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Evaluation of a Recombinant Multi-Epitope Sperm Protein Vaccine for Immunocontraception in Female Mice

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Abstract

Unintended pregnancy remains a significant global health concern, often leading to abortion and associated maternal morbidity. Current contraceptive strategies suffer from limitations including side effects, irreversible sterility, and unreliable efficacy, prompting the development of immunocontraceptive vaccines that target gamete-specific antigens. This study aimed to assess the contraceptive efficacy and safety of a recombinant vaccine comprising three sperm proteins, IZUMO1, SACA3 and PH-20 in female mice. Bioinformatic analyses guided the design of a fusion protein, which was synthesized, expressed in E. coli, and purified for immunization. Female mice were immunized with the recombinant protein and monitored for antibody production, mating outcomes, and tissue pathology. Quantitative enzyme-linked immunosorbent assay (ELISA) revealed significantly increased systemic and mucosal IgG responses compared with control animals. Mating tests demonstrated a 66.6% reduction in pregnancy rate among immunized mice, while in vitro fertilization assays confirmed a serum titer-dependent impairment of fertilization. Histopathological examination of major organs showed no gross histopathological changes during the study period. These findings indicate that epitope-based recombinant vaccines can significantly reduce fertility in female mice without detectable short-term systemic effects. The results support further investigation into safe, reversable immunocontraceptive strategies targeting sperm antigens for non-hormonal fertility control.

Background

Unintended pregnancy is a global health issue that affects families, society, and, the medical well-being of women and their children. One of the most serious and common consequences of unintended pregnancy is abortion (1). According to World Health Organization (WHO) reports, around 73 million induced abortions occur worldwide each year. Six out of 10 (61%) of all unintended pregnancies result in induced abortion. The proportion of unintended pregnancies ending in abortion was 51% in 1990–1994, and it remained roughly the same through 2000 to 2004. It then increased to 61% by 2015–2019 (2–3). The proportion of unintended pregnancies ending in abortion reached as high as 68%, even among countries that completely prohibited abortion. It should be noted that 45% of abortions are unsafe and lead to maternal death and morbidity (4). Physical health risks associated with unsafe abortion include incomplete abortion, hemorrhage, infection, and uterine perforation. In addition, unsafe abortion can lead to complications such as tears within the genital tract and internal organs due to the insertion of dangerous objects into the vagina or anus (5). Therefore, preventing unintended pregnancies is of great importance for women and society. The main cause of accidental or unintended pregnancies is contraceptive failure (6). Contraceptive methods are classified into four main groups. Natural methods, such as tracking ovulation days are among the most unreliable. Hormonal methods, including pills, patches, vaginal rings, and implants, prevent pregnancy by disrupting sexual hormones, ovulation, and menstruation, but long-term use can cause side effects such as mood disorders, skin complications (e.g., acne or irritation), vomiting, dizziness, flushing, and potential effects on infertility (7–8). Physical barrier methods, such as condoms, diaphragms, and intrauterine devices (IUDs), create obstacles to fertilization; however, IUDs may damage uterine tissue, be displaced, causing unintended pregnancies,

increase leukocyte activity for sperm phagocytosis, and stimulate the immune system, resulting in hemorrhage, cramps, and irregular bleeding (9). The most invasive group involves male or female sterilization, which is generally irreversible and raises risks like ectopic pregnancy in women or other health issues in men. Consequently, there is a recognized need for effective, long-term reversible contraception with minimal side effects to better prevent unintended pregnancies (10). Currently, scientists are working on immunocontraceptive methods commonly referred to as contraceptive vaccines. These methods are based on immune reactions between the immune systems and conception -related factors (11–12). The antigen used for designing the contraceptive vaccines are categorized into three main classes. The first class includes gamete production factors such as FSH, LH and GnRH. The second class includes gamete function factors, such as surface antigens of sperm or oocytes, which play a critical role in fertilization. The third class encompasses gamete outcome factors, such as Beta-human chorionic gonadotropin (β HCG). Each category has its own advantages and disadvantages. Blocking sex hormones is widely used for population control in wildlife; however, it is not a suitable approach for humans. FSH, LH and GnRH play critical roles in the secretion of sex hormones, such as androgens and progesterone in humans. In addition to regulating reproduction, sex hormones play critical roles in maintaining cardiovascular, skeletal, neural, muscular, immune, adipose and, hematopoietic system function (11). The female reproductive system is part of the mucosal immune system, which provides protection through a large amount of immunoglobulins (13). If sperm antigens stimulate the female immune system, anti-sperm antibody (ASA) production can occur. As a result, ASA may be present in the genital tract. ASA can prevent fertilization by interfering with processes, including sperm motility, sperm capacitation, sperm-oocyte adhesion, acrosomal reaction, and sperm-oocyte penetration (14). IZUMO1 is a sperm-specific transmembrane protein with an immunoglobulin-like (Ig-like) domain, located on the sperm head surface. IZUMO1 has three domains: intracellular, intermembrane (or transmembrane?), and extracellular Ig-like. The Ig-like domain mediates sperm-oocyte adhesion and fusion via interaction with JUNO, while the intracellular domain influences IZUMO1 expression levels. Mice lacking the IZUMO1 gene are healthy but sterile, as their sperm can bind to the egg but fail to fuse with it (15). SACA3 (also known as SPACA3 or SLLP1) is a sperm-specific intra-acrosomal protein, similar to a lysosome-like protein found in mammalian spermatozoa. Additionally, SACA3 is present at all stages of ovarian follicle development in some mammals. SACA3 functions as a catalyst in sperm-oocyte adhesion and fusion (16). PH20, also known as SPAM1, is a protein found in the testis, epididymis, luminal fluid, and sperm. Together with SACA3, PH20 facilitates sperm-oocyte adhesion and fusion. PH20 plays three key roles in the fertilization process: it acts as a hyaluronidase to help sperm transition through the cumulus layers of the oocyte, binds to hyaluronic acid in the zona pellucida, which increases intracellular calcium levels, and stimulates the acrosome reaction (17). The aim of this study is to evaluate the pregnancy rate in female mice immunized with the multiepitope IZUMO1-PH20-SACA3 vaccine, the in vitro fertilization rate of sperm treated with anti-IZUMO1-PH20-SACA3 antibodies. In addition, the study assesses the presence of histopathological changes in major organs, and the hypothesis that anti-sperm antibodies can be effective in females, as observed in infertile women.

