Technology Report

Silkworm recombinant bovine zona pellucida protein 4 (bZP4) as a potential female immunocontraceptive antigen; impaired sperm-oocyte interaction and ovarian dysfunction

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Abstract. Porcine zona pellucida proteins (ZPs) have been utilized as female immunocontraceptive antigens. The purpose of this study was to explore the potential use of silkworm recombinant bovine ZP4 as an alternative. When the protein was injected with monophosphoryl lipid A (MPL) – an immuno-stimulative agent – into two female goats, marked elevation of the anti-ZP4 titer was detected. Application of the purified specific IgG to a porcine *in vitro* fertilization system reduced the sperm penetration rate. In one goat, the cyclic profile of serum progesterone disappeared as the anti-ZP4 titer increased. Histological examination of the ovaries revealed degeneration of antral follicles with sparse infiltration of inflammatory cells in the theca, indicating that autoimmune oophoritis had been induced. Together, the present results suggest that recombinant ZP4 disturbs fertilization and exerts a pathogenic effect on follicle development in goats, thus indicating its potential as a female immunocontraceptive antigen.
Key words: Bovine ZP4, Goat, Immunogen, Monophosphoryl lipid A (MPL), Silkworm recombinant protein

Immunocontraception is a non-killing, humane method for suppressing the reproduction of overabundant feral and native animals, and thus reducing their populations [1]. Vaccination with native porcine zona pellucida protein (pZP) has been used for fertility control in various wild and zoo animals, including deer species, horses, elephants, and gray seals [2]. As the antigen is specific to the ovary, the vaccine is considered to affect reproduction, but not animal health. A single shot of the native antigen combined with AdjuVac, a mycobacterium-based adjuvant [3], has been reported to achieve multiyear contraceptive effectiveness in white-tailed deer [4], suggesting the practicality of the vaccine for control of wildlife populations. In mammals, the zona pellucida forms a capsule around oocytes and mediates several critical aspects of fertilization, such as sperm recognition, prevention of polyspermy, and mechanical protection of ovulated oocytes and embryos until implantation. The zona pellucida contains several ovary-specific glycoproteins – ZP1, ZP2, ZP3 and ZP4 – and that in pigs and cattle comprises ZP2, ZP3 and ZP4, also referred to as ZPA, ZPC and ZPB, respectively [5]. In contrast, the mouse zona pellucida contains ZP1, ZP2 and ZP3 [6]. ZP2 and ZP3 are common to all mammals, but ZP1 and ZP4 have been identified as the products of different genes [7]. In our preliminary study, we found that extracts from the bovine zona pellucida also had the same immunocontraceptive antigenicity in goats. However, these native proteins are not suitable for large-scale application due to the limited amounts available and concerns about the inadvertent spread of infectious diseases.

Recently, the immunocontraceptive efficacy of recombinant pZPs has been investigated in female equines [8–10]. The antigen consisted of two chimeric fusion proteins expressed in bacteria: porcine ZP3 fused with the T-cell epitope of tetanus toxoid and porcine ZP4 fused with the T-cell epitope of bovine RNase. Multiple injections of the antigen with modified Freund's adjuvants rendered females infertile and elicited an antibody titer against the native proteins [8, 10]. Since those studies shed light on the availability of recombinant proteins for immunocontraception, we tried to produce ZPs using a silkworm protein expression system. Fragments of bovine *ZP3* or *ZP4* DNA corresponding to amino acid sequences 23-346 (GenBank NP_776399) and 18-461 (NP_776400), respectively, were introduced into the silkworm genome. The resulting level of the bovine ZP4 (bZP4) expression was intense in the middle silk gland, but not in cocoons. On the other hand, the level of the ZP3 expression was very faint in both the silk gland and cocoons (data not shown). We obtained 0.4 mg of the purified bZP4 per head (Supplementary Fig. 1). The antigenicity of bZP4 was assessed in two female Shiba goats (Nos. 506 and 509) by injection of 2 mg of the protein with a water-in-oil adjuvant supplemented with an immuno-stimulative material, monophosphoryl lipid A (MPL). Two weeks after injection, a sharp increase of the serum anti-bZP4 titer was detected in both animals, and this reached a plateau at 4 weeks (Fig. 1a). This rapid immunoreaction may have been introduced, at least in part, by the stimulative effect of MPL, a detoxified form of lipopolysaccharide (LPS, [11]). Recently we have reported the effectiveness of LPS on the humoral immune response in rats [12]. Several species, including

