Animal Reproduction Science 120 (2010) 151–157

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

ZP-binding peptides identified via phage display stimulate production of sperm antibodies in dogs

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ARTICLE INFO

Article history:
Received 29 January 2010
Received in revised form 22 March 2010
Accepted 6 April 2010
Available online 13 April 2010

Keywords:
Dogs
Phage display
Canine sperm antibody
Zona pellucida
Fertilization
Contraception

ABSTRACT

Zona pellucida (ZP) glycoproteins play a central role in sperm–oocyte binding and fertilization. Sperm protein sequences that are involved in sperm-ZP recognition and have an important role in fertilization represent attractive targets for development of contraceptive vaccines, yet are currently unknown. To identify peptide sequences that recognize and bind to ZP proteins, we developed a novel selection procedure from phage display libraries that utilizes intact oocytes surrounded by ZP proteins. The major advantage of this procedure is that ZP proteins remain in their native conformation unlike a selection protocol previously published that utilized solubilized ZP on artificial solid support. Several peptides of 7 and 12 amino acids with binding specificity to canine ZP proteins were identified. Four of them (LNSFLRS, SSWYRGA, YLPIYTIPSMVY, and NNQSPILKLSIH) plus a control ZP-binding peptide (YLPVGGLRRIGG) from the literature were synthesized and tested for antigenic properties in dogs. NNQSPILKLSIH peptide stimulated production of anti-peptide antibodies. These antibodies bind to the acrosomal region of the canine sperm cell, demonstrating ability to act as sperm antibodies. The identified ZP-binding peptides (mimicking sperm cell surface antigens) may be useful in the design of immunocontraceptive agents for dogs.

1. Introduction

The zona pellucida is a protective glycoprotein membrane surrounding the oocyte in all mammalian species. A sperm cell must bind to the ZP to fertilize the oocyte. Such binding is recognized as a receptor–ligand interaction; however, the exact epitopes participating in sperm-ZP binding leading to fertilization remain unknown (Chamley and Clarke, 2007; Suri, 2005a). To identify sequences of peptides that bind to ZP, Naz et al. (2000) applied a diverse library of random peptides presented in the format of a biological display to ZP proteins that had been removed from human oocytes and immobilized to a matrix. One of the selected peptides appeared to bind to a sperm–egg interactive site and, when used as a synthetic antigen, stimulated production of sperm antibodies that reduced fertility in mice (Naz and Chauhan, 2002).

To identify sperm antigens that bind to ZP and stimulate production of sperm antibodies, we have developed a novel procedure that identifies peptides via selection from phage display peptide libraries on intact oocytes surrounded by ZP proteins. The major advantage of this procedure is that the ZP proteins remain on oocytes in their native conformation, unlike the previously published selection protocol (Naz et al., 2000) that used solubilized (possibly denatured) ZPs immobilized on an artificial solid support. Using the selection procedure on canine oocytes, we found several ZP-binding peptides and characterized

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doi:10.1016/j.anireprosci.2010.04.002

them as to their ability to stimulate the production of sperm antibodies in dogs. Antibodies generated by this protocol have the potential to reduce fertility in dogs and, possibly, in other animals. The ultimate goal is to develop peptide-based immunocontraceptives to humanely control feral/unowned animal populations.

2. Materials and methods

2.1. Ova and semen collection and evaluation

All protocols involving live animals were approved prior to use by the Auburn University Institutional Animal Care and Use Committee. Ovaries were obtained from female dogs (6 months of age or older) undergoing elective ovariohysterectomies at a veterinary clinic. Ovaries were frozen at −20 °C until required for experiments. To isolate oocytes, ovaries were thawed, sliced thinly and then washed extensively with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). The released oocytes were then retrieved by visual inspection at 25× magnification. Upon microscopic evaluation, selected oocytes had intact ZP and did not have cumulus cells attached to the surface, making zona sites available for binding with phage in phage selection procedures. Oocytes were washed two additional times, pooled and frozen at −20 °C in PBS, 1% BSA before further use.

