Investigation of Recombinant Mouse Sperm Protein Izumo as a Potential Immunocontraceptive Antigen

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Introduction
The development of a contraceptive vaccine (CV) has attracted considerable interest in the field of population control for many years. The molecules that are being explored for CV development target gamete production [luteinizing hormone-releasing hormone (LHRH)/GnRH, FSH], gamete function [sperm antigens and oocyte zona pellucida (ZP)], and gamete outcome (HCG).¹ Unlike applicable anti-infection vaccines, the effective and safe CV using autologous or isologous antigens should result in voluntary, long-lasting but reversible infertility with the occurrence of limited side-effects.

There was evidence that many sperm antigens can stimulate production of autoantibodies in certain individuals,² leading to a condition of immunological infertility¹–⁵ and also occurring in a majority of men.

Method of study
The recombinant mIzumo fused with 6His tag (6His-mIzumo) was purified by immobilized Ni²⁺ affinity chromatography. Enzyme-linked immunosorbent assay and Western blot were used to detect anti-6His-mIzumo activities of serum from the mice immunized with 6His-mIzumo. Inhibition of the anti-6His-mIzumo antibody on mouse sperm–egg fusion in vitro was performed using the zona free oocytes and acrosome reacted sperm. Fertility of the 6His-mIzumo immunized male and female mice was compared with control mice.

Results
The recombinant mIzumo was successfully produced. Female and male mice inoculated with 6His-mIzumo developed a specific serum antibody and the highest antibody titer lasted at least 6 weeks. The serum anti-6His-mIzumo antibody almost completely blocked mouse sperm–egg fusion in vitro. However, there was no significant reduction in fertility for both male and female mice immunized with 6His-mIzumo compared with control mice.

Conclusion
The circulated anti-mIzumo antibody can block mouse sperm–egg fusion in vitro but has no effect on fertility in vivo. It seems that application of Izumo as a candidate antigen in development of contraceptive vaccine needs further investigation.
who have had a vasectomy.\textsuperscript{6,7} In addition, normal fertility can be inhibited by immunization directly with spermatozoa.\textsuperscript{8} Thus, these investigations had indicated a potentiality for the antisperm contraceptive method. However, the whole spermatozoon per se cannot be used for the development of a CV because there are several antigens of sperm cell that are likely to be shared with various somatic cells.\textsuperscript{9} Therefore, the utility of a CV targeting only one sperm-specific antigen may represent an effective, valuable, and available approach to control fertility. Over the last more than 20 years, many sperm antigens with potential immunocontraceptive properties have been described in various laboratories and some of them are still being investigated. Among these candidates, lactate dehydrogenase-C4 (LDH-C4),\textsuperscript{10} cleavage signal-1 (CS-1),\textsuperscript{11} fertilization antigen-1 (FA-1),\textsuperscript{12} NZ-1, -2,\textsuperscript{13} NZ-3,\textsuperscript{14} contraceptive vaccinogen,\textsuperscript{15} YLP\textsuperscript{12,16} testis-specific antigen-1 (TSA-1),\textsuperscript{17} PH-20,\textsuperscript{18} P26\textsuperscript{19,20} and sperm associated antigen (SPAG9)\textsuperscript{21} have been studied in detail, but no single recombinant sperm antigen has shown a complete block of fertility in all the vaccinated animals.\textsuperscript{22}

Recently, a novel sperm protein, Izumo has been characterized in mice and human, becoming detectable on sperm surface only after the acrosome reaction.\textsuperscript{23} In that study, mouse Izumo (mIzumo), described as a testis (sperm)-specific 56.4-kDa protein by Western blotting, was required for sperm to fuse with eggs. A gene-disrupted mouse line, Izumo\textsuperscript{−/−} male mice were healthy but sterile, and their normal-looking sperm could bind to and penetrate the zona pellucida but were incapable of fusing with eggs. Furthermore, it was demonstrated that the sequence of Izumo has an extracellular immunglobulin-like domain that contains one putative glycosylation site. The computerized homology analysis revealed that amino-acid sequence of mizumo is homologous to human Izumo with 57\% identity over the entire sequence. For reasons of tissue-specific expression and a vital role in sperm–egg fusion process, Izumo may be a new candidate for development of CV for family planning or pest animal control. To understand whether Izumo protein is strongly immunogenic and capable of stimulating the production of specific circulating antibodies, which can affect the fertility of the immunized animals, we investigated the production of recombinant mIzumo in bacteria and assessed the effect of immunization of this protein on fertility of both female and male mice.

