Inhibition of Fertilization by Zona Pellucida Antibody

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The correlation between the process of fertilization and immunological reaction has been reviewed in the past and the control of fertility by immunological approaches has been discussed by many authors[1]. Recently, it has been reported that anti-ovary antiserum could inhibit the fertilization in the hamster, mouse and rat[2]. It has been considered, by using absorption test of anti-ovary serum, that the inhibitory effect of anti-ovary serum on fertilization was due to antibody(ies) to zona pellucida[3]. This estimation was proved by testing the effect of antiserum to isolated zonae pellucidae on fertilization[4,5]

In this paper the following data will be discussed; (1) production of antisera to isolated zonae pellucidae, (2) tissue specificity of antiserum, (3) inhibitory effect of antiserum on fertilization and (4) species specificity of antiserum.

Antiserum production

1) Preparation of antigen

(1) Mouse: Mature ddY mice were superovulated with PMSG*1) and HCG*2), and were then sacrified 14 to 18 hr after injection of HCG. The eggs in the cumulus clot were recovered from the ampulla and treated with 0.05% hyaluronidase in PBS*3) (pH 7.4). After the dispersion of follicular cells, the denuded eggs were washed three times in PBS without hyaluronidase. By sucking the denuded eggs in PBS through a fine glass pipette, eggs were ruptured and the isolated zonae pellucidae were washed several times for antigen.

(2) Pig: Pig zonae pellucidae were collected from ovaries which were obtained from a slaughterhouse and kept frozen at −20°C. The thawed ovaries were minced by using a meat grinder (Japan Mincer Co.,). The minced material in PBS was filtered through gauze to remove tissue debris and the filtrate was refiltered through 210, 177, 105, 88, 63 and 53 μ stainless mesh. The washed solution of the residue on 105, 88 and 63 μ mesh was mixed up and centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and the sediment was resuspended in PBS and centrifuged again (3,000 rpm for 10 min). After this was repeated two more times, the sediment was suspended in a small volume of PBS and the suspension was placed in a watch glass and the debris of cumulus cells from the mixture was removed by stirring with a brush made of human hair. Eggs and isolated zonae were collected and washed several times in PBS. By sucking the eggs in PBS through a fine glass pipette, eggs were ruptured and isolated zonae were collected, washed several times for the antigen of immunization[6].

(3) Cow: Cow zonae pellucidae were also obtained from ovaries. Follicular fluid was aspirated from folliculs in ovaries and the follicular fluid was centrifuged at 3,000 rpm for 10 min. The supernantant was discarded and the sediment was resuspended in PBS

* 1) Pregnant mare serum gonadotrophin
* 2) Human chorionic gonadotrophin
* 3) Phosphate buffered saline

and centrifuged again. After this was repeated two more times, the sediment was suspended in a small volume of PBS and the follicular eggs were collected under a stereomicroscope. The ovaries aspirated of follicular fluid were then homogenized with a meat grinder and eggs or isolated zonae were collected following the method mentioned in the above (2). By sucking the eggs through a fine glass pipette, isolated zonae were collected and then washed several times.

2) Immunization format

Isolated zonae pellucidae from each species were frozen and thawed ten times and homogenized in a glass homogenizer. The homogenate of zonae (900 zonae for the mouse, 15,000 zonae for the pig and 600 zonae for the cow), with Freund's complete adjuvant (FCA, 1:1) was injected to rabbits (for the mouse zonae) and goats (for the pig and cow zonae), and the homogenate of zonae of same doses with Freund's incomplete adjuvant (FIA) was injected 7 to 11 times with 10 to 17 days interval. Then, the injections were continued for 12 to 20 months at an interval of 10 to 44 days in combination with one time FCA and three times FIA. A total number of about 27,000 zonae for the mouse, 390,000 zonae for the pig and 12,000 zonae for the cow were used. Control rabbits and goats received PBS and adjuvants.

3) Antiserum evaluation

(1) Titration: The titer of antisera was mainly assayed by zona precipitate[3]. The antisera, of which titer were 2^6 to 2^{15}, were used in the present study.

