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Evidence for the inhibition of fertilization *in vitro* by anti-ZP3 antisera derived from DNA vaccine

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ABSTRACT

Previously we have found that DNA vaccine, pCMV4-rZPC' can generate specific antibodies against rabbit ZPC (amino acid 263-415, rZPC'), which binds to ovarian ZP and leads to a significant reduction of fertility *in vivo*. The purpose of this study was to evaluate the effect of antisera from pCMV4-rZPC′-immunized mice on sperm–oocyte interaction *in vitro*. The effect of antisera from DNA vaccine-immunized mice on fertilization and early embryonic development was studied using an *in vitro* fertilization system. The results showed that the antisera supplemented in fertilization medium (10%, v/v) significantly decreased the rate of fertilization compared to that of control groups ($P < 0.05$); whereas the antisera showed no significant effect on the rate of fertilization when ZP-free eggs were used. Moreover, the antisera pre-neutralized with mouse soluble zona pellucida lost the capacity to inhibit fertilization when compared with that of control groups. In addition, the antisera showed no detrimental effect on early developmental potential of mouse embryos *in vitro*. Taken together, our study provided herein direct evidence showing that antisera generated by DNA vaccine can block sperm–egg recognition during fertilization via targeting the oocyte ZP proteins.

1. Introduction

The zona pellucida (ZP), an extracellular coat surrounding the plasma membrane of mammalian eggs and pre-implantation embryos, was composed of three or four glycoproteins designated ZP1, ZP2, ZP3, and ZP4, respectively [1–3]. ZP glycoproteins, by virtue of their tissue specificity and critical roles during mammalian fertilization, have been proposed as candidate antigens for non-steroid contraceptive [4–6]. Indeed, considerable efforts have been expended towards developing ZP glycoprotein-based contraceptive vaccines for control of either human population or that of wild life [7–11].

Moreover, previous studies demonstrated that anti-ZP antibodies were strongly associated with unexplained infertility [12], and were more likely to be detected in women who failed in *in vitro* fertilization procedures [13]. There was also evidence showing that antibody to ZP3 peptide inhibited human sperm–zona binding *in vitro* [14,15]. However, the potential association and underlying mechanisms of anti-ZP immunization-induced infertility remains elusive. A better understanding on above-mentioned processes will help in designing a safe, reversible and effective contraceptive vaccine for human use.

The immunological cross reactivity among ZP glycoproteins from different species owing to their sequence homology has led to the possibility of heterologous immunization. In this respect, our previous studies have demonstrated that DNA vaccine targeting rabbit ZPC can effectively prevent the fertility without interfering with the normal follicular development in mice [16]. Furthermore, we have also observed that the anti-fertility efficacy of DNA vaccines targeting the same ZP protein show substantial variations among those targeting with different encoding sequences [17]. Although ZP DNA vaccines can significantly reduce the fertility of immunized animals *in vivo*, whether the antibodies from ZP-based DNA vaccines would inhibit the process of fertilization and/or subsequent embryonic development needs further investigation. Thus, we surmised herein that the cause of infertility by DNA vaccine was an interrupted sperm–egg interaction resulted from anti-ZP3 antisera.

In the present study, we applied an *in vitro* fertilization and embryo culture system to investigate whether anti-ZP3 antisera would inhibit the process of fertilization and subsequent embryo development. The rates of fertilization, early embryo cleavage, and blastocyst formation were used to elevate the effects of ZP3 antis-

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era on the process of zygote formation and preimplantation embryo development.

2. Materials and methods

2.1. Animals

Kunming and BALB/c mice purchased from the Laboratory Animal Center, Academy of Military Medical Sciences were used for superovulation experiments and preparation of antisera, respectively. Mice were housed in a 12-h light/dark cycle and given food and water *ad libitum*. All experiments were conducted according to the guidelines of the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

2.2. Preparation of antisera

DNA vaccines, pCMV4-ZPC', encoding 6–8 exons of rabbit ZPC were constructed as we previously described [16]. BALB/c mice were immunized with saline, pCMV4 and pCMV4-ZPC', respectively. The procedure of immunization was performed as described elsewhere [18]. Pre-immune serum was also collected.

