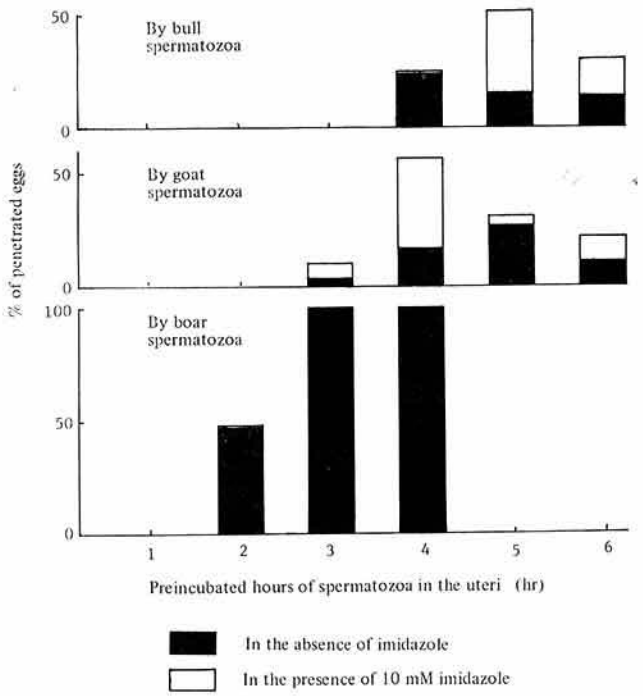

Fig. 2. Penetration of zona-free hamster eggs *in vitro* by bull, goat and boar spermatozoa preincubated in rabbit uteri from mature does

females or in the uterus *in situ* of oestrous rabbit.[10,11] The capacitation of goat spermatozoa was also suggested to be induced by incubation of spermatozoa in isolated uterus from a gilt.[12] For convenience in handling we used the isolated uteri from mature rabbits without any hormonal pretreatment as sperm preincubators and found that this procedure for bull, boar and goat spermatozoa worked rather well in subsequent penetration study with zona-free hamster eggs *in vitro* (Fig. 2). Minimum effective time for fertilization of porcine eggs *in vivo* is known to be 2 to 3 hr which can be regarded as the time intervals for the achievement of boar sperm capacitation[9]. The same time intervals in the isolated rabbit uteri were found to be effective for boar spermatozoa to penetrate into zona-free hamster eggs *in vitro*. Longer time intervals of sperm preincubation in rabbit uteri were needed to obtain the maximum proportions of eggs penetrated by bull and goat spermatozoa than by boar spermatozoa (5 h and 4 h vs 3 h). In addition, the presence of 10 mM imidazole, a cyclic nucleotide phosphodiesterase stimulator, in the incubation medium (BWW medium[1]) after uterine preincubation had some beneficial effect on the increase of penetrated eggs in the study with bull and goat spermatozoa.

The problem involved in these semi-*in vivo* methods for the induction of sperm capacitation is the inconstancy and low repeatability of experimental results owing to sampling variation of female reproductive tracts. Accordingly it is desirable to develop new methods which do not use biological materials but use chemically defined media for the induction of sperm capacitation.

The acrosome reaction of mammalian sperm cells is known to be calcium-dependent[19] and its artificial induction is reported by many

