An attempt to induce antibody production for immunocontraception in the Hokkaido sika deer (Cervus nippon yesoensis) by immunization with a porcine zona pellucida synthetic peptide

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Short communication

An attempt to induce antibody production for immunocontraception in the Hokkaido sika deer (*Cervus nippon yesoensis*) by immunization with a porcine zona pellucida synthetic peptide

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Conflicts between humans and wildlife are becoming a major issue throughout the world. In Japan, overabundance of the Hokkaido sika deer (*Cervus nippon yesoensis*) causes many problems, including agricultural, forestry, and ecological damage (Kumagai and Onoyama 1988; Akashi and Nakashizuka 1999; Nomiya et al. 2003; Kanda et al. 2005). The Japanese government has implemented deer management plans focused on shooting (Matsuda et al. 1999), but the efficiency of this method is inadequate and the problem remains serious. The development of new methods for deer population control is imperative (Hokkaido Government 2012).

One of the reasons for overabundance of the Hokkaido sika deer is their high reproductive rate. With good nutrition, the pregnancy rate is more than 90% in females which is over two years of age (Suzuki and Ohtaishi 1993); therefore, it is necessary to kill huge numbers of deer to achieve satisfactory population control. However, the decreasing numbers of hunters and unsuitable regulatory and administrative systems for deer management make it difficult to control the deer population by shooting alone (Hamasaki et al. 2011). Thus, a form of management that incorporates both shooting and reproductive control should be considered as an effective tool for population control.

Immunocontraception is a method of inhibiting reproduction through the animal's own immune system (Naz et al. 2000; Miller et al. 2004; Wang et al. 2008). As a means of population control, various studies have been conducted regarding the use of contraceptive vaccines that stimulate the production of antibodies against a specific target that is critical for reproduction. Population management based on contraceptive vaccines has been studied in several species including bison, possums, and koalas (Miller et al. 2004; Duckworth et al. 2007; Kitchener et al. 2009). Porcine zona pellucida glycoprotein (PZP) is the most common target antigen for immunocontraception (Gupta et al. 1997; Paterson et al. 2000). Contraceptive vaccines based on purified PZP have been tested to develop a new method for fertility control in many species including African elephants, domestic cats, and domestic ewes (Fayrer-Hosken et al. 2000; Gorman et al. 2002; Stoops et al. 2006). When PZP is administered with an adjuvant, the antibody against PZP binds to autologous zona pellucida (ZP) of the target species (Harrenstien et al. 2004). The finding that the antibody against the target protein from the source species binds with autologous ZP, indicates that some epitopes are same in different species.

In previous studies, anesthesia or immobilization was required when the immunocontraceptive vaccine was administered to wildlife (Kirkpatrick et al. 2011). Administration of immunocontraceptive vaccines by capture and immobilization requires much effort and high cost in free-ranging populations (Boulanger et al. 2012), necessitating the development for an effective method of population control that allows administration without anesthesia. Previous studies showed that synthetic peptide vaccines have the potential for oral administration (Carcaboso et al. 2003). However, when an oral immunocontraceptive vaccine is used, both target species and non-target species can ingest the vaccine and become vaccinated. Therefore, ensuring species specificity of the vaccine to avoid affecting non-target species is a key priority in developing a immunocontraceptive vaccine in free-ranging wildlife.

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Because immunocontraceptive vaccines using purified PZP antigen are effective in many species (Shideler et al. 2002), purified PZP may be a common interspecies antigen. Thus, species specificity would not be expected in immunization using purified PZP. Synthetic peptide vaccines can be used to establish species specificity because they can target short segments of amino acid sequences (Samoylava et al. 2012). Although a synthetic peptide is short and specific, it is able to cross react between different species (Hasegawa et al. 2002). Antibody production against a synthetic peptide based on the B-cell epitope of PZP, which has the potential to inhibit fertilization, has been reported in white-tailed deer (Miller and Killian 2002). However, its ability to bind to ZP and its species specificity in cervids remain unknown.

As a first step in the development of an immunocontraceptive vaccine, the present study was conducted to elucidate antibody production against a synthetic peptide based on the B-cell epitope of PZP in the Hokkaido sika deer and the ability of the antibody to bind to the ZP.