2.3. Oocyte collection and removal of ZP

The collection of oocytes of Kunming mice was performed as previously described [19]. Also, the removal of ZP was performed as described elsewhere with some modifications [20]. Briefly, eggs were incubated with acidic Tyrode's solution and monitored under a stereo-microscope and transferred to HTF medium.

2.4. Preparation of MSZP

The collection of mouse eggs was performed as described above. On the day of each experiment, ZP was collected by vigorously pipetting oocytes with small-bore glass pipette and washed three times in Dulbecco's Phosphate-Buffered Saline (DPBS). Following centrifugation, the ZP pellet was resuspended in DPBS and heat-solubilized at 70 °C for 90 min. After centrifugation, the supernatant was designated as MSZP and its concentration was determined using the Bradford protein assay (Bio-Rad).

2.5. Sperm

Spermatozoa were collected from Kunming mice of proven fecundity as described elsewhere [21]. Briefly, the cauda epididymides were isolated and epididymal contents were squeezed out to allow the sperm disperse for 10 min in HTF medium. Then, the dispersed spermatozoa were transferred to HTF medium for capacitation for 1 h at 37.5 °C in a CO_2 incubator.

2.6. ELISA

Enzyme-linked immunosorbent assay was performed to detect the generation and specificity of anti-ZP3 antisera. The procedure of detection was performed as described elsewhere with some modifications [22]. Briefly, microplates were coated with 50 μl MSZP in coating buffer at 4 °C overnight.

2.7. Immunofluorescence

Indirect immunofluorescence was performed to detect the generation and specificity of anti-ZP3 antibodies as previously described [23]. Briefly, superovulated oocytes were fixed in 4% paraformaldehyde solution. After blocking in 2% BSA for 3 h, oocytes were incubated with antisera (diluted 1:50) at 4 °C overnight. After rinsing in PBS, the oocytes were incubated in fluorescein isothiocyanate-conjugated anti-mouse IgG (1:200) for 2 h at 37 °C. Finally, nuclei were stained with 10 μg/ml propidium iodide for 10 min. The fluorescence was detected under an inverted confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

Direct immunofluorescence was performed to detect the presence of specific serum antibodies binding to the ZP of oocytes used for *in vitro* fertilization. The oocytes from Section 2.8.1 were fixed with paraformaldehyde at 24 h post insemination at room temperature for 1 h and blocked with 1% BSA. Then, anti-mouse fluorescein isothiocyanate-conjugated antibody diluted 1:200 was used as the secondary antibody at 37 °C for 1 h. The samples were examined by an inverted epi-fluorescent microscope (Eclipse Ti, Nikon, Japan). The cell nuclei were counterstained with propidium iodine.

2.8. In vitro fertilization

2.8.1. Experimental design 1

To investigate the effect of anti-ZP3 antisera on the fertilization and subsequent preimplantation development of fertilized eggs, *in vitro* fertilization experiments were performed. Antisera were treated by heating at 56 °C for 30 min to inactivate the complement and added to the fertilization medium at a 10% concentration. Pre-immune serum and serum from saline-immunized mice were used as negative controls, while anti-ZP3 antibody (Santa Cruz, SC25802) corresponding to amino acids 23–322 mapping within an extracellular domain of ZP3 of human origin was added in the fertilization medium as a positive control (3 μl/100 μl). Superovulated oocytes were randomly distributed in the positive control, pCMV4-rZPC′, pCMV4, and negative groups, respectively. Capacitated sperm suspension was gently added into fertilization droplets to give a motile sperm concentration of 1.5–2.0 × 10^6/ml. The rates of fertilization, cleavage and blastocyst were assessed.

2.8.2. Experimental design 2

To evaluate whether anti-ZP3 antisera would inhibit the fertilization by interrupting the interaction between the sperm and oocytes, ZP-free oocytes were used as a substitute for ZP-intact oocytes, and the rate of fertilization was calculated.

2.8.3. Experimental design 3

To confirm that the bioactivity of anti-ZP3 antisera during the course of *in vitro* fertilization of ZP-intact oocytes, anti-ZP3 antisera pre-neutralized by 3 μl MSZP (200ng/μl) for 3 h before insemination were used as a substitute for the corresponding antisera used in Section 2.8.1.

2.9. Embryo culture

Eggs were transferred into CZB (MR-019-D, Millipore) droplets at 6 h after insemination and cultured to the stage of blastocyst. Successful fertilization was examined by detecting the existence of two pronuclei at 9–10 h after insemination. The number of cleavaged eggs and blastocysts was recorded at 24 h and 96 h after insemination, respectively.