Materials and Methods

Bioinformatics Evaluation and Analysis of Fusion Protein

The proteins IZUMO1 (UniProt: Q9D9J7, Gene ID: 73456), SACA3 (UniProt: Q9D9X8, Gene ID: 75622), and PH-20 (UniProt: P48794, Gene ID: 20690) were selected for the design of the fusion construct. Epitope prediction and structural analyses were performed using multiple bioinformatics tools, including IEDB, I-TASSER, ProtParam, ASA-View, and Chimera (v1.14). Candidate regions were selected based on predicted epitope specificity, solvent accessibility, molecular size, antigenicity, and topological features. The three-dimensional structure of the chimeric protein was modeled using MODELLER (v11). The predicted construct was subsequently evaluated for toxicity, antigenicity, and immunogenicity before proceeding to experimental validation.

Cloning of Synthetic Gene Constructs

A codon-optimized synthetic gene encoding IZUMO1-Linker1-SACA3-Linker2-PH-20 was synthesized (General Biosystems) and subcloned into the pET21b expression vector. A 3' terminal His-tag was included to facilitate protein purification. The recombinant plasmid was transformed into competent *Escherichia coli* (*E. coli*) BL21 (DE3) cells using the heat shock method. Positive transformants were selected on LB agar plates supplemented with 100 μg/mL ampicillin. Recombinant plasmid DNA was isolated from selected colonies, and correct gene insertion was confirmed by PCR using gene-specific primers (Forward: 5’-GCTTTCCACAGCCATTTCAGG-3’; Reverse: 5’-GTAGGTTGAGGCCGTTGAGC-3’).

Heterologous Expression and Inclusion Body Isolation of Recombinant Protein

Recombinant E. coli BL21 (DE3) cells harboring the verified plasmid were cultured in 10 mL of LB medium containing 100 μg/mL ampicillin and incubated overnight at 37°C with shaking at 180 rpm. The overnight culture was diluted 1:100 into fresh LB medium, and protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density at 600 nm (OD600) reached 0.5–0.7. Following induction, cells were harvested by centrifugation at 8,000 rpm for 10 min at 4°C. The cell pellet was resuspended in 20 mL of lysis buffer (50 mM Tris, 150 mM NaCl, 5% v/v Triton X-100, pH 8.0) and disrupted by sonication on ice (70% amplitude, 0.5-second pulses, 30-second on/off cycles, total 5 min). The lysate, supernatant, and pellet fractions from induced and uninduced cultures were analyzed by SDS-PAGE, confirming that the target protein was localized in the inclusion body fraction. Inclusion bodies were subsequently isolated, washed repeatedly to remove soluble contaminants, and solubilized in 8 M urea for further purification.