Materials and methods

Three captive female Hokkaido sika deer raised in a zoo (Obihiro Zoo, Hokkaido, Japan) were used for immunization. All were over 5 years of age and all were mated with one sexually matured male. In the present study, two were treated with synthetic peptide vaccine, and one was treated with adjuvant only as a control. Natural mating was observed in late October. Subsequently, the deer were immobilized with xylazine hydrochloride (Selactar 4 mg/kg: Bayer, Leverkusen, Germany) using blowgun darts or dart guns. After vaccination and blood sampling, recovery from sedation was promoted using atipamezole (Antisedan 4 mg/kg: Orion, Espoo, Finland). This experiment was approved by the Animal Experiment Committee of the Obihiro University of Agriculture and Veterinary Medicine, Japan.

The synthetic peptide used in this study was designed on the basis of the amino acid sequence of PZP1 B-cell epitope. Eighteen amino acids were selected for the synthetic peptide antigen, because an increase in the antibody titer was observed in white-tailed deer treated with this peptide (Miller et al. 2002). The amino acid sequence of the synthetic peptide was CTYVLDPENLTLKAPYEA. The synthetic peptide vaccine was manufactured and conjugated with keyhole limpet hemocyanin (KLH) by Hokkaido System Science Co. Ltd (Sapporo, Hokkaido, Japan). The vaccine was injected four times at an interval of approximately 2 weeks. An emulsion of 500 μg/500 μl of the synthetic peptide mixed with 400 μl of adjuvant (Titer Max Gold: Titer Max Georgia, USA) was injected into the muscles of the right and left thigh (a total of 4 injection sites). In the control deer, only the adjuvant was administered at the same volume.

To evaluate the condition of the deer and antibody production, blood samples were collected from the jugular vein of each deer just before each administration of the vaccine and 80 days after first vaccination. Complete blood count using whole blood and biochemical analysis using a plasma sample were performed to evaluate the side effects of the vaccination. To collect serum for analysis of antibody production, blood was kept warm for 1 h at 35°C until it was coagulated, and centrifuged at 2,500 g for 20 min. Sera were stored at –20°C until antibody analysis.

To evaluate antibody production in response to the administered synthetic peptide, the antibody titer was measured using enzyme-linked immunosorbent assay (ELISA). Two immunoplates (NUNC, Roskilde, Denmark) were coated with PZP synthetic peptide and incubated overnight at 4°C. After washing three times with phosphate-buffered saline (PBS, 200–300 μl/well), 0.05% Tween-20 and 3% skim milk in PBS were added to each plate for blocking any non-specific reaction. The plates were incubated for 2 h at room temperature and washed three times with PBS containing 0.05% Tween-20. Each antiserum was diluted (1 to 10^{-9}) with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS, pH 7.4), and added as the primary antibody, followed by incubation overnight at 4°C. Subsequently, the plates were washed three times with PBS containing 0.05% Tween-20. Peroxidase-labeled rabbit anti-deer IgG antibody (5.0 μg/ml, Funakoshi, Tokyo, Japan), diluted with 1% BSA in TBS was added as the second antibody. Then, a chromogenic reaction was performed using tetramethyl benzidine (TMB Substrate Reagent Set, BD, Frannklin Lakes, New Jersey, USA). After 20 min of incubation, the chromogenic reaction was stopped by the addition of 1 M H_2SO_4. Absorbance was measured at 405 nm using a micro plate reader (MTP-120, Corona electronic Co., Ltd., Ibaraki, Japan).

To verify the binding of the antibody to ZP, immunohistochemistry using deer and porcine ovarian tissues was performed. Deer and porcine ovarian tissues were embedded in Tissue-Tek O. C. T. compound (Sakura Finetechnical Co. Ltd. Tokyo, Japan), frozen in liquid nitrogen, and stored at –80°C. The deer ovary was obtained from a