2.10. Parallel experiments with pCMV4-rZPC'

Contraceptive DNA vaccines, pCR3.1-ZP3-T and pCR3.1-ZP3-L encoding different sequences of rabbit ZP3 were made by our lab as previously described [17]. The generation and specificity of anti-ZP3 antisera was examined using Western blotting. The effects of antisera from pCR3.1-ZP3-T and pCR3.1-ZP3-L-immunized mice on the fertilization and subsequent preimplantation development of fertilized eggs were also examined as described above.

Table 1
Effects of anti-ZP3 antisera on fertilization and early development of mouse ZP-intact eggs *in vitro*.

Group	No. of eggs for IVF	No. of fertilized egg (%)	No. of cleavage embryo (%)	No. of blastocyst (%)
Mock	100	89(88.7 ± 2.7)	80(90.0 ± 2.9)	43(48.6 ± 1.7)
Saline	95	83(87.7 ± 2.4)	73(87.5 ± 3.1)	42(50.6 ± 5.1)
pCR3.1	103	89(86.6 ± 4.1)	79(89.0 ± 1.6)	44(49.3 ± 6.7)
pCMV4	97	84(86.3 ± 2.5)	73(87.1 ± 1.4)	40(47.8 ± 1.1)
pCR3.1-ZP3-T	105	50 (47.8 ± 3.7)	43(86.4 ± 4.5)	26(51.6 ± 4.7)
pCR3.1-ZP3-L	104	52 (49.8 ± 2.7)	46(88.7 ± 2.6)	26(50.2 ± 3.1)
pCMV4-rZPC'	97	49 (50.4 ± 2.8)	42(85.2 ± 3.4)	23(47.4 ± 2.6)
Positive	105	60 (57.4 ± 2.6)	56(93.3 ± 1.7)	31(51.7 ± 3.3)

Note: values with different superscripts within a column differ significantly ($P < 0.05$).
Each fertilization mircodrop supplemented with 10% (v/v) antisera from mice immunized with saline, pCR3.1, pCMV4, pCR3.1-ZP3-T, pCR3.1-ZP3-L, and pCMV4-rZPC', respectively; pre-immune serum from BALB/c mice was added in mock group as negative control. Anti-ZP3 antibody (Santa Cruz, SC25802, 3 μl/100 μl) was used as a positive control. Ratio of cleavage embryo: no. of cleavage embryo/fertilized eggs. Ratio of blastocyst: no. of blastocyst/fertilized eggs. Data are presented as mean ± S.D.

2.11. Statistical analysis

The percentages were subjected to an arc-sine transformation, and the significance of the difference was analyzed by one-way ANOVA employing SPSS 15.0 software. Values were reported as the mean ± S.D. *P* values below 0.05 were considered to be statistically significant.

3. Results

3.1. Generation and specificity of antisera

3.1.1. ELISA

The concentration of IgG specific to mouse soluble zona pellucida in sera samples of the immunized mice was detected by standard ELISA after serial dilution. As shown in Fig. 1A, the titer of ZP3 specific antibody elicited by pCMV4-rZPC' were significantly higher compared with that elicited by pCMV4 ($P < 0.05$).

3.1.2. Immunofluorescence

To identify whether antisera generated by the DNA vaccine, pCMV4-rZPC' could bind to the ZP in situ, immunofluorescence analysis was performed. The representative result was shown in Fig. 1B and C. It was demonstrated that mice immunized with pCMV4-rZPC' generated specific antisera, which could bind to mouse ZP.

3.2. Effects of anti-ZP3 antisera on the fertilization rate and preimplantation development of mouse eggs in vitro

As shown in Table 1 and Fig. 2A, B, the fertilization rate of pCMV4-rZPC' group was significantly lower when compared with that of pCMV4 group (50.4% *versus* 86.3%, $P < 0.05$), whereas no apparent detrimental effects of antisera on the cleavage and early development of mouse eggs were observed (Fig. 2C–F).

