



Detection of sperm-reactive antibodies in wild sika deer and identification of the sperm antigens

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ABSTRACT. Antisperm antibodies potentially inhibit sperm functions causing the sterility in humans and experimentally treated animals. However, there is no information about antisperm antibodies emerging spontaneously in wildlife. In this study, we searched for the sperm-reactive antibodies, spontaneously produced in wild sika deer (*Cervus nippon*), and identified the sperm antigens. We collected 529 fecal masses of sika deer in Japanese cities, from which we extracted the mucosal antibodies to test them for reactivities to deer sperm proteins by ELISA. Two of the extracts contained IgAs that were highly reactive to the sperm proteins. The molecular weights of the active IgAs, partially purified by DEAE-sephadex A-50, were estimated at more than 100 kDa, suggesting that the IgAs evaded drastic digestion in the gastrointestinal tract. Two-dimensional electrophoresis and immunoblotting detected three major antigens, and the following LC-MS/MS analysis identified them as alpha-enolase, phosphoglycerate kinase 2 and acrosin-binding protein. The antibodies were cross-reactive to a recombinant human acrosin-binding protein. To our knowledge, this is the first research to find that the sperm-reactive antibodies are produced spontaneously in wildlife and they recognize a common antigen found in humans.

KEY WORDS: *Cervus nippon*, sika deer, sperm antigen, sperm-reactive antibody

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Sperm have both auto- and iso-antigenic potentials to induce antisperm antibodies in mammalian species [26]. Antisperm antibodies sterilize the animals by affecting the pre-fertilization events, such as sperm viability, motility, transport, capacitation, acrosome reaction and interaction with zona pellucida, and the post-fertilization events, such as early embryonic development, implantation and fetal development [29]. A lot of studies indicated that various animals including humans become infertile by the immunization with testicular or seminal materials [26]. Furthermore, the contraceptive effects of some antigens have been demonstrated in mice, rats, rabbits and guinea pigs [15]. In primates, the inoculation of two sperm antigens (LDH-C₄ and Eppin) were shown to suppress the reproduction in baboons and macaques respectively [17, 20].

Antisperm antibodies occasionally emerge without such an active immunization. Repeat-breeding cows are frequently exposed to sperm antigens and produce sperm-reactive antibodies [23]. Some of the antibodies agglutinate and/or immobilize bull sperm, presumably reducing their fecundities. In humans, sperm-reactive antibodies are detected in 9 to 36% of infertile couples, 8 to 21% of infertile men and 6 to 23% of infertile women [14]. The production of sperm-reactive antibodies is normally suppressed, because sperm and the precursor cells are prevented from contact with the immune system by anatomical barrier and T regulatory (T_{reg}) cells serve for the immunological tolerance to male antigens [22, 30]. However, the testicular injuries, such as testicular torsion, surgery and trauma, can possibly cause the exposure of sperm antigens to immune cells [1, 9]. The infectious agents can also invalidate the T_{reg}-dependent tolerances [31].

The antibodies may be the key for infertility treatment and contraception. However, the knowledge in wildlife is insufficient because no attempt has been performed to find the antibodies naturally emerge in wildlife. Therefore, we searched for the sperm-reactive antibodies, spontaneously produced in wild sika deer (*Cervus nippon*), and identified the sperm antigens.

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MATERIALS AND METHODS

Preparation of sperm stock

Two male deer were hunted in a forest in Nikko, Japan by the Japanese hunting association (Ryoyukai) in conformity with the Specified Wildlife Conservation and Management Plan of Tochigi prefecture, and the testes with attached epididymides and vasa deferentia were excised by them and provided us. The testes were transported on ice to a laboratory in Dokkyo Medical University. Five ml phosphate buffered saline (PBS, Wako, Osaka, Japan) was infused into the lumen of vas deferens and caudal epididymis by a syringe. After the swollen cauda epididymis was washed with PBS to avoid the contamination of sperm with blood, it was then cut with a surgical knife to flush the sperm into a test tube. The sperm from two deer were mixed and washed three times with PBS by centrifugation ($250 \times g$, 5 min, 20°C), and the final precipitate was stored at -60°C before use. We checked that sperm had normal shape and motility, and the contamination of sperm with blood was negligible by observing it under a microscope at $1,000 \times$ magnification. The Institutional Animal Care and Use Committee of Dokkyo Medical University approved all procedures.