hunting exercise and the porcine ovary was obtained from a slaughterhouse. The tissues were sectioned at a thickness of 10 μm at –20°C using a cryostat, placed onto MAS-coated slides (Matsunami grass Ind., Ltd, Osaka, Japan), and air-dried for 2 h. After fixing with refrigerated methanol for 10 min, the slides were washed with TBS, boiled for 10 min with 0.01 M sodium citrate (pH 6.0) to retrieve antigenicity, and then washed again with TBS. They were then soaked in 0.18% H_2O_2 in methanol for 1 h to inactivate the endogenic peroxidase. To block non-specific binding, the slides were incubated with 3% normal rabbit serum (CLSD403-R Cedarlane Laboratories Ltd; Canada) at room temperature for 30 min. Serum from each deer, collected 48 days after the first vaccination, was diluted 200-fold with 1% BSA in TBS. The sera were replaced with 1% BSA in PBS as the negative control. The slides were incubated with diluted deer sera over night at 4°C in a moist chamber for the first reaction. After washing with TBS, the second reaction was performed by addition of peroxidase-labeled rabbit anti-deer IgG (Funakoshi) diluted with 1% BSA in TBS (2.0 μg/ml) followed by incubation for 1 h at room temperature. The slides were washed again, and a chromogenic reaction was conducted with 3,3''-diaminobenzidine (DAB) and 0.6% H_2O_2. Finally, the slides were soaked in hematoxylin solution for nuclear staining.

Pregnancy diagnosis was performed every 6 days in November by transrectal ultrasonography (HS-1500V, HONDA ELECTRONICS, Aichi, Japan) of the uterus.

Results

Blood cell count and biochemical analysis showed no apparent abnormalities. The administration of peptide vaccines and the frequency of anesthesia did not affect the health on the animals.

Each deer treated with PZP synthetic peptide and adjuvant produced a high antibody titer. The titer reached a maximum at four weeks after the first vaccination and remained stable for at least 80 days (Fig. 1). In contrast, the antibody was not produced in the control deer, which was treated with adjuvant alone.

Many growing follicles were observed in the frozen sections of porcine and deer ovaries. Immunohistochemistry of deer ovarian sections showed that anti-sera from both immunized and control deer were not able to bind to the ZP (Fig. 2A, B). In the porcine ovary, anti-sera from deer immunized with the synthetic peptide and adjuvant were able to bind to ZP *in situ* (Fig. 2D). In contrast, anti-

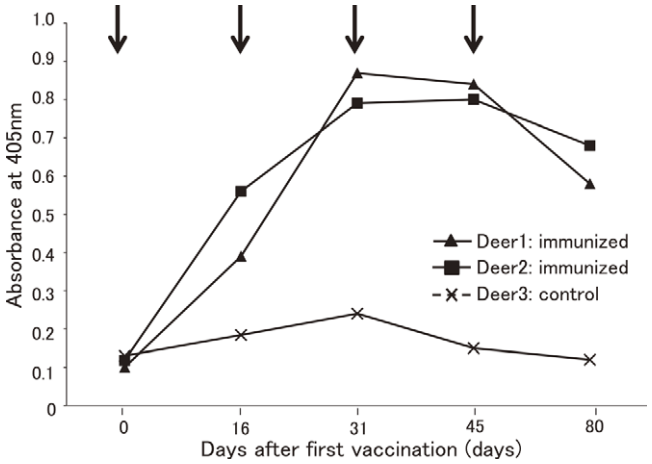

Fig. 1. Transition of antibody titer after vaccination in sika deer. Squares and triangles indicate antibody titer of vaccinated deer. And cross mark indicates the antibody titer of control deer. Vaccination and blood sampling were performed on 12 Aug, 28 Aug, 12 Sep, and 26 Sep. Blood sample were also collected on 31 Oct. Arrows indicate vaccination.

sera from the control deer immunized with adjuvant alone was not able to bind to PZP (Fig. 2E). In the negative control of both deer (Fig. 2C) and porcine (Fig. 2F) ovaries, no positive staining was observed.

One deer that had been immunized with the synthetic peptide and adjuvant was found to be pregnant using transrectal ultrasound on November 22 (Fig. 3). Fetal length assessment indicated this pregnancy appeared to be a result of mating in late October (Yanagawa et al. 2009) when there was still a high antibody titer. Another immunized deer was not pregnant and the control deer was found to be pregnant.