Animal Immunization and Sample Collection

Fifteen female mice were allocated to the treatment group, and eight female mice served as controls. Animals were randomly assigned to experimental groups. All animals were quarantined prior to immunization. Immunizations were performed using two subcutaneous injections followed by one intraperitoneal booster. The first and second doses consisted of 100 µg and 50 µg of recombinant protein, respectively, each emulsified with an equal volume of complete Freund's adjuvant. The booster dose contained 50 µg of recombinant protein formulated with incomplete Freund's adjuvant and was administered on days 0, 14, and 28, respectively. Blood samples were collected from the facial vein, and vaginal washes were performed using 80 µL PBS under anesthesia prior to each blood collection and two weeks after the final immunization (day 42). Sample collection and analysis were conducted blinded to group allocation. Samples were centrifuged at 3,000 g for 15 minutes; and the resulting serum and vaginal wash supernatants were aliquoted and stored at -20°C for subsequent analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Sera from control and immunized mice were analyzed for antigen-specific IgG and IgA antibodies. Maxisorp ELISA plates (Nunc, Denmark) were coated with 5 µg per well of purified recombinant protein in 100 µL of carbonate-bicarbonate buffer (0.2 M $NaHCO_3$, 0.2 M Na_2CO_3, pH 9.0) and incubated overnight at 4°C. Wells were then blocked with 100 µL of 5% (w/v) non-fat skim milk prepared in PBST (PBS containing 0.05% Tween-20) at 37°C for 2 hours, followed by three washes with PBST. Serially diluted serum samples (1:50 to 1:1,600,000 for IgG; 1:5 to 1:600 for IgA) were added in triplicate (100 µL per well) and incubated at 37°C for 1 hour. Negative control wells containing PBST alone and sera from control animals were included on each plate. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgG (or IgA, where appropriate) (Sigma, Germany) at a 1:5,000 dilution, incubated for 1 hour at 37°C, followed by three PBST washes. Color development was achieved using O-phenylenediamine dihydrochloride (OPD; Sigma, Germany) as the substrate for 15 minutes at 25°C in the dark. The reaction was stopped with 2.5 M sulfuric acid, and absorbance was measured at 492 nm using a microplate reader.

Mating Test

To assess in vivo reproductive outcomes, immunized female mice were paired with untreated, sexually mature male mice (one male per two female) in individual cages. Mating pairs were maintained together for up to 7 days or until the presence of a vaginal plug was observed, which was recorded as evidence of successful mating. Females in which a vaginal plug was detected were separated from males and monitored for pregnancy. Control female mice were mated with untreated male mice under identical conditions to ensure that experimental handling did not affect fertilization rates. Pregnancy was confirmed by parturition, and litter size was recorded at birth. Each male mouse was used for a single mating pair to avoid bias related to male fertility.

Pathological Evaluation

Vital organs including the heart, liver, kidneys, lungs, uterus, ovaries, and spleen were collected from female mice following completion of the mating experiments., Tissues were fixed in 10% formalin, processed using standard histological procedures, embedded in paraffin, and sectioned at approximately 5 µm thickness. Sections were stained with haematoxylin and eosin (H&E) and examined by light microscopy. Histopathological evaluation was performed by an experienced pathologist blinded to treatment group allocation. Organs were assessed for evidence of inflammation, tissue damage, necrosis, or other pathological alterations, and findings from immunised mice were compared with those from control animals.

Oocyte fertilization evaluation

To investigate the effect of ASA on oocyte fertilization, immune mouse serum was substituted for fetal bovine serum (FBS) during in vitro fertilization (IVF). Female mice (7–8 weeks old-NMRI) were superovulated with equine chorionic gonadotropin (eCG), followed 46–48 hours later by human chorionic gonadotropin (hCG). Oocytes were collected from the oviducts 12–14 hours post-hCG, washed in M2 medium, and prepared for IVF. For fertilization, 10–15 morphologically normal oocytes (grade A) were placed in droplets of TCM-199 medium.

Sperm were obtained from 10–12-week-old male NMRI mice following three days of isolation and capacitated in TCM-199 medium supplemented with 10% BSA before being added to the oocyte-containing droplets. The oocytes were assigned to three experimental groups: (1) control (TCM-199 supplemented with 10% FBS), (2) treatment (TCM-199 supplemented with 10% immune mouse serum), and (3) serum substitution control (TCM-199 supplemented with 10% non-immune mouse serum). The third group was included to differentiate the effects of serum substitution from those attributable to FBS.

Following 4 hours of co-incubation at 37°C in a humidified atmosphere of 5% CO_2, presumptive zygotes were denuded, washed in KSOM medium, and cultured. Embryonic development was assessed by evaluating progression to the two-cell stage 36 hours after fertilization.

Result

Recombinant plasmid detection

Bacterial colonies grown on ampicillin-containing medium were analyzed to confirm the presence of a recombinant plasmid using PCR. Correct insertion of the gene construct was further confirmed by Sanger sequencing. The DNA template consisted of a segment of the pET21b+ vector and the complete sequence of the recombinant gene. Successful insertion of the gene into the vector was indicated by an amplicon length of approximately 1160 bp, confirmed to match the designed fusion construct, as shown in Fig. 1.