Preparation of fecal extracts

Four hundred ninety-two fecal masses were collected in Nara, Japan in February 2013 and 37 fecal masses in Kushiro, Japan in March 2013. Deer fecal mass is composed of many round feces, which do not stick together and are normally strewn on the ground after evacuation. We picked up some feces probably evacuated together and handled them as a "fecal mass". The freshness of fecal masses was estimated by their moisture and solidity. Dry crumbly fecal masses were not collected. Feces were homogenized and mixed with 3-fold volume of PBS for 15 min at room temperature. After centrifugation ($15,000 \times g$, 20 min, 4°C), the supernatants were collected and stored at -60°C before use.

Partial purification of sperm-reactive antibodies by DEAE-sephadex A-50

Fifteen ml fecal extract (K24 from Kushiro or N459 from Nara) was extensively dialyzed with a dialysis device, Float-A-Lyzer G2 (3.5–5 kDa cutoff, Spectrum Laboratories, Rancho Dominguez, CA, U.S.A.) against 20-fold diluted PBS ($\times 1/20$ PBS) at room temperature. Fifteen ml of DEAE-sephadex A-50 (GE Healthcare, Buckinghamshire, U.K.) rehydrated in $\times 1/20$ PBS, was added into the dialyzed extract and they were mixed overnight at room temperature. The supernatant was collected as "Flow through" fraction. The remaining resin was washed four-times with 25 ml of $\times 1/20$ PBS. The first washing solution was collected as "Wash" fraction. The substances bound to the resin were eluted twice with three solutions, i.e. 2-fold diluted PBS ($\times 1/2$ PBS), PBS and 5-fold concentrated PBS ($\times 5$ PBS), successively.

Separation of sperm-reactive antibodies by ultrafiltration

Five hundred μl of the "Flow through" fraction was concentrated up to 15 μl by a Nanosep centrifugal device with 100 kDa cutoff (Pall Life Sciences, Ann Arbor, MI, U.S.A.) and diluted again with 485 μl PBS (" >100 kDa" fraction). Twenty-four μl of 20-fold concentrated PBS ($\times 20$ PBS) was added to the solution that passed through the filter membrane (" <100 kDa" fraction). These processes adjusted the salt concentration of " >100 kDa" and " <100 kDa" fractions to that of PBS.

Measurement of IgA amount by ELISA

The fecal extracts and separated fractions were 4-fold diluted using 0.05 M carbonate buffer (pH9.6). Fifty μl of this solution was then added to the wells of a 96-well ELISA plate (IWAKI, Tokyo, Japan) and kept overnight at 4°C . The wells were washed once with PBS containing 0.05% Tween 20 (PBST) and blocked by 100 μl PBST containing 3% skim milk (blocking solution) for 1 hr at 37°C . They were washed once with PBST and treated for 1 hr at 37°C with 50 μl of Horseradish peroxidase (HRP)-conjugated anti-bovine IgA antibody produced in sheep (Bethyl Laboratories, Montgomery, TX, U.S.A.) which was 2,000-fold diluted with the blocking solution. After the wells were washed six times with PBST, color development reaction was induced by ELISA POD Substrate TMB Kit (Nacalai Tesque, Kyoto, Japan), and the light absorbance at 450 nm (OD_{450}) was measured by a spectrophotometer, Infinite 200 Pro (Tecan Trading AG, Männedorf, Switzerland).

Because HRP-conjugated anti-bovine IgA antibody was similarly reactive to the extracts of bovine feces and deer feces, but not reactive to the extract of rat feces, we concluded that this antibody was available for the detection of deer fecal IgA.

Evaluation of the reactivities of IgAs to sperm proteins by ELISA

The sperm stock containing 2.7×10^8 cells were thawed, suspended in 2 ml H_2O and sonicated with Sono Cleaner (Kaijo, Tokyo, Japan) for 30 min at room temperature. The supernatant was collected by centrifugation ($15,000 \times g$, 20 min, room temperature), and added to 28 ml of 0.05 M carbonate buffer (pH9.6). Fifty μl of the antigen solution was added to the wells of a 96-well ELISA plate and kept overnight at 4°C . The wells were washed once with PBST and blocked by 100 μl of blocking solution for 1 hr at 37°C . They were washed once with PBST and treated with 50 μl of primary antibodies, i.e. the fecal extracts or the partially purified fractions, which were added 0.05% Tween 20 before the treatment. The wells were washed six times with PBST, and reacted for 1 hr at 37°C with 50 μl of HRP-conjugated anti-bovine IgA antibody, which was 2,000-fold diluted with the blocking solution. After the wells were washed again six times with PBST, the HRP color development reaction was induced by ELISA POD Substrate TMB Kit, and the OD_{450} was measured by Infinite 200 Pro.

The relative reactivity of each extract was calculated using the following formula:

$$\text{Relative reactivity} = (\text{OD}_{450} - \text{OD}_{450\text{ave}}) / \text{SD}$$

OD_{450ave} was the average OD₄₅₀ of all extracts tested simultaneously, and SD was the standard deviation.