Materials and methods

Construction of Prokaryotic Expression Vector

The pCXN2-mIzumo plasmid DNA, which contains the complete coding sequence of mIzumo, was kindly provided by Dr Naokazu Inoue (Genome Information Research Center, Osaka University, Japan). The mIzumo cDNA coding region was subcloned into pET28a(+) vector (Merck KGaA, Darmstadt, Germany), where the inserted mIzumo cDNA was fused in-frame with N-terminal six-histidine (6His) nucleotide sequence. The restriction enzyme EcoRI was used to digest pET28a (+) vector and isolate mIzumo cDNA from pCXN2-mIzumo plasmid, and then the former was dephosphorylated by calf intestinal alkaline phosphatase (Fermentas International Inc., Burlington, ON, Canada). Next, the linearized pET28a (+) vector and mIzumo DNA fragment were mixed at 1:3 to 1:10 molar ratio in a ligation reaction and incubated at 16\(°\)C overnight. The ligation mixture was used directly to transform the competent Escherichia coli BL21 (DE3) cells. The right orientation and authenticity of the mIzumo coding sequence were confirmed by both restriction digestion and sequencing.

Expression and Purification of Recombinant mIzumo

Expression of the recombinant mIzumo was conducted according to standard protocol in Molecular Cloning: A Laboratory Manual, 3rd edn. The bacteria containing the plasmid with right insert were cultured overnight at 37\(°\)C with vigorous shaking. The culture was diluted (1:50), and continued until OD\textsubscript{600} reached 0.4–0.6. Then, isopropyl \(\beta\)-D-thiogalactopyranosides (IPTG) was added to get a 1 m\textsuperscript{m} final concentration, and the culture was incubated with vigorous shaking for an additional 6 hr at 37\(°\)C.

The recombinant mIzumo fused with 6His tag (6His-mIzumo) was purified by immobilized Ni\textsuperscript{2+} affinity chromatography. The bacterial cells were harvested by centrifugation at 10000 \(\times\) \(g\) for 10 min, and the cell pellet was resuspended in ice-cold Binding Buffer (0.5 m NaCl, 20 m Tris–HCl, 5 m imidazole, 6 m Urea, pH 7.9). After repeated sonication and resuspension of the pellets in Binding Buffer, the supernatant with \(\beta\)-mercaptoethanol (3 m final concentration) in it was incubated on ice for 30 min. The recombinant 6His-mIzumo was then affinity
purified using HisBind Purification Kit (Merck KGaA) under denaturing conditions according to the manufacturer’s instruction. The eluting purified 6His-mIzumo was analyzed in SDS–PAGE procedures. The concentration of purified 6His-mIzumo was measured using Bradford Protein Quantitation Kit (Tiangen Biotech, Beijing, China). Finally, the purity and authenticity of the purified recombinant protein was analyzed in SDS–PAGE and Western blot procedures.

Mouse strain

C57BL/6 mice at 6–8 weeks of age were obtained from Institute of Laboratory Animal Science, Chinese Academy of Medical Science, and maintained in the laboratory animal center under the animal welfare guidelines of our college. All animal experimental procedures were approved by the Animal Experimentation and Ethics Committee of Shantou University.

Immunization and Mating Protocols

An intraperitoneal (i.p.) inoculated-route using Freund’s adjuvants was chosen to immunize mice in our study, because this method could induce high antibodies titer to self-antigen in mice.24,25 C57BL/6 mice (6–8 weeks old) were divided into two trials to study the antibody response (trial I) and the fertility (trial II) of the mice immunized with 6His-mIzumo. In trial I, three female and three male mice were immunized systemically by i.p. with purified 6His-mIzumo protein as described elsewhere.25 Each animal received a total of four injections at 2-week intervals. Each injection consisted of 25 µg 6His-mIzumo in 100 µL PBS emulsified with an equal volume of complete (first injection) or incomplete (three booster injections) Freund’s adjuvant (Bio Basic Inc., Markham, ON, Canada). Mice were bled from the suborbital sinus before each immunization and 2, 4, and 6 weeks after the final boost. The control animals (6–8 weeks old; n = 2 female, 1 male) were immunized with adjuvant containing PBS alone using the same procedures described above.