(2) Tissue specificity: Tissue specificity was tested in antiserum against pig zonae pellucidae by immunoelectrophoresis. The freeze-dried powders of pig liver, kidney, lung, uterus, oviduct, spleen, brain, heart, tongue, follicular fluid and eye extracts, and pig isolated zonae pellucidae were solubilized with 8M urea. Electrophoresis was conducted on 1% agarose in barbital buffer (pH 8.4). Immunoelectrophoresis of antiserum showed that antiserum produced 2 to 5 precipitin bands against zonae pellucidae and failed to react with all other tissue extracts. The present study demonstrated that zona pellucida was tissue specific, but had several different antigens.

Effect of zona antibody on fertilization

1) Inhibition of fertilization in own species

(1) Mouse: To examine the effect of passive immunization with anti-mouse zona antibody on fertilization in the mouse, 0.2 ml of control rabbit serum and antiserum or 0.1 to 1 mg of control IgG of control serum and antiserum were intraperitoneally injected to females at the time of PMSG injection. HCG was injected 44 to 52 hr after the injection of PMSG, and mated or artificially inseminated via the cervix with epididymal spermatozoa. The females were killed 1 day after insemination for the examination of fertilization.

The fertilization was inhibited in passive immunization with unabsorbed or antiserum absorbed with liver and kidney (Table 1). The proportion of fertilized eggs after passive immunization with antiserum absorbed with ovary was not different from that obtained after injection of normal serum. A complete inhibition of fertilization was also observed after passive immunization with antiserum IgG at the dosages of 0.4 mg or more. A significant ($P<0.001$) reduction of fertilization was observed after injection of 0.2 mg and 0.3 mg antiserum IgG compared with that obtained after injection of control serum IgG.

(2) Pig: Whole anti-pig zona serum or control goat serum (1 to 3 ml/kg/body weight) was intramuscularly injected into mature females 3 to 6 days before mating. The females were slaughtered 3 to 11 days after mating, and the oviducts and/or uteri were flushed with Ringer's solution containing 10% inactivated cow serum for the recovery of eggs. The eggs were stained with 1% orcein in acetic acid and normally cleaved eggs with or without zonae with stained

Table 1. Inhibition of fertilization after passive immunization with antizona antibodies

Species	Serum	Fraction of serum	Dosage	Percentage of eggs fertilized
Mouse	Control	Whole serum	0.2ml/mouse	79
	Antiserum		do	0
	Absorbed with L+K		do	0
	Absorbed with ovary		do	97
Mouse	Control	IgG	1 mg/mouse	92
	Autiserum		1	0
			0.5	0
			0.4	0
			0.3	54
			0.2	74
			0.1	92
Pig	Control	Whole serum	1–2 ml/kg	100
	Antiserum		1–3	6
Cow	Control	Whole serum	1 ml/kg	84
	Antiserum		1	50
			2	0

nuclei of uniform appearance were considered to be fertilized.

All eggs recovered from both pigs injected with control goat serum showed normal cleavage (Table 1). The normally cleaved eggs recovered from pigs injected with antiserum was 0 (for 1 ml/kg) and 1 (for 2 ml and 3 ml/kg) and all others were fragmented or uncleaved. In pigs, it has been reported that up to 80% of unfertilized eggs recovered from uterus show varing degree of fragmentation, indeed some of them may resemble to normally cleaved eggs[7]. Abnormally cleaved (fragmented) eggs recovered from females passive immunized with antiserum should be considered unfertilized. But we were unable to determine normally cleaved eggs from pigs injected with antiserum were fertilized. Nevertheless, it is clear that passive immunization with anti-pig zona serum inhibits fertilization in pigs since fertilization rate was 6% (2/32) even when normally cleaved eggs are considered to be fertilized.

(3) Cow: Mature Japanese Black and Brown cattle and Holstein were superovulated in combination with PMSG, estradiol and HCG or Prostaglandin $F_{2\alpha}$ and HCG according to the procedures which have been routinely used in our laboratory[8]. Whole anti-cow serum or control goat serum (1–2 ml/kg/body weight) were intraperitoneally injected to the females at the time of estradiol or prostagrandin injection (1 to 4 days before mating). Cows were slaughtered 2 to 7 days after detection of ovulation and ovuducts or uteri were flushed with Ringer's solution containing 10% inactivated cow serum for the recovery of eggs.