Expression and purification of the recombinant protein

Subsequent SDS-PAGE analysis confirmed expression of a 34 kDa protein, consistent with the predicted molecular weight of the synthetic fusion protein. Maximum protein expression was detected 8 hours after induction with IPTG (Fig. 2a). The recombinant protein was predominantly localized to bacterial inclusion bodies; however, solubilization with urea enabled recovery of the protein in a soluble form (Fig. 2b). The recombinant protein containing a hexahistidine (6xHis) tag was purified by affinity chromatography under denaturing conditions and SDS-PAGE analysis following purification confirmed the presence of a protein band at approximately 34 kDa (Fig. 2c, d).

1. (a) SDS-PAGE analysis of recombinant protein expression in E. coli BL21 (DE3). Lane 1: molecular mass marker. Lane 2: protein sample before IPTG induction. Lanes 3–11: protein samples collected at 2-hour intervals following IPTG induction.
2. (b) SDS-PAGE analysis of bacterial cell fractions following sonication, showing localisation of the recombinant protein in insoluble and urea-solubilised fractions.
3. (c) SDS-PAGE analysis of recombinant protein purification by affinity chromatography. Lane 1: molecular mass marker. Lane 2: protein sample prior to purification. Lane 3: flow-through. Lane 4: wash fraction. Lanes 5–13: eluted fractions containing the purified recombinant protein.
4. (d) SDS-PAGE analysis of the recombinant protein before and after dialysis, demonstrating recovery of the protein following removal of denaturant

Effect of immunization on fertility outcomes

Following three rounds of immunization, treated female mice were housed with non-immunized male mice to evaluate pregnancy outcomes. The pregnancy data are summarized in Table 1. Immunized females exhibited a 66.6% reduction in pregnancy rate compared with the control group.

Table 1
Effect of immunization with the IZUMO1 PH20 SACA3 fusion protein on fertility outcomes in female mice

Groups	**Number of females per group**	**Number of pregnant females**	**Total number of newborns per mother** **(number of mothers: number of newborns)**	**Fertility rate (%)**	**fertility inhibition rate (%)**
Control	8	8	5.875 (1:10,2:8,1:6,1:5,2:4,1:2)	100%	0
Treatment	12	4	1.91 (2:8,1:4,1:3,8:0)	33.4%	66.6%

Histopathological Evaluation of Immunized Mice

Histopathological examination of tissues from immunised and control mice is shown in Figs. 3, 4, and 5. No gross histopathological abnormalities were observed in the examined organs, including the ovaries, following administration of the recombinant protein and adjuvant.

Serum and Mucosal IgG Induction Following Immunization

Antigen specific IgG responses to the recombinant protein were quantified by ELISA. Increased serum IgG levels were observed in immunised mice following the third immunisation. Both serum and mucosal IgG responses were detected, however, serum IgG levels were higher compared to mucosal IgG levels (Fig. 6a, b).

Effect of Immunized Serum on In Vitro Fertilization Rates

Fertilization efficiency was evaluated based on the proportion of embryos reaching the two-cell stage following in vitro fertilization. As illustrated in Fig. 7, fertilization rates in the control groups and in groups supplemented with serum from non-immunized mice did not differ. In contrast, incubation with sera from immunized mice resulted in reduced fertilization rates, compared with control conditions, with the strongest inhibition observed in the presence of undiluted immunised serum (Table 2). These findings indicate that serum derived from immunized mice can inhibit fertilization.

Table 2
In vitro fertilization (IVF) results following incubation of oocytes with sera from immunised and non-immunised mice at different dilutions

Group	Initial number of healthy oocytes	Number of 2-cell embryos	Fertilization rate (%)
Control (no mouse serum, containing 10% FBS)	44	40	90.9%
10% control mouse non-immunised serum instead of FBS	54	44	81.5%
10% immunized mouse serum instead of FBS	96	0	0%

Discussion

Sperm antigens can induce infertility in both males and females. In many species, including humans, sperm-mediated immunogenesis has been reported to induce the production of anti-sperm antibodies, resulting in infertility. In a study conducted by Mortazavi et al. in 2021, the recombinant protein was used

to immunized male mice. The results shown that the recombinant protein was able to reduce the fertility rate in male mice up to 80% (18). This study evaluated the immunocontraceptive potential of a recombinant protein vaccine containing three epitopes derived from sperm surface proteins in female mice. After three rounds of immunization, mating assays demonstrated a 66.6% reduction in pregnancy rate among immunized females compared with controls, suggesting effective inhibition of fertility through humoral immune responses targeting sperm antigens (19). This finding is consistent with multi-epitope immunocontraceptive approaches in which combining species-specific sperm peptides has resulted comparable or improved contraceptive efficacy. For example, multi-antigen peptide vaccines incorporating sperm epitopes have been shown substantial fertility inhibition in mice by inducing antisperm antibodies that interfere with gamete interactions (20).