Discussion

Antibody production in response to administration of a synthetic peptide based on ZP was previously reported in rabbits and white-tailed deer (Hasegawa et al. 2002; Miller et al. 2002), however, the function of the antibody has not yet been elucidated. The present study clearly showed that production of antibodies that bind to PZP is induced by administration of a synthetic peptide based on the amino acid sequence of the B-cell epitope of PZP1. Consistency between immunohistochemical antibody binding and reduction of fertility has been previously demonstrated (Gorman et al. 2005). Production of an antibody that binds to ZP, after administration of the synthetic peptide demonstrated the possibility of developing an immunocontraceptive vaccine using a synthetic pep-

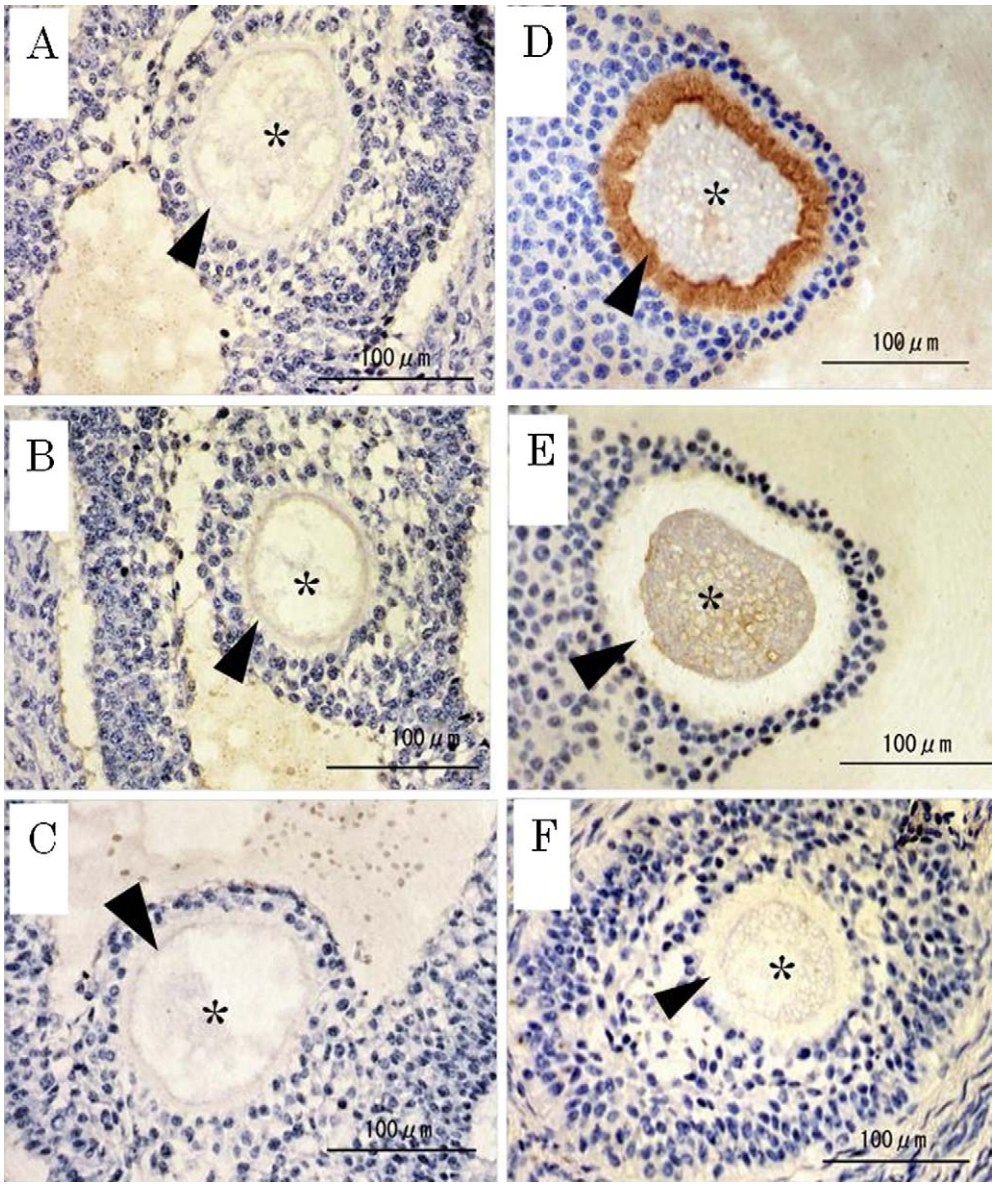

Fig. 2. Zona pellucida binding in immunohistochemistry. A: Sika deer ovary with antiserum from anti-PZP synthetic peptide vaccinated deer. B: Sika deer ovary with antiserum from control deer. C: Sika deer ovary with BSA as negative control. D: Porcine ovary with antiserum from anti-PZP synthetic peptide vaccinated deer. E: Porcine ovary with antiserum from control deer. F: Porcine ovary with BSA as negative control. Arrow heads: zona pellucida, *: oocyte.

tide antigen.

However, the antibody produced in this study was not able to bind ZP of the Hokkaido sika deer. In addition, the pregnancy of immunized deer was observed. Thus, the present results suggest that the B-cell epitope of ZP that has a role in fertilization is different between porcine and the Hokkaido sika deer.