3.3. Effects of anti-ZP3 antisera on fertilization rate of ZP-free eggs

As shown in Table 2 and Fig. 2G, H, when ZP-free oocytes were used, no significant difference in the rate of fertilization between pCMV4-rZPC' and pCMV4 group was found (84.5% *versus* 85.1%) ($P > 0.05$). The depletion of ZP did not result in an increase of multispermic fertilization or fragmentation of eggs.

3.4. Effects of anti-ZP3 antisera neutralized by MSZP on fertilization rate

To further investigate the effect of anti-ZP3 antisera on fertilization of mouse eggs, antisera neutralized with mouse soluble zona pellucida was used in *in vitro* fertilization experiments. As shown in Table 3, there was no significant difference in the rate of fertilization between pCMV4 and pCMV4-rZPC' groups after MSZP pretreatment (74.8% *versus* 75.8%) ($P > 0.05$).

Table 2
Effect of anti-ZP3 antiserum on the rate of fertilization *in vitro* of mouse ZP-free eggs.

Group	No. of eggs for IVF	No. of fertilized eggs (%)
Saline	70	59(84.2 ± 3.0)
pCR3.1	103	92(89.1 ± 3.2)
pCMV4	71	63(88.8 ± 1.3)
pCR3.1-ZP3-T	101	86(85.1 ± 3.3)
pCR3.1-ZP3-L	75	63(84.0 ± 2.3)
pCMV4-rZPC'	72	60(83.4 ± 2.2)
Positive	72	61(84.5 ± 4.0)

Note: values without different superscripts within a column did not differ significantly ($P > 0.05$).
Each fertilization mircodrop supplemented with 10% (v/v) antisera from mice immunized with saline (negative control), pCR3.1, pCMV4, pCR3.1-ZP3-T, pCR3.1-ZP3-L, and pCMV4-rZPC', respectively. Anti-ZP3 antibody (3 μl/100 μl, Santa Cruz, SC25802) was used as positive control. Ratio of fertilized eggs: no. of fertilized eggs/no. of eggs for IVF. Data are presented as mean ± S.D.

Table 3
The effect of anti-ZP3 antisera neutralized by mouse soluble zona pellucida on the rate of fertilization *in vitro* of mouse ZP-intact eggs.

Group	No. of eggs for IVF	No. of fertilized eggs (%)
Mock	83	67(80.7 ± 2.1)
Saline	79	64(80.7 ± 4.4)
pCR3.1	77	61(79.5 ± 1.3)
pCMV4	83	63(75.8 ± 1.1)
pCR3.1-ZP3-T	85	61(71.9 ± 2.1)
pCR3.1-ZP3-L	85	66(77.5 ± 3.5)
pCMV4-rZPC'	80	60(74.8 ± 1.6)
Positive	77	59(76.6 ± 0.9)

Note: values without different superscripts within a column did not differ significantly ($P > 0.05$).
Each fertilization mircodrop supplemented with 10% (v/v) antisera from mice immunized with saline, pCR3.1, pCMV4, pCR3.1-ZP3-T, pCR3.1-ZP3-L, and pCMV4-rZPC', respectively, and neutralized with mouse soluble zona pellucida (3 μl MSZP, 200ng/μl) for 3 h before insemination; pre-immune serum from BALB/c mice was added in mock group as a negative control; Anti-ZP3 antibody (Santa Cruz, SC25802) was used as a positive control. Ratio of fertilized eggs: no. of fertilized eggs/no. of eggs for IVF. Data are presented as mean ± S.D.

3.5. Effects of antisera generated by DNA vaccines, pCR3.1-ZP3-T and pCR3.1-ZP3-L on fertilization in vitro

In order to investigate the relationship between the ZP encoding sequences and the relative antifertility efficiency of the anti-ZP3 DNA vaccines, effects of anti-ZP3 antisera from two other DNA contraceptive vaccines, pCR3.1-ZP3-T and pCR3.1-ZP3-L were