humans, rabbits, and ruminants, are highly sensitive to LPS [13]. To our knowledge, this is the first study to have investigated MPL as an adjuvant for immunocontraception. The application of MPL as a vaccine adjuvant in the field of veterinary medicine is expected in the future.

The immunocontraceptive mechanism of ZP injection is thought to include disturbance of fertilization by the resulting antibody, which would target the surface of the zona pellucida and/or sperm in the female reproductive tract, thus interfering with the association between the oocyte and the sperm. Instead of subjecting the treated females to a mating test, we used a porcine *in vitro* fertilization (IVF) system to investigate whether the anti-ZP4 antibody disturbed fertilization. The amino acid sequence of bZP4 (19-461) shows 78.9% identity to pZP4. First, we applied both pre-immune and immune serum to the IVF system, but this made the oocytes adhere to the bottom of the dish, resulting in complete inhibition of penetration in all of the groups. The reason for this was unclear, but we thought that some serum components might have caused the adhesion. Therefore, we purified anti-ZP4-specific IgG from the immune serum of the females (Fig. 1b), then added it to the fertilization medium at a final concentration of 50 µg/ml. Both of the specific IgGs reduced the sperm penetration

rate in comparison with control IgG obtained from pre-immune serum (Table 1). The heterocomplex of ZP3 and ZP4, but not the free proteins, has been shown to have sperm-binding activity in both pigs and cattle [14, 15]. The present results indicated that the antibody against ZP4 alone could interfere with sperm-egg association, in accordance with the reported inhibitory effect of antibodies against pZP4 on sperm-oocyte binding in bovine IVF ([16], referred to as ZP3α in the report). Therefore, we speculated that immunization with the ZP4 antigen might interfere with fertilization in females.

The serum progesterone level in goat No. 509 showed a cyclic profile for 5 weeks after the ZP4 injection, and thereafter became undetectable (Fig. 1c). At the study endpoint (12 weeks after injection), no corpus luteum was evident macroscopically in the ovaries. In sagittal sections of the ovaries (Fig. 2a), several antral follicles, accompanied by sparse lymphocyte infiltration in the theca interna, were evident, but there were no significant changes in the granulosa cell layer (Fig. 2c and d, arrows). Weak eosinophilic components, probably derived from degenerated thecal cells, were located near the granuloma cell layer in the theca interna (Fig. 2d, asterisk). Follicles that showed obvious signs of atresia, including shedding of granulosa cells into the lumen, had the eosinophilic component
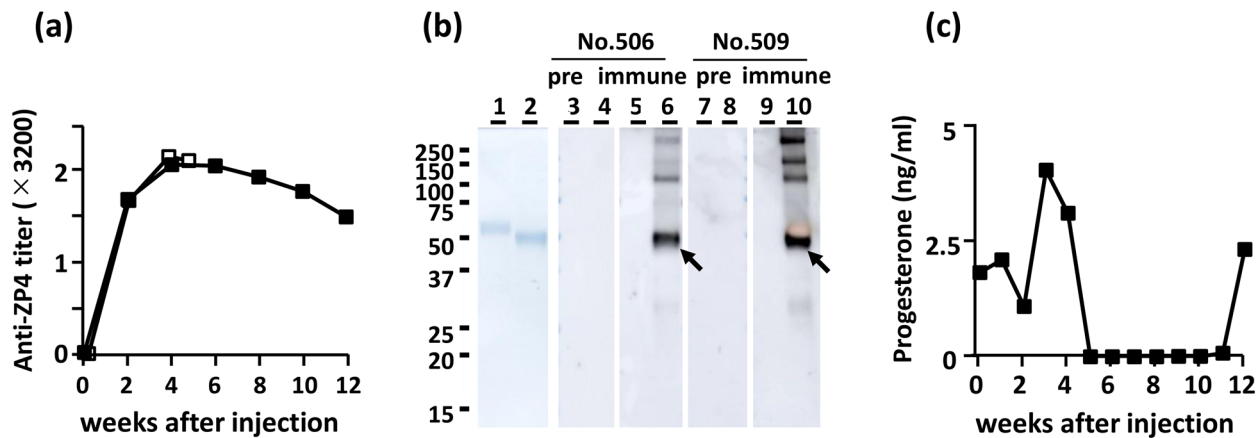