Amino acid sequences and cross-immunity were previously examined in humans, rabbits, and mice by administration of an 18 amino acids synthetic peptide based on the B-cell epitope of human ZP1 (Hasegawa et al. 2002). That study suggested that the species differences between the B-cell epitope of ZP are caused by differences in the amino acid sequence. The species difference in the antigenicity of the B-cell epitope observed in the present study may be caused by a difference in the amino acid sequence between PZP and Hokkaido sika deer ZP. Sequencing of the B-cell epitope of ZP of the Hokkaido sika deer and immune challenge using a synthetic peptide based on the amino acid sequence of the Hokkaido sika deer zona pellucida is required to develop a specific immunocontraceptive vaccine for the Hokkaido sika deer.

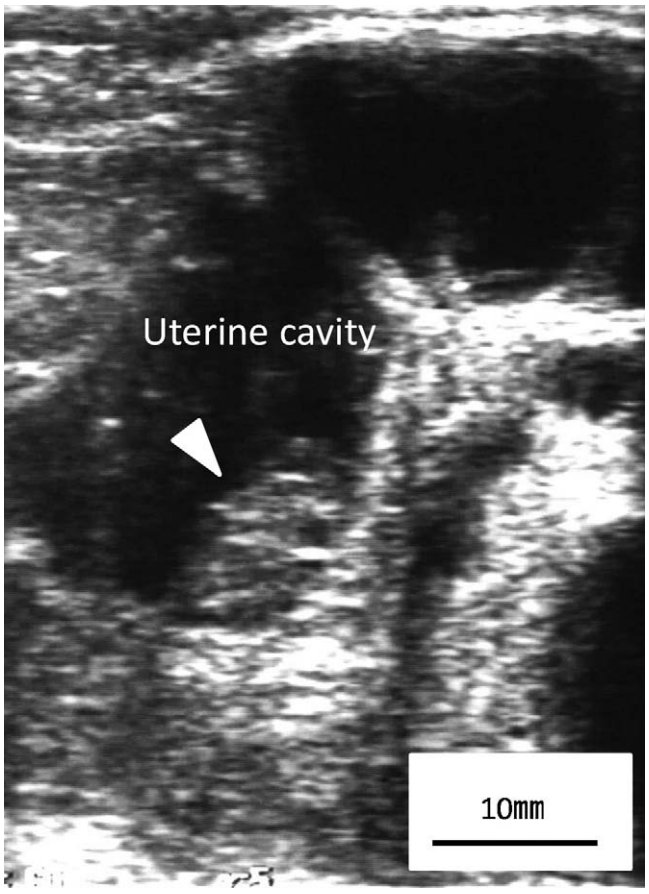

Fig. 3. Ultrasonographic image of the uterus with a fetus in the vaccinated deer. Arrow head indicates fetus.

The present study showed that a synthetic peptide vaccine can induce the production of a specific antibody and that the functioning of this antibody can be confirmed by immunohistochemistry. Therefore, the following is proposed as an effective method for the generation of a species-specific antigen for immunocontraception: 1) designing the synthetic peptide based on the amino acids sequence of a target antigen that is critical for fertilization and 2) immunohistochemical evaluation of the ability of the antibody to bind to ZP, using ovaries of the target species.

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