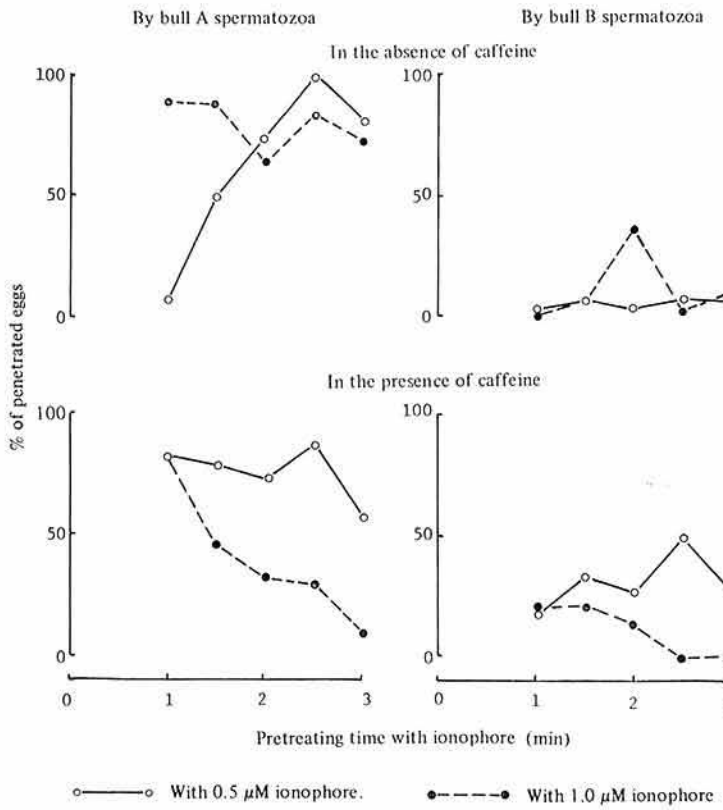

Fig. 3. *In vitro* penetration of zona-free hamster eggs by bull spermatozoa pretreated with two concentrations of ionophore A23187 in BSA-free BO medium in the presence or absence of 2 mM caffeine

workers to be possible by treating spermatozoa from various species with calcium in the presence of divalent cation ionophore A23187. This substance binds to Ca^{+2} and transports it across lipid barriers, including cell membranes. It has been recognized that too much influx of Ca^{+2} may destroy the mitochondrial calcium pump and abolish sperm motility. On account of this disadvantage the ionophore has not been used frequently in the study of *in vitro* fertilization. However, it was reported recently that porcine eggs[17] and zona-free sheep eggs[14] can be fertilized *in vitro* by homologous spermatozoa after their treatment with the ionophore.

We have also found that bull spermatozoa pretreated with ionophore A23187 in the presence of 2.25 mM calcium chloride can penetrate into zona-free hamster eggs *in vitro*[15]. In the study, ejaculated bull spermatozoa were washed three times and suspended at a concentration of 25×10^6 cells/m*l* in a chemically defined medium (BO medium[2]). Ionophore solution (5 μl) was added to 1 m*l* sperm suspension to give its final concentration at 0.5 to 2.0 μM. After treatment for 1 to 15 min the suspension (50 μl) was introduced into 350 μl medium which contained 0.3% bovine serum albumin (BSA) and zona-free hamster eggs and incubated for 3 hr in a CO_2 incubator.

The proportions of penetrated eggs differed according to the conditions of ionophore treatment of spermatozoa. In general, ionophore

treatment of spermatozoa in the medium without BSA was of great advantage over the treatment in the medium with BSA. In the former, the effective concentration and treating time was able to reduce, and thereby maintained good sperm motility for longer hours after insemination. In the latter, the binding of BSA to the ionophore diminished the effect of ionophore, hence higher concentration and longer treating time was necessary for sperm penetration into the eggs, and this caused quick loss of sperm motility. The typical whip-lash movement of spermatozoa, which is known as a figure of capacitated spermatozoa[18], was noticeable before their penetration into the eggs. First appearance of penetrated egg was observed at 1.5 hr after insemination and the peak of penetration was obtained at 3 hr incubation. These observations suggest that the capacitation and the acrosome reaction of bull spermatozoa can be induced artificially in relatively short hours by their treatment with the ionophore.

The proportions of penetrated eggs were also affected by individual variation within bulls. As shown in Fig. 3, bull A spermatozoa penetrated rather easily into the eggs where the proportion was highest with spermatozoa treated by 0.5 μM ionophore for 2.5 min in the medium without BSA. On the contrary, the proportions were always low by bull B spermatozoa at any ionophore concentration and its treating time. The reason of this individual variation between bulls may be explained from the data obtained by ionophore treated spermatozoa in the presence of 2 mM caffeine. Caffeine can act as cyclic nucleotide phosphodiesterase inhibitor and hence affect the intracellular level of cAMP by inhibiting its enzymic breakdown. The shortened effective time of ionophore treatment (0.5 μM) of bull A spermatozoa and the increased rates of penetrated eggs by bull B spermatozoa after their treatment with 0.5 μM ionophore indicate the presence of close relationship between the cAMP content in sperm cells and the occurrence of sperm acrosome reaction which is triggered by the intracellular accumulation of Ca^{+2}. A certain level of cAMP may be necessary for the acrosome reaction, and the enzyme activity which regulates the intracellular level of cAMP may be different in the spermatozoa from different individuals.

Concluding remarks

The number of studies on *in vitro* fertilization in domestic farm animals has been limited because of the difficulty in the induction of sperm capacitation and the acrosome reaction *in vitro*. As introduced here, we found that zona-free hamster eggs can be used as substitute eggs for the study of these important physiological events. Although chemical induction of these events is possible, further improvements are needed for the development of reliable induction methods. Additional work is also needed on the mechanism and way of oocyte maturation before the establishment of *in vitro* fertilization techniques which will be useful for the improvement of reproductive efficiency of farm animals in future.

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