Comparable results have also been reported using recombinant fusion proteins containing sperm antigens along with zona pellucida glycoprotein-3 (ZP3) fragments and gonadotropin-releasing hormone (GnRH), achieving up to 90% contraception in female mice following dual booster doses (21). However, although such hybrid vaccine designs enhance contraceptive efficacy, vaccines targeting GnRH have been associated with systemic hormonal effects, including reduced levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and sex steroids, which can lead to reduced bone density, altered immune responsiveness, behavioral changes, and long-term metabolic disturbances (22–23).

Nevertheless, the 66.6% reduction observed in this study exceeds the efficacy reported in some earlier models, such as the 37.5% pregnancy success following testicular nuclear autoantigenic sperm protein (tNASP) immunization, which exhibited persistent infertility even after antibody declined (24). These findings highlight the vaccine's efficacy in hindering sperm function in vivo, similar to recombinant FA-1 (rFA-1) antigens that reversibly inhibited female fertility through sperm-specific immune responses (25). Epitope-based immunocontraceptive strategies therefore offer promising non-hormonal alternatives, though optimization for complete reversibility remains an important challenge, as indicated by variable recovery (about 71%) seen in Eppin-based vaccines in primates (25).

The use of selected protective epitopes derived from three sperm proteins identified in this study may provide an advantage over whole protein vaccines by reducing the risk of immune tolerance and off target immune responses (18).

Enhancement of mucosal immunity in addition to humoral responses is considered crucial for effective immunocontraceptive vaccination targeting sperm surface antigens. Local antibody production within the female reproductive tract, facilitates direct neutralization of sperm at the site of fertilisation. In this study, subcutaneous and intraperitoneal immunisation induced both systemic and mucosal antibody responses, with increased IgG and IgA levels detected in serum and vaginal washes. These immune responses were correlated with reduced fertility but were not accompanied by detectable adverse effects. This finding aligns with previous reports indicating that mucosal vaccination strategies can enhance genital tract antibody responses, particularly IgA production, compared with systemic immunization alone (26–27).

Histopathological examination further supported the safety profile of the vaccine, showing no pathological alterations in vital organs, including ovaries, following administration of the recombinant protein and adjuvant. Normal tissue morphology confirmed the absence of off-target inflammation and autoimmune reactions (28). These findings are consistent with other epitope-focused immunocontraceptive studies including vaccines based on urokinase type plasminogen activator and Izumo derived antigens, which demonstrated fertility inhibition without systemic toxicity (29–30). Similarly, CatSper derived vaccines have been reported to induce reversible contraceptive effects without histopathological damage (31).

In vitro fertilization (IVF) assays indicated a dose-dependent reduction in two-cell embryo formation in the presence of sera from immunized mice, while controls showed no inhibition. The antibody-mediated suppression of fertilization is likely due to interference with sperm-egg binding or acrosome reactions. Similar findings have been reported following Izumo1 peptide immunization, where antisera inhibited sperm-egg fusion and reduced fertility rates (32). Immunization with recombinant sperm DE protein has also shown to impair sperm motility and zona penetration in both sexes, confirming the role of antibody titers in IVF inhibition (33). In contrast, YLP12 peptide vaccines achieved reversible immunocontraception through local antibody mediated neutralization, suggesting that modulation of antibody levels may allow controlled contraceptive efficacy (34).

Conclusion

This recombinant multi-epitope vaccine demonstrates potential as a safe and effective immunocontraceptive approach in female mice, inducing both humoral and mucosal immune responses that substantially reduce fertility without detectable organ integrity. Although the observed efficacy (66.6%) is promising, future studies should aim on optimizing mucosal delivery strategies—such as Peyer’s patch targeting—and confirming full reversibility to support further translational development. With further refinement, epitope-based recombinant vaccines may offer a versatile, non-hormonal contraceptive option applicable to both animal models and potentially human use.

Declarations

Ethics approval and consent to participate

All procedures were approved by the institutional animal ethics committee and conducted in accordance with relevant guidelines and regulations (approval number: IR.NIGEB.EC.1404.9.29. E).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Rozhia Zangeneh: Investigation, Data Curation,Mojtaba Dashtizad: Methodology, Formal analysisSeyed Ali Ghorashi: Visualization, review and editing the publicationGhazaleh Zandi: Investigation, Writing original draftFarid Heidari: Supervision, Methodology, Project administration, Funding acquisition.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

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Figures

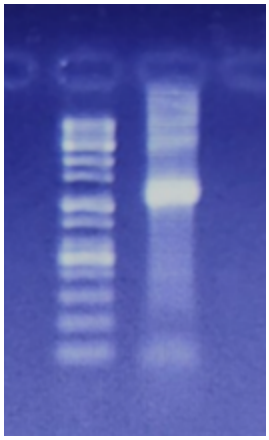


Figure 1

Agarose gel electrophoresis of the PCR amplicon corresponding to the recombinant fusion gene IZUMO1–PH20–SACA3. The expected amplicon size was approximately 1160 bp.