In trial II, test mice (6–8 weeks old; n = 19 female, 9 male) and control animals (6–8 weeks old; n = 15 female, 10 male) were given the same inoculation as above. Also, serum samples were collected from all mice immediately prior to the first immunization and 2 weeks after the final boost. When the serum antibody titers reached the highest level 2 weeks after the final immunization (data shown below), the pairs of immunized female mice were mated with single normal male and the pairs of normal females were mated with each of the immunized males. All males were removed after 2 weeks and the females were allowed to deliver in the next 3 weeks. The number of pups was counted, and pups were killed by CO2 asphyxiation.

Assessment of Antibodies

Serum IgG antibody levels against recombinant antigens were tested by enzyme-linked immunosorbent assay (ELISA) using 200 ng purified 6His-mIzumo per well as antigen. The microplates (Nunc A/S, Kamstrupvej, Roskilde, Denmark) were coated with 100 µL of 6His-mIzumo in coating buffer (15 mM Na2CO3, 3.5 mM NaHCO3, pH 9.6) per well overnight at 4°C. The plates were washed three times with PBS (pH 7.4) and blocked further with 5% skimmed milk in PBS and the normal mouse serum was used at dual wavelength 450 and 630 nm as a reference. The coated wells treated identically with 5% skimmed milk in PBS served as blank and negative controls. The coated wells were washed six times and treated with 100 µL peroxidase-conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich, Inc., St Louis, MO, USA) diluted 1:10,000 in PBS for 1 hr at 37°C. After blocking, the plates were washed three times again and 100 µL immunized mice sera (1:100 dilution in PBS) was added to the wells. After 1 hr at 37°C, the plates were washed six times and treated with 100 µL solubilized mono-component tetramethyl benzidine (TMB) substrate solution (Tiangen Biotech) per well was added to develop color. After 30 min at room temperature, 100 µL of 2 m H2SO4 was used to end the color reaction and the absorbance (OD) was measured at dual wavelength 450 and 630 nm as a reference. The coated wells treated identically with 5% skimmed milk in PBS and the normal mouse serum instead of the antisera served as blank and negative controls. The OD reading was converted to standard deviation (SD) units by following formula: SD units = (OD (test) – OD (blank control))/ (OD (negative control) – OD (blank control)).26 The sample with
SD units ≥+3 were considered positive. This ELISA procedure was performed in triplicate on different days.

**Protein Extracts and Western Blot Analysis**

Membrane protein extracts of mouse caudal epididymal sperm (insert total number of sperm used for the protein extract) were prepared by using ProteoExtract Native Membrane Protein Extraction Kit (Merck KGaA).

The purified 6His-mIzumo and membrane protein extracts of mouse caudal epididymal sperm were subjected to SDS–PAGE, and transferred onto a nitrocellulose membrane (American Membrane Corporation, Ann Arbor, MI, USA) by Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Briefly, the nitrocellulose membranes were blocked with blocking buffer [5% skimmed milk in TBS-0.05% Tween-20 (TBST)] for 1 hr at room temperature (RT). After blocking, the membranes were incubated with immunized mice sera diluted at 1:100 in blocking buffer for 1 hr at RT. The membranes were washed twice with TBST and then once with TBS for 7 min and incubated with peroxidase-conjugated goat anti-mouse IgG secondary antibody diluted at 1:1000 in blocking buffer for 1 hr at RT. Finally, after being washed three times as above, the membranes were visualized using Western Blotting Luminol Reagent according to the manufacturer’s instructions (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