To measure the reactivities of IgAs to human acrosin-binding protein (hACRBP, OriGene Technologies, Rockville, MD, U.S.A.), we used 5 µg/ml hACRBP in 0.05 M carbonate buffer (pH9.6) to immobilize the antigen on the ELISA plate.

Two-dimensional electrophoresis

Sperm (1.3×10^8 cells) were suspended in H₂O containing 1% 3-(3-cholamidopropyl) dimethylammonio-1-propanesulphonate (CHAPS, Dojindo Molecular Technologies, Kumamoto, Japan) and mixed for 15 min at room temperature. The sperm proteins solubilized in the supernatant were collected by centrifugation ($15,000 \times g$, 10 min, 20°C) and then chilled on ice. The proteins were precipitated with 500 µl of cold 10% trichloroacetic acid (TCA) and washed three times with 500 µl of cold acetone. The precipitate was dissolved in 50 µl of the sample buffer (7M urea, 2M thiourea, 4% CHAPS, 100 mM dithiothreitol, 60 mM Tris (hydroxymethyl) aminomethane (Tris) pH 8.8) and was added to 5 µl of 100 mM acrylamide for propionamidation of cysteine residues. An agarose gel strip, agarGEL (pH3–10, 75 mm) (ATTO, Tokyo, Japan) was used to separate proteins in the first dimension. The separation was achieved with an isoelectric machine (DiscRun-R (ATTO)) using a stepwise output (50 V for 10 min, 100 V for 10 min, 300 V for 210 min). The agarGEL was incubated in 2.5% TCA for 3 min and washed five times with H₂O. It was soaked in the equilibration buffer (2% sodium dodecyl sulfate (SDS), 0.001% bromo phenol blue, 50 mM Tris pH 6.8) and the proteins in it were resolved in the second dimension on SDS-polyacrylamide gel electrophoresis with 12.5% acrylamide gel. To detect whole proteins separated by the two-dimensional electrophoresis, the acrylamide gel was stained with Coomassie Brilliant Blue R-250.

Immunoblot

The gel of two-dimensional electrophoresis was soaked in a transfer buffer (192 mM Glycine, 25 mM Tris, 20% Methanol) and the proteins were transferred onto a PVDF membrane, BioTrace PVDF (Pall life Sciences) by “rapid mode” of the western blotting transfer system, PoweredBLOT ONE (ATTO), for 20 min. The membrane was blocked by ELISA blocking solution for 1 hr at room temperature, and agitated for 1 hr in 12 ml of the “Flow through” fraction containing 0.6 ml of $\times 20$ PBS and 126 µl of 5% Tween 20. It was intensively washed in Tris buffered saline (TBS, 137 mM NaCl, 3 mM KCl, 10 mM Tris pH6.8) with 0.05% Tween 20 (TBST), and treated for 1 hr with HRP-conjugated anti-IgA antibody, 1,000-fold diluted with TBST containing 3% skim milk. The solution was completely washed out by TBST and colorimetric detection was performed by a TMB substrate for HRP, EzWestBlue (Nacalai Tesque).

Identification of sperm antigens

Each protein within a gel piece of two-dimensional electrophoresis was digested overnight at 37°C by 5 ng/µl trypsin from porcine pancreas Mass Spectrometry Grade (Wako) dissolved in 25 mM NH₄HCO₃. The peptide mixture was extracted from the gel piece once with 25 mM NH₄HCO₃ and twice with 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA), and these extracts were pooled. The mixture was separated by reverse-phase HPLC using a column Cadenza CD-C18 (Imtakt, Kyoto, Japan) with acetonitrile gradient of 5 to 50%, and was analyzed using LC-Q-TOF TripleTOF5600⁺ (ABSciex, Framingham, MA, U.S.A.). After conversion of data file in mgf format, the amino acid sequences of peptides were estimated by PepNovo⁺ with DeNovoGUI [7, 13]. The amino acid sequences with the score of 80 or higher were collected and were compared with sequences in a publicly available reviewed protein database of Swiss-Prot (<http://www.uniprot.org/downloads>) using fasts36.3.6 [12].