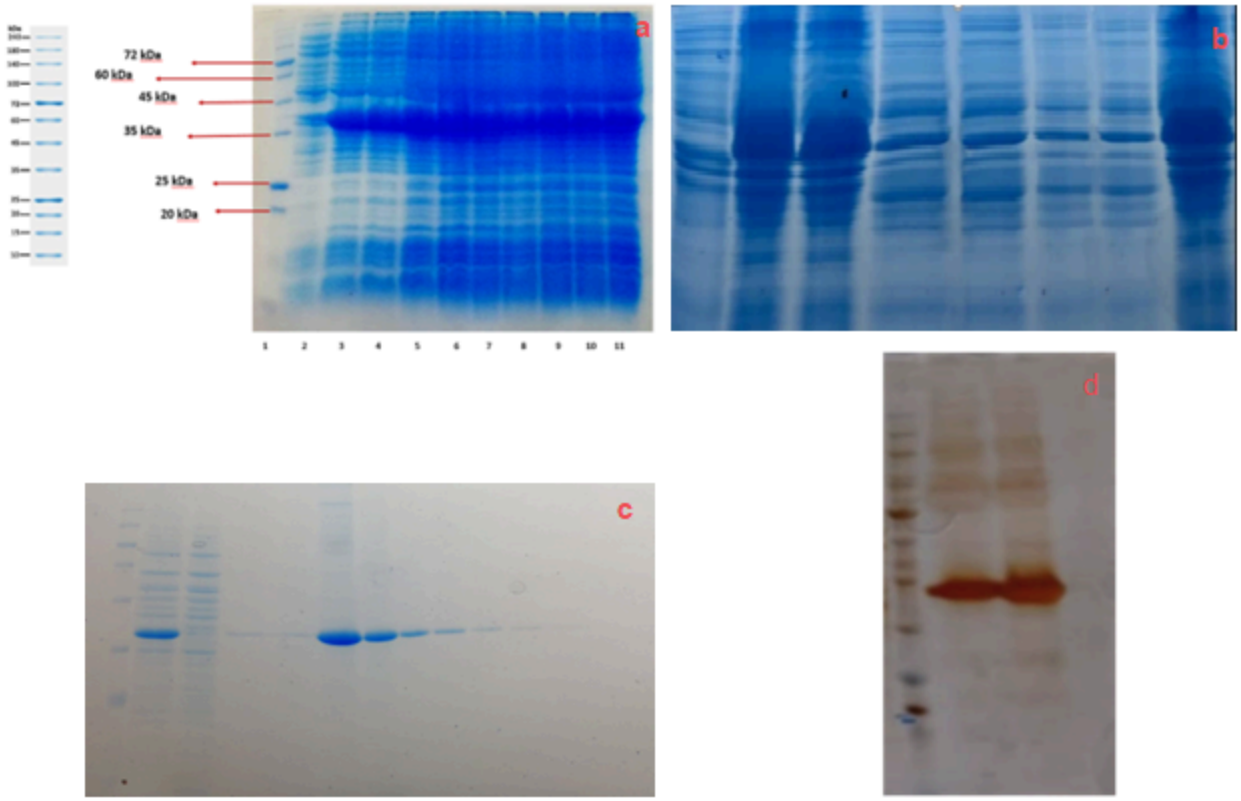


Figure 2

Expression, solubilisation, and purification of the recombinant IZUMO1–PH20–SACA3 fusion protein.