Fig. 1. Immunization of female goats with recombinant bovine ZP4. Individual data for the serum anti-ZP4 titer (a). The values are shown as OD 415 nm at 1:3,200 dilution. An injection of 2 mg of recombinant ZP4 with MPL, an immune stimulative material, was given to two female Shiba goats. Since one of the two (No. 506; □) became ill, the experiment was stopped 6 weeks after injection. In the other animal (No. 509; ■), the experiment was completed at 12 weeks after injection. Immunoblotting with IgG purified from serum indicated on the top (b). Lanes 5–6 and lanes 9–10 were reacted with IgG from serum collected 5 weeks after injection. ZP4 (even lanes) and BSA (odd lanes) were loaded. No positive band is seen in lanes 3–5 and 7–9. In lanes 6 and 10, the bands corresponding to the ZP4 are seen (arrows). The other bands (> 100 kDa) indicate silkworm proteins that had contaminated in the purified ZP4 used for both immunization and immunoblotting (see Supplementary Fig. 1). The volumes per lane were 400 ng (lanes 1 and 2. Stained with Coomassie dye) and 4 ng (lanes 3–10). Serum progesterone profiles of goat No.509 (c).

Table 1. Inhibition of *in vitro* fertilization by bZP4-specific IgG

Goat No.	IgG added	No. of assays	No. of oocytes (total)	Matured [1] (%)	Penetrated [2] (%)	Penetrated range (%)
–	–	3	143	67.1 ± 10.3 [a]	88.5 ± 7.8 [a]	82.5–100
506	pre-immune	3	142	64.8 ± 20.8 [a]	82.6 ± 12.3 [ab]	66.7–96.7
509	pre-immune	3	142	71.1 ± 10.3 [a]	79.2 ± 12.9 [ab]	64.5–96.2
506	immune	3	135	68.9 ± 15.6 [a]	29.0 ± 21.3 [bc]	4.0–56.0
509	immune	3	136	72.1 ± 15.3 [a]	40.8 ± 16.0 [c]	22.7–61.3