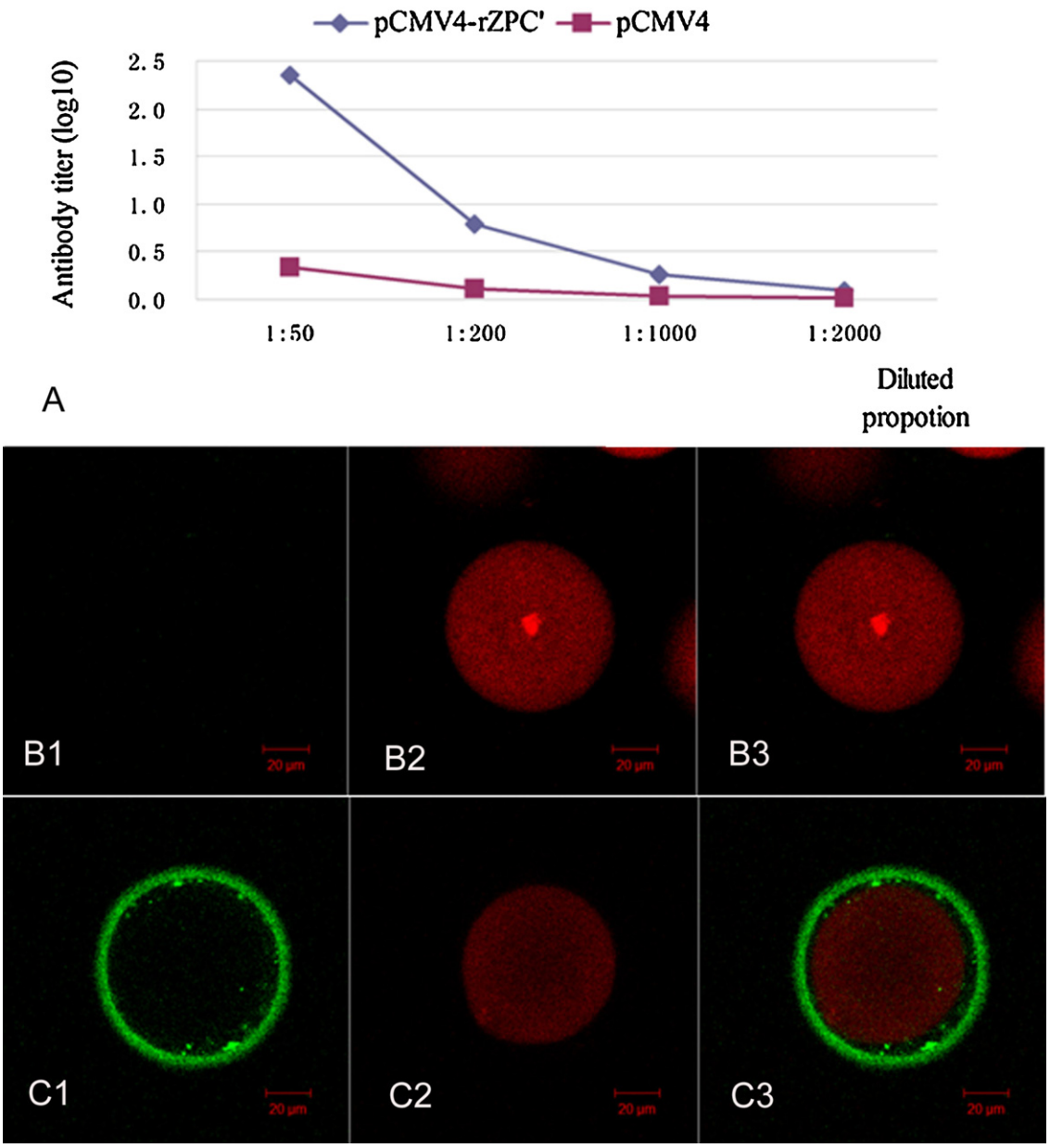

Fig. 1. Detection of the generation and specificity of antisera generated by DNA vaccine, pCMV4-rZPC' by ELISA and immunofluorescence. *ELISA*: mouse solubilized zona pellucida protein was chosen as coated antigen. Antisera from pCMV4 and pCMV4-rZPC'-immunized mice were serially diluted from 1:50 to 1:2000. The antibody titers were pooled and expressed as $\log_{10}$ titers. *Immunofluorescence*: different antisera from immunized mice were used as the first antibodies. Antiserum from pCMV4 and pCMV4-rZPC'-immunized mice was used as primary antibodies. The existence of anti-ZP3 antibody was probed with fluorescein isothiocyanate-conjugated secondary antibody (FITC). The samples were analyzed using an inverted confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany). B1, C1: FITC; B2, C2: propidium iodide; B3, C3: overlays. Magnification: 200×.