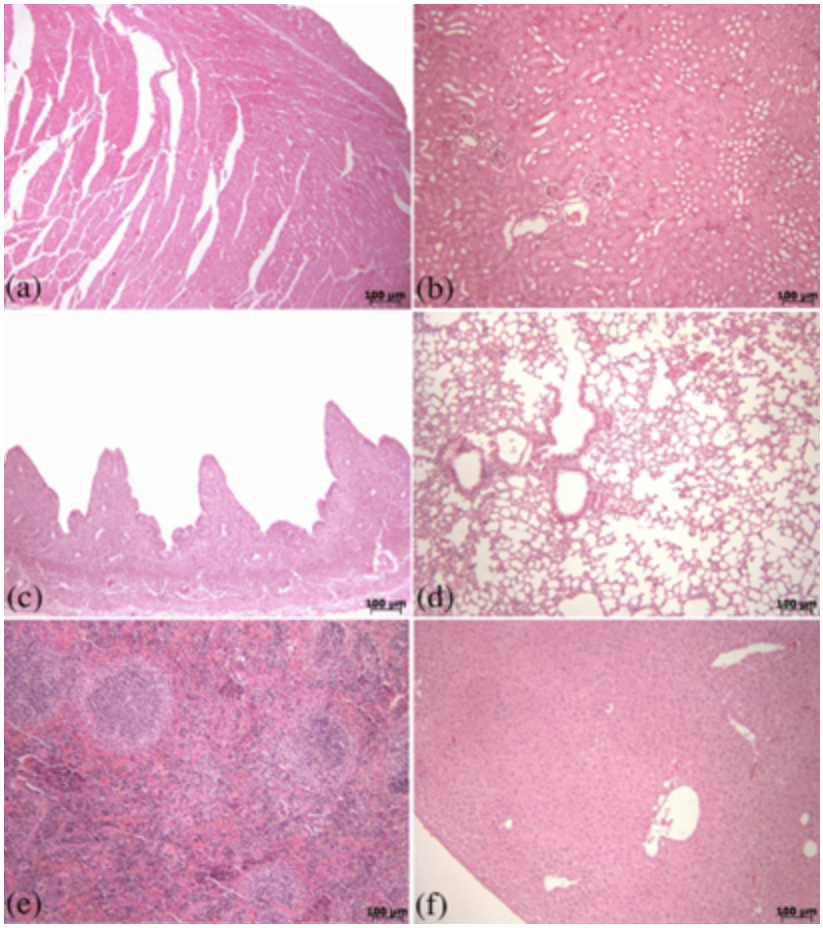


Figure 3

Representative histopathological sections from control female mice stained with H&E (a: heart, b: kidney, c: small intestine, d: lung, e: spleen, f: liver) (it would be good if you can add scale bar)

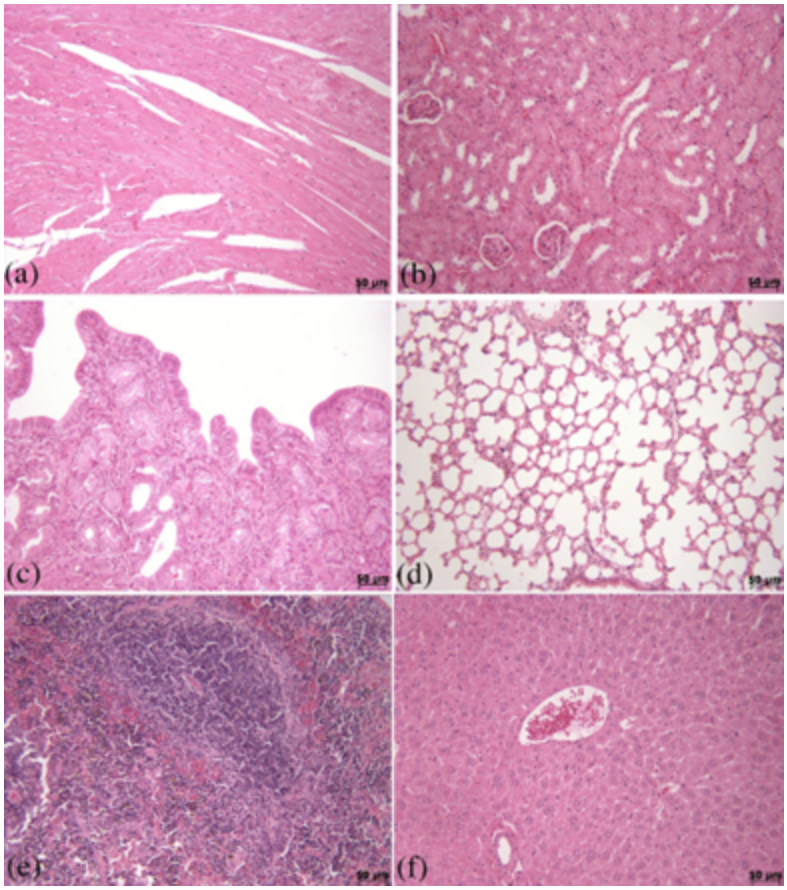


Figure 4

Representative histopathological sectionsfrom immunized female mice in stained with H&E (a: heart, b: kidney, c: small intestine, d: lung, e: spleen, f: liver)

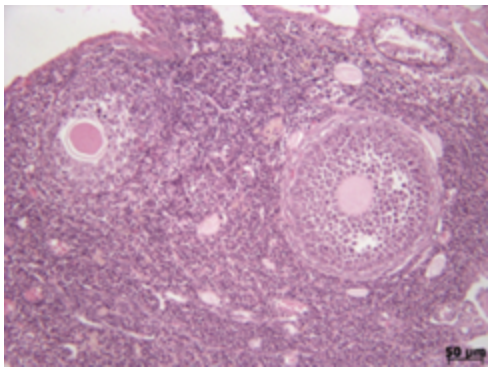


Figure 5

Histopathological results of the ovarian tissue in the treatment group mice (stained with hematoxylin and eosin).