In each group, about 50 oocytes (10 oocytes per 100 µl drop) were processed for maturation then subjected to *in vitro* fertilization. All the oocytes were fixed and stained with aceto-orcein then examined by phase-contrast microscopy.
[1] Maturation was defined when oocytes were at the MII stage or when sperm(s) was detected in the cytoplasm.
[2] No. of oocytes with sperm(s)/no. of matured oocytes. Data are presented as mean ± SD. Different superscripts denote significant difference ($P < 0.05$) by Duncan's multiple range test.
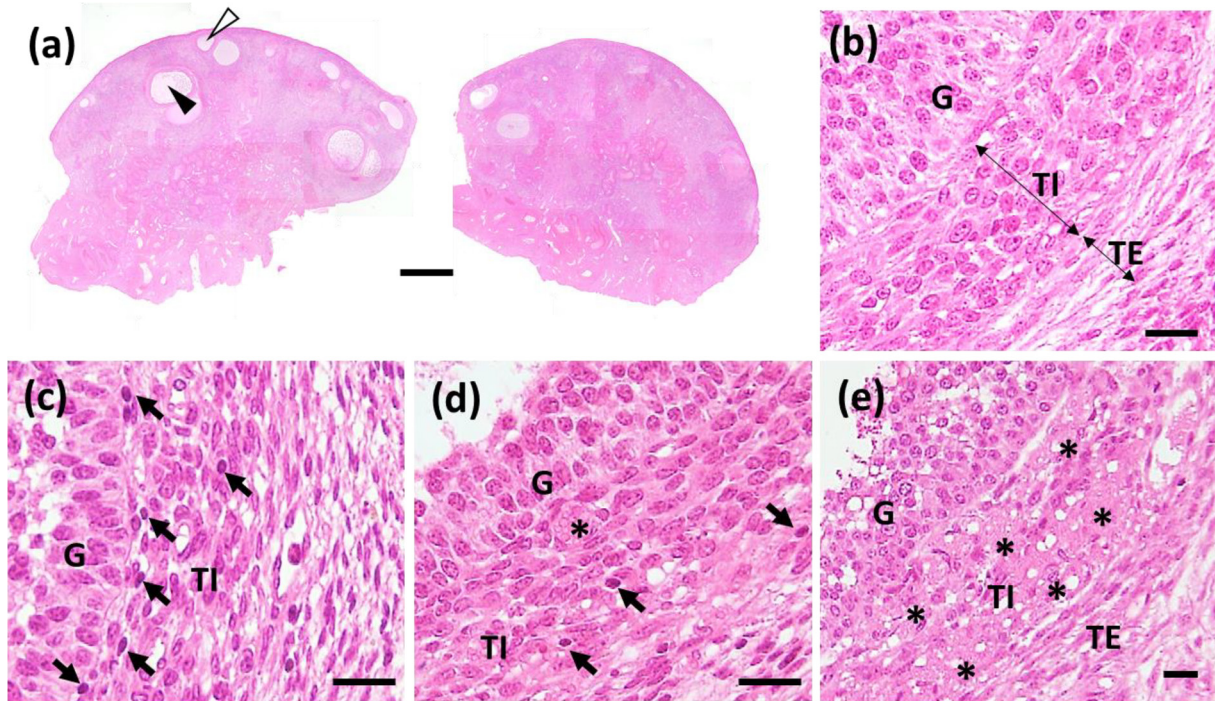
Fig. 2. Ovarian histology of the goat immunized with bZP4. Ovaries of goat No.509 obtained at 12 weeks after immunization. Hematoxylin and eosin staining. Sagittal sections (a). Several antral follicles are evident. None of these follicles were healthy. Parts of the follicle indicated with clear arrowhead are shown (c and d). Sparse infiltration of lymphocytes (arrows) is evident in the theca interna. Eosinophilic materials (asterisk, probably derived from degenerated thecal cells) are present near the granulosa cell layer. No marked change is evident in the granulosa cell layer of this follicle. Part of the follicle indicated with arrowhead is shown (e). Shedding of the granulosa cells is evident. The theca interna was occupied by eosinophilic material (asterisks). Normal antral follicle for reference (b). G: granulosa cell layer; TI: theca interna; TE: theca externa. Bars: 2 mm (a) and 20 μm (b–e).

that had completely spread to the theca interna (Fig. 2e, asterisks). No marked change was detected in primary and pre-antral follicles (data not shown). These histological characteristics suggested the induction of autoimmune oophoritis in the treated goat. Reduced ovarian endocrine function accompanying inflammatory lesions has been reported after immunization with native pZP in rabbits and sheep [17, 18], and with synthetic ZP peptides in monkeys [19]. In mice inoculated with a recombinant virus expressing mouse *Zp3*, the resulting IgG affected zona pellucida formation, and consequently disrupted follicle development [20–22]. Joonè *et al.* [23] have indicated that the protocol of vaccination (i.e. the amount of antigen, the frequency of booster injection, and the combination of adjuvants), species specificity and individual differences in immunoreaction can contribute to disease incidence. They suggested that the suppressive effect on ovarian function could be an inherent feature of ZP-based immunocontraception. This would be in agreement with the present results. Notably, elevation of the serum progesterone concentration was detected at the last point of the experiment, i.e. 12 weeks after injection. This might reflect the presence of functional corpora lutea in the ovaries, although no corpora lutea were evident macroscopically. Reversibility of ovarian dysfunction long after pZP treatment has been reported in pony mares [8]. Further study is necessary to clarify the recoverability of ovarian cyclicity after ZP4 immunization.