**Sperm–egg Fusion Assay**

The dye transfer technique was used to visualize sperm–egg fusion after fertilization. Sperm were obtained by swim-up procedure from mouse caudal epididymis and capacitated in modified Krebs-Ringer bicarbonate solution (TYH) containing 1.5% bovine serum albumin (BSA; Sigma-Aldrich, Inc.) at 37°C in 5% CO₂ for 2 hr. To induce the acrosome reaction, sperm before capacitation were treated with 10 μM calcium ionophore A23187 (Sigma-Aldrich, Inc.) at 37°C in 5% CO₂ for 30 min and then rinsed with TYH containing 0.3% BSA. The sperm suspension was diluted to make the insemination droplets (100 μL) containing 0.5–1 × 10⁶ motile sperm/mL covered with mineral oil and pre-incubated with 10% (v/v) antisera or pre-immune sera in TYH containing 0.3% BSA for 30 min. The oocytes were collected from female mice 16 hr post-HCG injection and then cumulus oophorus were removed by 0.1% hyaluronidase in TYH containing 0.3% BSA. Next, the zona pellucida (ZP) was completely dissolved in acid Tyrode’s solution (pH 2.5). The oocytes were pre-incubated in TYH medium containing 0.3% BSA for 1 hr as this pre-incubation could enhance fusibility of ZP-free egg with sperm. Zap-free oocytes were loaded by Hoechst-33342 (1 μg/mL; Sigma-Aldrich, Inc.) in TYH containing 0.3% BSA for 10 min at 37°C in 5% CO₂, then rinsed thoroughly in TYH containing 0.3% BSA and immediately transferred into the insemination droplets. After 30 min of insemination, the oocytes were washed with TYH and then fixed with 2.5% glutaraldehyde in PBS for 30 min. Fixed oocytes were mounted on slides and the presence of fused sperm was examined under a fluorescence microscope (DM2500; Leica Microsystems, GmbH, Wetzlar, Germany).

**Statistical Analysis**

Results are presented as mean ± S.E.M.. The data were analyzed by the Student’s t-test and chi-squared test using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at P < 0.05.

**Results**

**Identification of the Prokaryotic Expression Plasmid**

Restriction fragment length analysis was performed to identify the structure of the pET28a(+) vector where the complete coding sequence of mIzumo was subcloned into. The recombinant expression plasmid DNA which contains the mIzumo gene expression cassette in the correct orientation was confirmed by detection of DNA fragments of anticipated sizes using EcoR I (1209 bp), BamH I (1097 bp), and NheI/Stul (1178 bp) digestion (Fig. 1). According to the sequencing report of the recombinant plasmid, the coding sequence of mIzumo subcloned into pET28a(+) vector demonstrated the sense orientation and identical sequence, which matched completely with the original mIzumo coding sequence and there was an additional nucleotide sequence of 120 bp including a 6His tag before the start codon of mIzumo cDNA. Therefore, the predicted molecular weight of
6His-mIzumo was slightly larger (approximately 4.2 kDa) than that of native mIzumo deduced from the primary amino acid sequence.

Properties of Recombinant Mouse Izumo

6His-mIzumo produced by *E. coli* BL21 (DE3) cells using the pET prokaryotic expression vector formed inclusion bodies in cytoplasm. Therefore, 6His-mIzumo was purified under denaturing conditions using high concentration (6 M) of urea as denaturing agent and refolded by dialyzing with PBS. The affinity-purified 6His-mIzumo appeared as a single band with an apparent molecular weight of ≈60 kDa on a 10% SDS–PAGE gel in the presence of 6 His tag (Fig. 2). The sera also all detected a single band on sperm extracts run on parallel immuno-blots. No visible bands were detected by Western blot against 6His-mIzumo and sperm extracts in pre-immune serum samples. In addition, no visible bands appeared in pre-immune and post-immune sera from the PBS-control mice.

Serum Immune Responses of Mice Inoculated with Recombinant mIzumo

In trial I, seven serum samples of each animal were obtained at 2-week interval from prior to first immunization to 6 weeks after the final boost and ELISA were performed to examine the time course of mean serum IgG responses in inoculated mice. The IgG response started to rise at 2 weeks after the first boost of immunization with 6His-mIzumo in both caudal epididymal sperm on Western blots (Fig. 3).
female and male mice compared with the PBS-control animals. The peak antibody titers in both female and male were observed at 2 weeks after the final boost (Fig. 4).

In trial II, all female and male mice inoculated with recombinant 6His-mIzumo antigen developed detectable serum anti-6His-mIzumo antibody (SD units +3) at 2 weeks after the final boost as determined by ELISA (Fig. 5). SD units that served as the titer of the antibody against 6His-mIzumo ranged from 6.7 to 21.2 for females and 10.2 to 18.5 for males, with mean SD units (±S.E.M.) of 13.1 ± 1.0 for females (PBS control 1.6 ± 0.1) and 14.5 ± 0.9 for males (PBS control 1.9 ± 0.2).