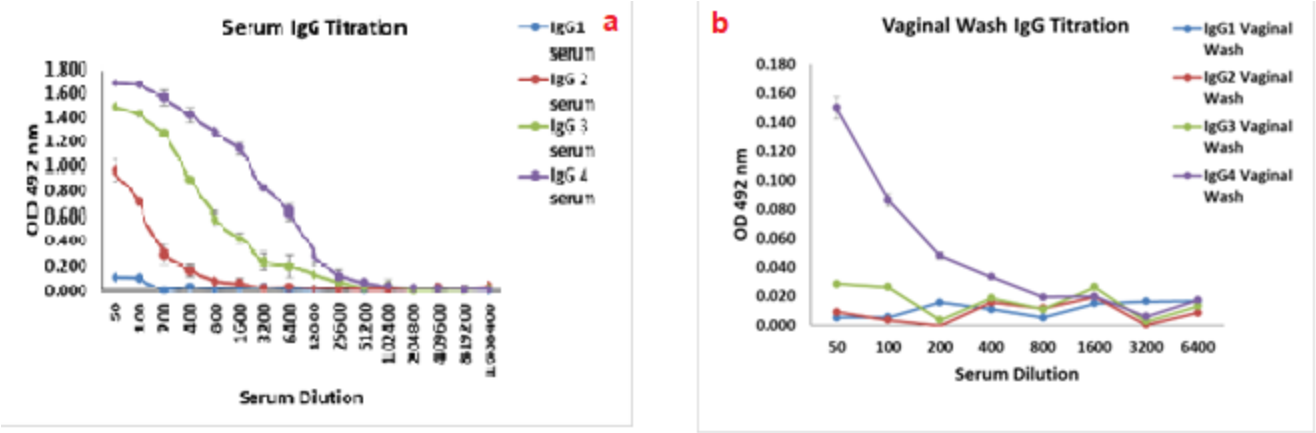


Figure 6

Antigen specific IgG antibody titres measured by ELISA in serum (a) and vaginal wash samples (b) from immunised female mice following vaccination.

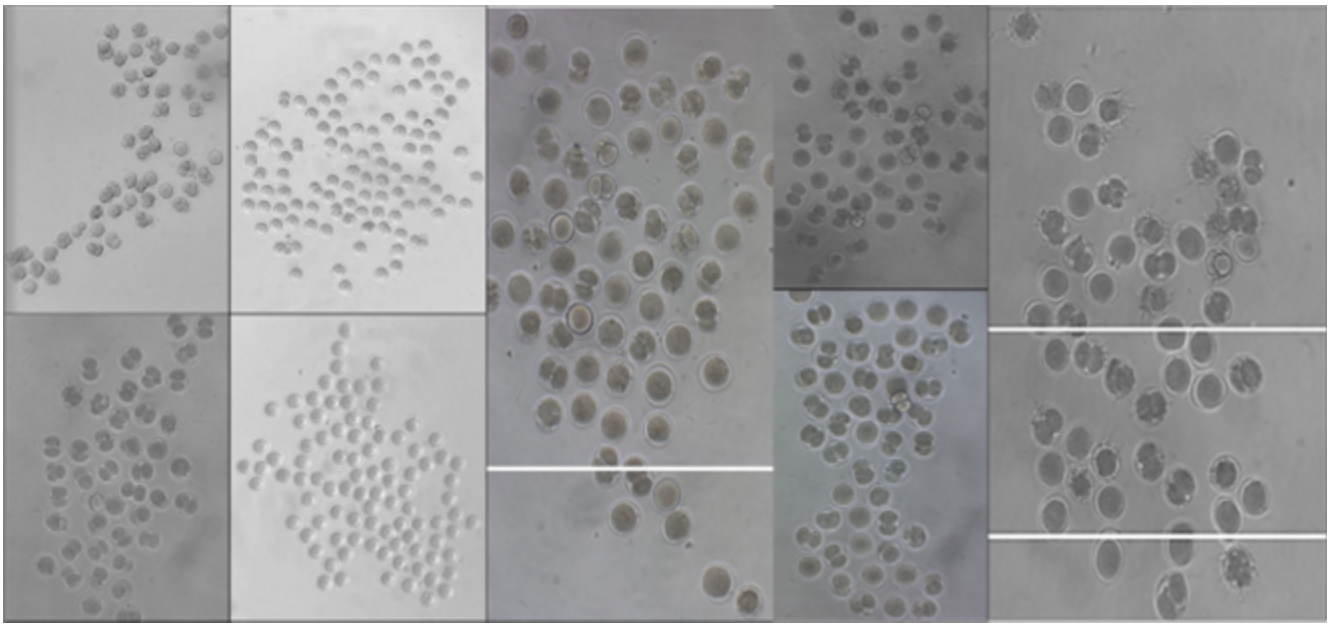


Figure 7

Representative microscopic images of oocytes following in vitro fertilisation under different serum conditions. (a) Control group (medium containing 10% FBS), (b) medium supplemented with 10% serum from non-immunised mice, (c) medium supplemented with 10% serum from immunised mice, (d) immunised mouse serum diluted 1:50, (e) 1:100, (f) 1:200, (g) 1:400, and (h) 1:1600.