Semen samples were obtained by digital stimulation from healthy adult male mixed breed laboratory dogs. Semen quality was estimated based on sperm concentration, sperm motility, and assessment of morphological sperm alterations. Ejaculates with sperm motility >70% and no more than 10% of abnormal spermatozoa were used for sperm ELISA and immunocytochemical localization of sperm antibodies.

2.2. Selection of ZP-binding peptides from phage display libraries

Peptides that specifically bind to canine ZP proteins were identified using two phage display libraries purchased from New England BioLabs (Beverly, MA, USA), including PhD-C7C Peptide 7-mer Library Kit and PhD-12 Peptide Library Kit. Four rounds of selection were performed using each library. To remove phage clones that bind to the plastic material of test tubes, an aliquot (10^{11} pfu) of the primary library was diluted in 1 mL of blocking buffer (PBS, 0.1% BSA) and added to an empty test tube for 1 h incubation at room temperature prior to incubation with oocytes. After that, the buffer containing the library was transferred to a tube containing 1000 intact canine oocytes (each surrounded by ZP) and incubated with gentle agitation for an additional 1 h at room temperature. Following incubation, phages expressing peptides not bound to oocytes were washed away with a washing buffer (PBS, 0.1% BSA, 0.1% Tween-20) using centrifugation (5900 × *g*, 30 s). The bound phages were recovered with a lysis buffer (2% sodium deoxycholate, 2 mM EDTA, 10 mM Tris–HCl, pH 8.0), amplified in bacteria, purified, and used for the next selection round. Following four selection rounds, phage DNAs were isolated and sequenced. Translation of foreign oligonucleotide inserts in phage DNA provided sequences of the peptides that bound to ZP. All general methods of handling phage, including phage propagation, purification, and isolation of phage DNA are described in detail in the New England BioLabs manuals provided with the library kits.

2.3. Peptides

Five different peptides were synthesized: two of the peptides were cyclic 7-mers identified in our study, another two peptides were linear 12-mers from our study, and one ZP-binding peptide sequence (12 amino acids long) was taken from the literature (Naz et al., 2000) to serve as a putative positive control. The peptides were conjugated to keyhole-limpet hemocyanin (KLH), a carrier protein, to assure maximum immunogenicity. The sequences were rationally modified as follows. A cysteine amino acid was added to all sequences to allow conjugation to KLH. A spacer of two glycines was inserted between the peptides and the carrier protein. A lysine amino acid was added to both 7-mer sequences to increase their solubility in water. Additionally, unconjugated peptides of the same sequences were synthesized for use in ELISA experiments. The peptides were synthesized and conjugated to KLH by Global Peptide Services (Fort Collins, CO, USA) at a yield of 10–20 mg having >85% purity. Table 1 shows the peptide sequences identified as well as the sequences of synthetic peptides that were used to immunize dogs.

2.4. Immunization of dogs with ZP-binding peptides

For vaccinations, each antigen was formulated to include: 250 μg of KLH-conjugated peptide mixed at 1:1 ratio with aluminum hydroxide adjuvant (Alhydrogel “85”, Brenntag Biosector, Frederikssund, Denmark) and 100 μg immunostimulatory oligodeoxynucleotide (CpG, 5′-TCG TCG TTG TCG TTT TGT CGT T-3′) (Wernette et al., 2002) synthesized by Integrated DNA Technologies (Coralville, IA, USA). The five synthetic peptides were injected intramuscularly into one to three years old female dogs. Booster immunizations were given at 3 wk and again at 7 wk following initial immunization to achieve high titer antisera. Each immunized dog served as its own control since none were expected to have preexisting antibodies to the selected peptides. Immunizations did not result in any adverse reactions in any of the dogs.

2.5. ELISA detection of anti-peptide and anti-sperm antibodies

Serum samples were collected from all dogs prior to immunization and biweekly or monthly thereafter during a 12-month period. The samples were tested for the presence of anti-peptide and sperm antibodies in ELISA format. For detection of anti-peptide antibodies, synthetic peptides were diluted (10 μg/mL) in 50 mM carbonate/bicarbonate buffer. The diluted peptide antigens were added to 96-well plates (100 μL/well) for overnight incubation at 4 °C. After incubation, the peptide solutions were removed from the plates and 175 μL of 1% BSA in PBS were added to the wells