**Effect of Antisera to Recombinant mIzumo on Sperm–egg Fusion in vitro**

To examine whether the antibodies against 6His-mIzumo were potent in inhibiting sperm–egg fusion,
sperm–egg fusion assays using ZP-free mouse oocytes were performed. The sperm–egg fusion rate (percentage of eggs with at least one fused sperm) in the antisera-treated group (2.9%, 3/103) was significantly lower than that in the control group (57.5%, 73/127) with pre-immune sera \((P = 0.00)\) (Table 1). An average number \((\pm S.E.M.)\) of fused sperm per fused egg was 1.0 \pm 0.0 in antisera-treated group, which was markedly less than that in the control group (2.5 \pm 0.2, \(P = 0.00\)) (Table 1).

### Table I Effect of Antisera Against Recombinant 6His-mIzumo Protein on Sperm–egg fusion in vitro

<table>
<thead>
<tr>
<th>Sera</th>
<th>Total eggs</th>
<th>No. fused eggs</th>
<th>No. fused sperm</th>
<th>Fusion rate (%)</th>
<th>Fusion index (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-immune</td>
<td>103</td>
<td>3</td>
<td>3</td>
<td>2.9 (^a)</td>
<td>1.0 \pm 0.0 (^b)</td>
</tr>
<tr>
<td>Pre-immune</td>
<td>127</td>
<td>73</td>
<td>185</td>
<td>57.5 (^a)</td>
<td>2.5 \pm 0.2 (^b)</td>
</tr>
</tbody>
</table>

\(^a\)Fusion rate indicated the percentage of eggs with at least one fused sperm.

\(^b\)Fusion index showed the average number of fused sperm per fused egg.

\(^a,b\)There was significant difference of fusion rate and fusion index between the two groups \((P < 0.01)\).

Fertility of Mice Immunized with Recombinant mIzumo

Immunized C57BL/6 mice 2 weeks after the final boost were mated with contra-sexual animals to determine the effect of active immunization on mouse fertility. Fertility rates and litter sizes were compared between 6His-mIzumo-immunized and PBS-control mice. There was no significant reduction in the fertility rates of the test mated pairs (the immunized females to the untreated males, \(P = 0.49\), or the untreated females to the immunized males, \(P = 0.26\)) compared with that of the PBS-control mice (Table 2). Likewise, no significant difference of litter size between the test mated pairs (the immunized females to untreated males, \(P = 0.13\), or the untreated females to the immunized males, \(P = 0.96\)) and the PBS controls was observed (Table 2). There was no correlation between fertility and the level of antibody response of mice inoculated with 6His-mIzumo protein (result not shown).

### Table II Fertility of C57BL/6 Mice Immunized with Recombinant 6His-mIzumo Antigen

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Sex</th>
<th>No. mice</th>
<th>No. fertile mice</th>
<th>Total pups</th>
<th>Fertility rate (%) (^a)</th>
<th>Litter size (mean ± S.E.M.) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6His-mIzumo</td>
<td>F</td>
<td>19</td>
<td>14</td>
<td>46</td>
<td>73.7</td>
<td>3.2 \pm 0.4</td>
</tr>
<tr>
<td>PBS</td>
<td>F</td>
<td>15</td>
<td>12</td>
<td>52</td>
<td>80.0</td>
<td>4.3 \pm 0.5</td>
</tr>
<tr>
<td>6His-mIzumo</td>
<td>M</td>
<td>9</td>
<td>5</td>
<td>48</td>
<td>55.6</td>
<td>6.9 \pm 1.0</td>
</tr>
<tr>
<td>PBS</td>
<td>M</td>
<td>10</td>
<td>8</td>
<td>76</td>
<td>80.0</td>
<td>6.9 \pm 0.5</td>
</tr>
</tbody>
</table>

\(^a\)There was no significant difference between the fertility rate of the treated and control group in both female and male mice \((P > 0.05)\).

\(^b\)No significant difference in the mean litter size was presented between the treated and control group in both female and male mice \((P > 0.05)\).

Discussion

Mouse sperm–egg fusion protein Izumo was first reported by Inoue et al. in 2005. In this study, we demonstrated the successful expression and purification of the recombinant mouse Izumo fused with N-terminal 6his tag (6His-mIzumo) in bacteria. In our experiments, 6His-mIzumo was insoluble and formed inclusion bodies in bacterial cells probably because of the presence of a putative hydrophobic transmembrane region at C-terminal of mIzumo. This analogous situation has taken place in other studies on preparation of recombinant membrane protein in bacteria. In this study, the purified 6His-mIzumo appeared as a single band of \(\sim 60\) kDa on a 10% SDS–PAGE gel, which was slightly bigger than 56.4 kDa previously reported for native mIzumo by Western blot. The purified recombinant protein reported here was clearly mIzumo, as the antisera against 6His-mIzumo recognized the same single \(\sim 60\) kDa band on Western blot of mouse sperm. This discrepancy is probably attributed to the differences in concentration of SDS–PAGE gel and choice of protein marker between studies, which may also affect the apparent mobility of proteins between different SDS–PAGE protocols.