also examined. The generation and specificity of anti-ZP3 antisera from pCR3.1-ZP3-T and pCR3.1-ZP3-L-immunized mice were detected by Western blotting (Fig. 3). Antisera generated by the two DNA vaccines significantly decreased the rate of fertilization when compared to that of saline and pCR3.1 vector control groups (47.8%, 49.8%, 87.7%, and 86.6%, respectively, $P < 0.05$). Treatments of antisera from pCR3.1-ZP3-T and pCR3.1-ZP3-L-immunized mice showed no detrimental effect on the ability of early embryo cleavage and subsequent blastocyst formation (Table 1). The rate of fertilization of pCR3.1-ZP3-T and pCR3.1-ZP3-L groups using ZP-free eggs showed no significant difference with those of saline and pCR3.1 groups (85.1%, 84.0%, 84.2%, and 89.1%, respectively, $P < 0.05$) (Table 2). And the effect of the two antisera neutralized with mouse soluble zona pellucida on fertilization of mouse eggs *in vitro* was similar with that of antisera generated by pCMV4-rZPC'. There was no significant difference in the rates of fertilization of pCR3.1-ZP3-T and pCR3.1-ZP3-L groups using neutralized antisera when compared with those of saline and pCR3.1 groups (71.9%, 77.5%, 80.7%, and 79.5%, respectively, $P < 0.05$) (Table 3).

4. Discussion

The main scope of this study was to investigate whether the antisera generated by DNA vaccine, pCMV4-rZPC' would inhibit the fertilization and/or embryonic development *in vitro*. We designed an *in vitro* fertilization system to elevate the effect of the antisera