The present study has demonstrated the feasibility of silkworm recombinant bZP4 as a female immunocontraceptive antigen. The potential advantage of this recombinant protein over vaccines using native antigen would include production of a larger amount and risk of disease transmission. On the other hand, it has been reported that *Escherichia coli* recombinant pZPs have a relatively weaker effect on female fertility than the native antigen [8–10]. Although glycosylation of the expression system of insects differs from that in mammals, such post-translational modification might be advantageous for the silkworm expression system relative to the bacterial one, in terms of vaccine antigen production.

Methods

Antigen preparation

The bZP4 was produced using the transgenic silkworm technique [24]. The protein was expressed largely in the middle silk gland and little in the cocoon. The protein in the gland was extracted in PBS containing 1% Triton X-100, then purified by metal-chelation column chromatography using a His-tagged protein purification kit (TALON®; Clontech Laboratories, Mountain View, CA, USA), in accordance with the manufacturer's instructions. The eluate was dialyzed in PBS then concentrated using Amicon Ultra-15 (Merck, Darmstadt, Germany). The protein concentration was measured using a Coomassie® protein assay kit (Pierce Biotechnology, Rockford, IL, USA), then stored at –80°C until use.

Animal experiment and tissue specimen

The animal experiment procedure was undertaken in strict accordance with the recommendations in 'Standards Relating to the Care and Keeping and Reducing Pain of Laboratory Animals' (Ministry of Environment, Japan). Animal experiments were approved by the Institute of Livestock and Grassland Science, NARO Animal Care and Experiment Committee (No.20C002NILGS). The antigen (2 mg of bZP4 in 1 ml PBS) was emulsified with a same amount of

ISA 61VG (a gift from Seppic, Paris, France) supplemented with 0.2 mg of MPL (HC-4058; Hycult Biotech, Wayne, PA, USA), then injected into the rump region of female Shiba goats ($n = 2$, animal Nos. 506 and 509, 7 years old). Blood samples were collected from the jugular vein immediately before, and after the injection every 7 days. Since goat No. 506 became ill 6 weeks after the injection, presumably due to a sudden drop in the ambient temperature in December 2020, the experiment was discontinued at this point. The other goat (No. 509) was ovariectomized under anesthesia 12 weeks after the injection. The ovaries were fixed in Bouin's solution, then embedded in paraffin. Sections 4 µm thick were stained with hematoxylin and eosin for morphological examination.

Preparation of anti-ZP4 IgG

The antigen-specific IgG was purified by affinity chromatography using a NHS-activated Sepharose column (17071601; Cytiva, Marlborough, MA, USA) immobilized with bZP4 in accordance with the manufacturer's instructions. The immune serum (2 ml) was mixed with an equal volume of saturated ammonium sulfate solution (4.32 M), placed on ice for 1 h, and then centrifuged at $2,000 \times g$ for 30 min at 4°C. The precipitate was redissolved in PBS containing 0.1% Triton X-100 (PBS-T), then applied to the above-mentioned affinity column and incubated for 1 h. The resin was washed with PBS-T, then 5 ml of 0.1 M glycine, pH 2.5, was added to elute the bound IgG. The eluate was dialyzed and concentrated as mentioned above. From 2 ml of the immune serum of goats Nos.506 and 509, about 0.6 and 0.8 mg of the specific IgG was obtained, respectively. For the control IgG, pre-immune serum from each animal was subjected to ammonium sulfate precipitation, and then the redissolved precipitate was reacted with protein G Sepharose (1706180; Cytiva) for 1 h at room temperature. The ligated IgG was recovered and processed as described above.

Immunoblotting

The purified ZP4 and BSA (supplied with the Coomassie® protein assay kit) were subjected to SDS-PAGE under reducing conditions, followed by transfer of the proteins to Immobilon-P PVDF membranes (Merck). The membranes were incubated in 5% BSA-PBS supplemented with 0.05% Tween 20 for 1 h, then reacted with the IgGs described above (100 ng/ml). The secondary antibody used was donkey anti-goat IgG-HRP (A16104; Thermo Fisher Scientific, Waltham, MA, USA). The resulting immunoreactions were detected by enhanced chemiluminescence using ECL prime Western Blotting Detection Reagent (GE Healthcare Japan, Tokyo, Japan). Image acquisition was performed using an image analyzer (ImageQuant LAS 500, GE Healthcare Japan).