Table 1 shows the results of morphological examination of eggs recovered from cows passive immunized with control goat serum and anti-cow zona serum. As shown in the Table, 4 out of 5 females had normally cleaved eggs and 38 out of 45 eggs were normally cleaved (84%). All eggs recovered from the both cows injected with antiserum at the rate of 2 ml/kg uncleaved. All cows injected with antiserum at the rate of 1 ml/kg had normally cleaved eggs, but the rate of normally cleaved eggs was clearly low compared with that obtained in the cows injected with control serum (50% vs 84%). The results of the present study demonstrate that passive immunization with goat anti-cow zona serum inhibits fertilization in the cow.

Table 2. Reaction of anti-zona sera with zonae from various species and effect of passive immunization with antisera on fertilization of different species

Antiserum to	Tested in	Reaction to* immunofluorescence	Sera treated or injected	Dosage (ml/kg)	Percentage of eggs fertilized
Mouse zona	Rat	++	Control	1–2	66
			Antiserum	1–2	16
	Hamster	+	Control	0.5–0.6	100
			Antiserum	0.5–0.6	100
Pig zona	Cow	+++	Control	1	100
			Antiserum	1–2	13
	Rabbit	+++	Control	10	94
			Antiserum	10	13
				20	0
	Rat	+++	Control	10	96
			Antiserum	10	64
				20	64
	Mouse	+++	Control	10	94
			Antiserum	10	9
				20	0
Cow zona	Pig	++			
	Rabbit	++			
	Mouse	+			
	Rat	+			

* Detected by indirect immunofluorescence +++ = strong, ++ = moderate and + = weak reaction.

2) Inhibition of fertilization in different species

(1) Species specificity of zona antigen: The cross reaction of antisera with zonae of different species was determined at first by indirect immunofluorescence.

Each anti-zona serum strongly (+++) reacted with zonae from respective species used for collection of antigen. As shown in Table 2, each anti-zona serum reacted to some extent with zonae of all other species tested. In accord with our observation which indicates that zona antigens are not species specific, it has been reported that mouse anti-hamster zona serum cross reacted with zonae of mouse, rhesus monkey and squirrel monkey, and that rabbit anti-bovine zona serum cross reacted with zonae of rabbit, rhesus monkey, marmoset, dog, hamster and human[9].

(2) Effect on fertilization: Although it has previously been clearly demonstrated by immunofluorescence that zona antigen is not specific, the most critical test is the effect of anti-zona serum on fertilization in different species in vivo. For this purpose, passive immunization test was used; Respective whole antiserum or control serum (0.5 to 20 ml/kg/body weight) was intraperitoneally injected to the females of various species 1 to 6 days before mating (for the mouse, hamster, rabbit) or artificial insemination (for the rat and cow). The mice, hamsters and rats were killed 1 day after insemination for the recovery of eggs. The eggs were mounted on a slide and stained with lacmoid for the examination of fertilization. In these species, the term "fertilized" denotes the eggs which had enlarged sperm head or pronuclei with a fertilizing sperm tail. In rabbits, eggs were recovered 26 to 48 hr after mating and examined the cleavage. The cows were slaughtered 1 to 6 days after mating for the recovery of eggs. Eggs were stained with aceto-orcein and normally cleaved eggs were considered to be fertilized.

Results of fertilization obtained after pas-

sive immunization of other species with anti-mouse zona and anti-pig zona sera are presented in Table 2. Passive immunization with anti-mouse zona serum significantly inhibited the fertilization in the rat but not in the hamster. All eggs recovered from 4 cows injected with control goat serum showed normal cleavage. One of two cows injected with anti-pig zona serum at the rate of 1 ml/kg/body weight had no cleaved eggs, and the cow injected with antiserum at the rate of 2 ml/kg had no fertilized eggs. In the rabbit, rat and mouse, a significant or complete inhibition of fertilization was observed after passive immunization with antiserum compared with that obtained after injection of control goat serum. However, passive immunization with anti-pig zona serum was more effective for inhibition of fertilization in vivo in the rabbit and mouse than in the rat. In the rabbit and mouse, fertilization was completely inhibited by the injection of antiserum at the rate of 20 ml/kg/body weight, but not completely inhibited in the rat.

Concluding remarks

The present study demonstrates that passive immunization with heteroantiserum to isolated zonae pellucidae inhibits fertilization, not only in the same species but also in other species. This suggests that it may be possible to use pig and cow zonae pellucidae, which are available in large quantities from a slaughterhouse, for immunocontraception in women.

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