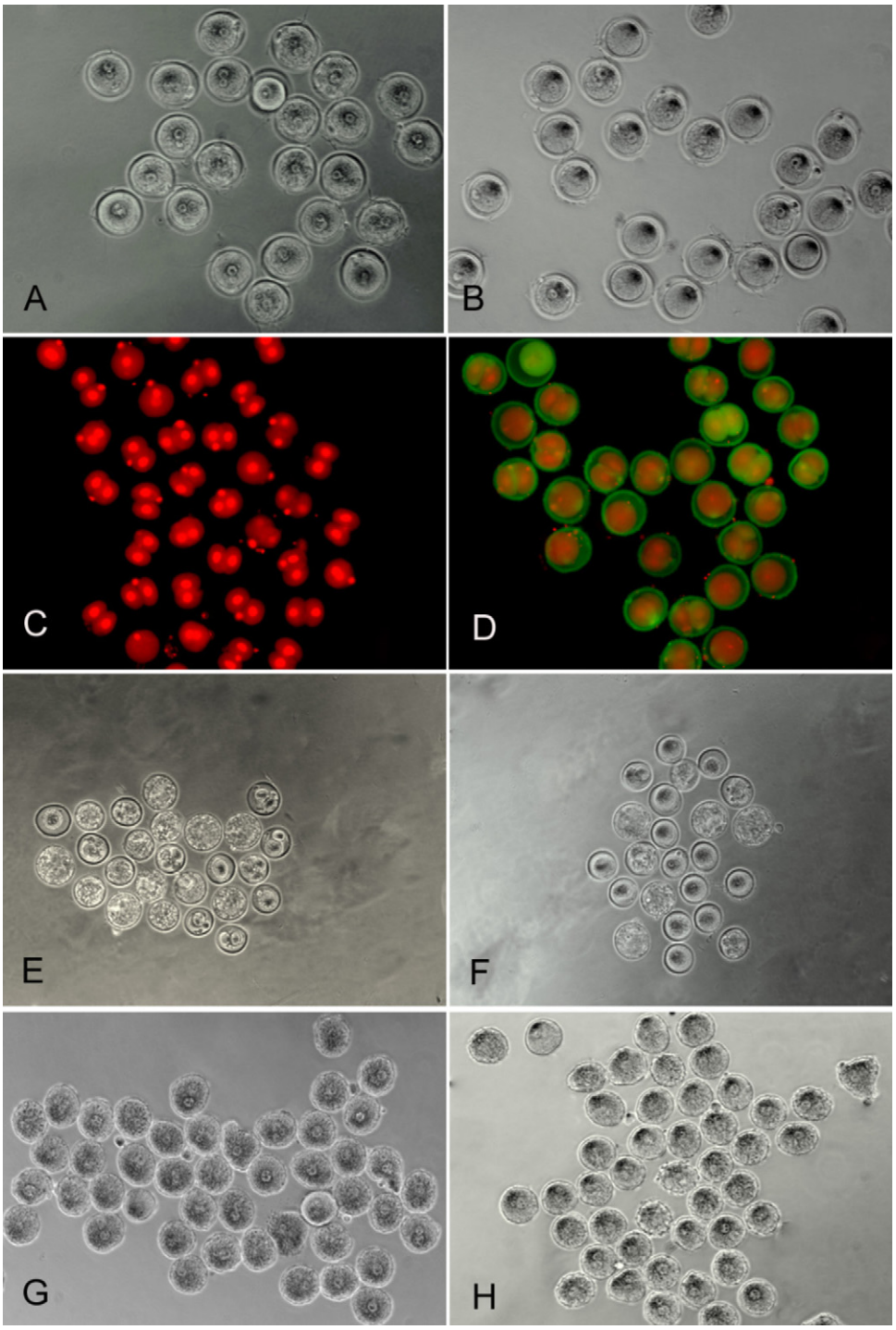

Fig. 2. Effects of anti-ZP3 antisera on fertilization and early development of mouse eggs *in vitro*. Different antisera (10%, v/v) from immunized mice were supplemented into the fertilization medium. Representative pictures of fertilized eggs derived from groups supplemented with (A) antiserum from pCMV4-immunized mice at 9–10 h after insemination; (B) antiserum from pCMV4-rZPC'-immunized mice at 9–10 h after insemination; (C) antiserum from pCMV4-immunized mice at 24 h after insemination; (D) antiserum from pCMV4-rZPC'-immunized mice at 24 h after insemination; (E) antiserum from pCMV4-immunized mice at 96 h after insemination; (F) antiserum from pCMV4-rZPC'-immunized mice at 96 h after insemination; (G) antiserum from pCMV4-immunized mice at 9–10 h after insemination of ZP-free eggs; (H) antiserum from pCMV4-rZPC'-immunized mice at 9–10 h after insemination of ZP-free eggs. Magnification in all panels: 100×.

from DNA vaccine-immunized mice on sperm–oocyte fertilization and preimplantation embryonic development *in vitro*. We observed that the supplement of the three antisera in fertilization medium led to a significant decline in the rate of fertilization of ZP-intact oocytes *in vitro*, suggesting that specific anti-ZP3 antibody generated by DNA vaccine can sufficiently inhibit the fertilization events in mice. This finding is consistent with our previous observation that the birth rates of mice immunized by pCMV4-rZPC', pCR3.1-ZP3-T, and pCR3.1-ZP3-L were reduced to 40%, 50%, and 67%, respectively, *in vivo* [16,17]. And the results are in consistent with previous studies which reported that both polyclonal and monoclonal antibodies to ZP3β, a homologue of human ZP3, inhibited *in vitro* binding of sperm to antibody-treated oocytes [24,25].

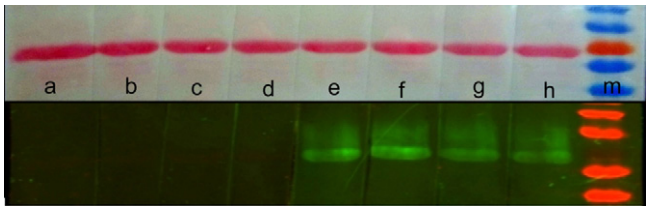

Fig. 3. Detection of the generation and specificity of antisera by Western blotting. Mouse heat-solubilized zona pellucida proteins were used as antigen. The membrane was incubated with serum diluted 1:50 from unimmunized mice (a), saline-(b), pCR3.1-(c), pCMV4-(d), pCR3.1-ZP3-T-(e), pCR3.1-ZP3-L-(f), and pCMV4-rZPC'-immunized (g) BALB/c mice, were used as first antibodies. Anti-ZP3 antibody (h) was used as a positive control. The membranes were scanned using the Odyssey infrared scanner (Li-COR Biosciences, Lincoln, NE). The presence of ZP3 protein was indicated by ponceau s staining. M: marker, 43, 55, 72, 95, and 130 (KD) in bottom-up order.