Sperm penetration assay

The *in vitro*-matured porcine oocytes were prepared as described previously [25]. Briefly, cumulus-oocyte complexes were collected from ovaries obtained at a local slaughterhouse, then cultured in maturation medium (North Carolina State University (NCSU)-37 solution [26] modified with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, and 50 mM β-mercaptoethanol). For the first 20–22 h of culture, 1 mM dibutyl cAMP, 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) and 10 IU/ml hCG (Gonatropin; ASKA Pharmaceutical) were added. The maturation culture was carried out at 39°C under 5% CO_2, 5% O_2 and 90% N_2. After 44–46 h of culture, the oocytes were freed from cumulus cells after temporary treatment with 0.1% (w/v) hyaluronidase. Then, after gentle pipetting, approximately 10 oocytes were transferred

to 100 µl of the fertilization medium containing frozen-thawed Meishan boar epididymal sperm [27] and the bZP4-specific IgG (2×10^4 sperm and 50 µg IgG per ml at final concentration, respectively). The concentration of the IgG was decided by reference to the previous report by Topper *et al*. [16]. The medium used for IVF was a modified Pig-FM medium [28] containing 10 mM HEPES, 5 mM caffeine (Sigma-Aldrich, St. Louis, MO, USA) and 5 mg/ml BSA (Fraction V; Sigma-Aldrich). The bZP4-specific antibody was added to the medium, then stored at 4°C overnight. In order to remove suspended matter, the medium was centrifuged at $170,000 \times g$ for 20 min at 20°C and the supernatant was used for *in vitro* fertilization. After insemination for 3 h, the oocytes were collected and washed to remove remaining cumulus cells and spermatozoa. The oocytes were then transferred to the *in vitro* culture (IVC) medium (IVC-PyrLac, [25]) and cultured for 7 h at 38.5°C under 5% O_2. They were then fixed with acetic alcohol (1:3), stained with 1% aceto-orcein (Sigma-Aldrich), and examined for sperm penetration under a phase-contrast microscope.

Anti-bZP4 titer

ELISA plates (467466, Thermo Fisher Scientific) were coated with bZP4 (1 µg/100 µl/well) overnight then blocked with 2% BSA in PBS for 2 h. After washing three times with PBS containing 0.05% Tween-20 (PBS-0.05T), 100 µl of the serum diluted 1:3,200 with PBS-0.05T was added and incubated for 1.5 h. After washing, 100 µl of biotinylated protein G (29988; Pierce Biotechnology) diluted with PBS-0.05T (1:1,000) and avidin labeled with HRP (A-2014; Vector Laboratories, Inc., Burlingame, CA, USA) diluted with PBS-0.05T (1:2,000) were sequentially reacted for 1.5 h. TMB substrate solution (100 µl/well; SeracareLife Sciences, Milford, MA, USA) was added as the chromogen, and the reaction was stopped with 1 M H_3PO_4. Absorbance at 415 nm was measured using an iMark Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Progesterone assay

Progesterone was extracted from 0.5 ml of each serum with 2.5 ml of hexane (Fujifilm Wako, Osaka, Japan), then redissolved in 0.5 ml PBS containing 1% BSA. The concentration of progesterone was measured using a progesterone EIA assay kit (a gift from Dr. K. Kimura, Okayama University). The assay range was 0.08–10 ng/ml and the intra-assay coefficient of variation was 3.8%. All of the samples were measured in the same assay.

Statistical analysis

Data on the proportions of oocytes (as percentages) that matured and were penetrated by sperm were arcsine-transformed before analysis, then subjected to one-way ANOVA using SAS® add-in 7.1 for Microsoft Office. The significance of differences among the groups was determined by Duncan's test ($P < 0.05$).

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