The recombinant mIzumo antigen raised an IgG antibody response in all mice of both sexes in the two trials. The circulating immune response appeared after the first boost and lasted with the highest antibody titer at least 6 weeks after the final immunization, according to the result of ELISA. Likewise, this change of mouse serum immune
response stimulated by recombinant allogenic antigen was reported elsewhere.\textsuperscript{24,35} The antisera from all female and male mice immunized with 6His-mIzumo recognized a single protein band in the extracts of mouse sperm by Western blot. We therefore concluded that the recombinant 6His-mIzumo fusion protein was strongly capable of inducing cross-reactive IgG antibodies to the native mIzumo. The antisera of 6His-mIzumo-immunized mice almost completely blocked sperm–egg fusion of the ZP-free oocytes \textit{in vitro} with no effect on sperm motility, suggesting the antibodies of mIzumo specifically inhibit sperm–egg fusion without detrimental effect on other sperm function such as motility. Unfortunately, no significant difference in either fertility rate or mean litter size between 6His-mIzumo-immunized and PBS-control mice was observed in our study.

Several factors may contribute to this result. Firstly, native mIzumo may have antigenic epitopes with immuncontraceptive potential not present in the recombinant form in this study. Mouse Izumo contains one putative glycosylation site. It is well known that glycosylation affects the antigenic properties of proteins\textsuperscript{36} and that it is involved in gamete recognition.\textsuperscript{37} Glycosylation of mIzumo may have taken place in native protein, but not in the bacterially recombinant fusion protein, as the predicted molecular weight of 6His-mIzumo should be slightly larger than that of native mIzumo, but the two different forms of mIzumo were detected as the same size band (\(\approx 60\) kDa) on Western blot. Otherwise, the recombinant mouse Izumo in this study contains an additional peptide and the putative signal peptide at N-terminal, which may affect protein folding, probably resulting in reduction of antibody production against discontinuous epitopes present in the native mIzumo.

Next, in this study, we did not examine the local antibody response in the genital tract where fertilization takes place. Although systemic inoculation with recombinant sperm antigens has been shown to induce both the circulating and the local immune response,\textsuperscript{16} the latter one is probably more important for antifertility effect. Lack of immunonnaceptive effect as a consequence of insufficient antibodies in the female and male reproductive tracts has been reported.\textsuperscript{38} Therefore, there may have been insufficient antibodies produced in the reproductive tracts to cause infertility in mice using the immunization method of the present study.

Finally, this would probably be because Izumo is not localized on plasma membrane of fresh spermatozoa but is hidden under plasma membrane and accessible after the acrosome reaction.\textsuperscript{23} The specific antibodies have to present at the particular time and space for binding the Izumo antigen to affect reproductive process.\textsuperscript{39} Some sperm membrane proteins involved in sperm–egg fusion have been shown to cause antifertility effect. Rat epididymal protein DE, for example, participated in sperm–egg fusion and immunization with it produced a significant reduction in rat fertility.\textsuperscript{40} During sperm maturation at epididymis, the antibodies would probably bind DE antigen and inhibit the recognition between DE and its specific receptor on oolemma. In this respect, especially for the male immunized mice, the antibodies against 6His-mIzumo might not reach inner acrosomal membrane, not recognize and bind the Izumo antigen, and not neutralize the biological function of Izumo before sperm fused to oolemma in reproductive tracts, resulting in the inability of antifertility effect in our study.

In conclusion, antibody against recombinant 6His-mIzumo can block sperm–egg fusion of the ZP-free oocytes \textit{in vitro} but no effect on fertility of both male and female mice immunized with recombinant 6His-mIzumo. Therefore, there was probably no contraceptive effect of immunization with recombinant mouse Izumo prepared by prokaryotic expression system on mouse model. It seems that application of Izumo as a candidate antigen in development of contraceptive vaccine needs further investigation.

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