Although anti-ZP antibody supplemented in cultures was harmful to the normal development of mouse preantral follicles and oocytes *in vitro* [26], some oocytes could complete maturation and developed to blastocyst after *in vitro* fertilization [27]. Our results provided herein direct experimental evidence supporting the hypothesis that ZP3-specific antisera from DNA vaccine immunized mice interrupt the normal sperm–oocyte recognition during fertilization without observable harmful effects on embryonic development. The role of anti-ZP3 antibody shown in the antiferilization process in this study was consistent with a previous report which indicated that the onset of infertility in immunized mouse was temporally related to the detection of antibody [28]. However, it was notable that the efficacy of anti-fertilization of anti-ZP antisera was much lower in our present study, compared with that previously reported using a combined approach with *in vitro* maturation and *in vitro* fertilization [26]. And our result cannot negate the possibility that the overall fertility may also be reduced due to abnormal oocyte maturation and development *in vivo*. This could be due to the fact that oocytes matured *in vitro* in cultures supplemented with anti-ZP antibody may completely lose their fertilization capacity, since anti-ZP antibody would interfere the normal oocyte development by impairing the bidirectional communication between oocytes and granulosa cells, which is essential for fertilization and embryo development [29].

Whether ZP3-specific antibody is solely responsible for inhibiting sperm–oocyte recognition remained unknown. In this respect, there was recent evidence showing that antibody may elicit infertility by disrupting oocyte development as well as inhibiting oocyte fertilization in rMCMV-mZP3 infected mice [30]. However, there was no convincing evidence for the direct cause of anti-ZP3 induced infertility. In order to investigate whether anti-ZP3 antisera inhibited the fertilization through interrupting the interaction between the sperm and ZP, *in vitro* fertilization using ZP-free eggs was performed. In contrast to ZP-intact eggs, ZP-free eggs did not show a decline in the rate of fertilization when antiserum (10%, v/v) was supplemented in fertilization medium. The results clearly demonstrate that the anti-fertilization bioactivity of anti-ZP3 antisera is achieved by targeting the oocyte coat, the ZP. This is further confirmed by our subsequent investigation showing that anti-ZP3 antisera pre-neutralized with mouse soluble zona pellucida completely lose their capacity to inhibit sperm–oocyte fertilization. It is conceivable that anti-ZP3 antibodies in the sera might combine with the zona pellucida which consequently blocks the epitope, essential for sperm binding. Taken together, anti-ZP3 antisera generated by DNA vaccine, pCMV4-rZPC' can disrupt the normal sperm–oocyte recognition during fertilization.

In order to identify relationship between the epitope and antifertility, we also tested the effect of two other DNA vaccines, pCR3.1-ZP3-T, pCR3.1-ZP3-L encoding different sequences of rabbit ZP3 on fertilization *in vitro* at the same time [17]. The generation and specificity of antisera was identified by Western blotting.

Although these ZP DNA vaccines showed different anti-fertility efficiency in mice *in vivo* [17], antisera from pCR3.1-ZP3-T, and pCR3.1-ZP3-L-immunized mice can inhibit the fertilization in a similar manner, showing no significant difference among the three DNA vaccines in the efficacy of inhibiting fertilization *in vitro*. One possible explanation for the inhibition of the interaction between the sperm and the egg in this study might be due to the block of the epitope encoded by mouse mZP3 exon-7, which was necessary and sufficient for the binding of mouse sperm *in vitro* by anti-ZP3 antisera [31]. And the variance on the efficacy of anti-fertilization between *in vivo* and *in vitro* might be due to the titer of antibody and the generation of complement-fixing antibodies [32]. In this study, the effect of anti-ZP3 antisera on the early developmental potential of mouse eggs was also examined. All three tested DNA vaccines showed no detrimental effect on the ability of early embryo cleavage and subsequent blastocyst formation.

In conclusion, the present study shows that DNA contraceptive vaccines generate specific anti-ZP3 antibodies, which can interrupt the interaction between the sperm and ZP-intact oocytes. However, the inhibition efficacy of fertilization *in vitro* of mouse eggs by anti-ZP3 antisera was far from satisfactory. Further exploration of the molecular mechanism of recognition and binding between the sperm and eggs are required to make a "perfect" non-steroid DNA vaccine for population control of wild life, even for human use.

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Conflict of interest statement: All authors disclose that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted.

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