RESULTS

Search for sperm-reactive antibodies in deer feces

Five hundred twenty-nine fecal masses of sika deer were collected from two places in Japan (Kushiro and Nara). We extracted the mucosal antibodies from each feces and evaluated whether the sperm-reactive antibodies were present in the extracts. Here, the extracts were examined for the reactivities to whole sperm proteins by ELISA, in which a labeled anti-IgA antibody was used as the secondary antibody, because IgA was the major immunoglobulin class in the mucosal immunity. Two of 529 extracts, K24 from Kushiro and N459 from Nara, were highly reactive to sperm proteins (Fig. 1). Their relative reactivities were 7.3 (K24) and 6.7 (N459) respectively, which meant that their OD₄₅₀ values were bigger than the average by 7.3 and 6.7 times of the standard deviation. In addition, the relative reactivities of 8 other samples, N16, 106, 107, 219, 224, 242, 381 and 462, were more than 3.0. We focused on K24 and N459 to characterize the naturally emerging sperm-reactive antibodies and investigate the sperm antigens.

Partial purification of sperm-reactive antibodies

Because the samples were just extracted directly from feces, they contained impurities. To improve the purity and avoid the unexpected effects of the impurities, the components of K24 and N459 were respectively separated by an anion exchange resin, DEAE Sephadex A-50. The components were separated into the fractions unbound to the resin (“Flow through” and “Wash” fractions) and the fractions bound (“ $\times 1/2$ PBS”, “ $\times 1$ PBS” and “ $\times 5$ PBS” fractions). We clearly found the IgAs were composed of the unbound and bound populations, because IgA amounts decreased once in “ $\times 1/2$ PBS” fraction and increased again in the following fractions (Fig. 2 upper graphs). Furthermore, IgAs in the unbound fractions reacted to sperm proteins (Fig. 2 lower graphs).

It is generally known that the molecular weights of mammalian IgA are about 170 kDa in the monomer and 390 kDa in the dimer. Therefore, we confirmed that the active IgAs in our extracts were as large as the general mammalian IgA. The components

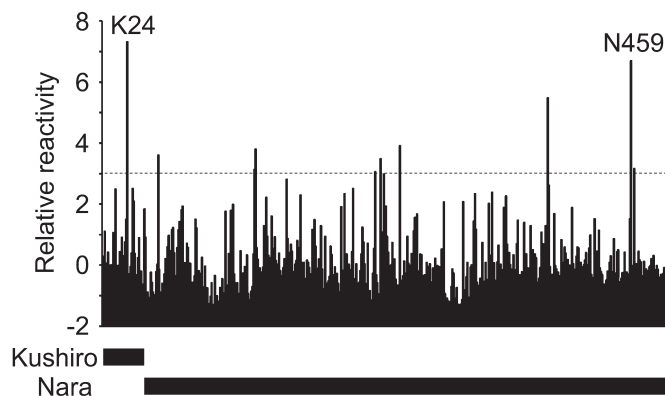


Fig. 1. Reactivities of fecal extracts to sperm antigens. Mucosal antibodies were extracted from 529 fecal masses (37 from Kushiro and 492 from Nara) with PBST, and the reactivities of the extracts to sperm proteins were measured by ELISA. Each value represents the average of two independent experiments. Two (K24 and N459) extracts strongly reacted to sperm proteins.

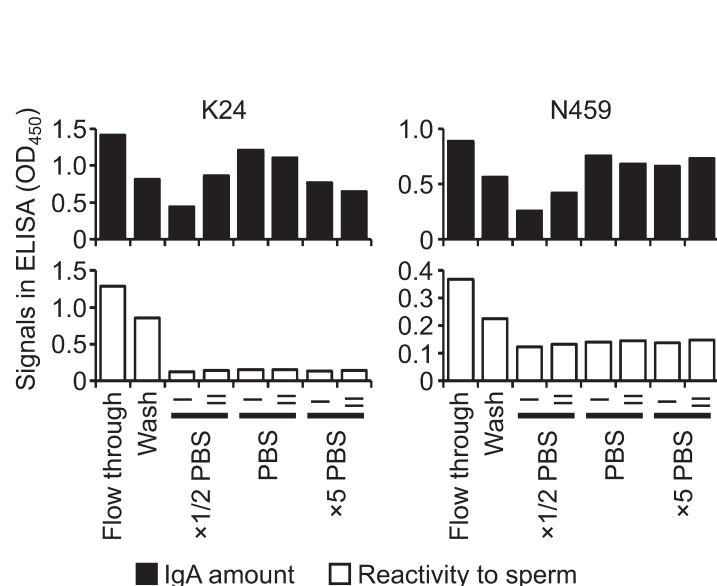


Fig. 2. IgA amounts and reactivities of partially purified fractions. K24 and N459 extracts were respectively applied for DEAE Sephadex A-50. The unbound substances were collected in “Flow through” and “Wash” fractions, and the bound substances were eluted twice by $\times 1/2$, $\times 1$ and $\times 5$ PBS. The first and second fractions of each solution were indicated as “I” and “II” respectively. The relative IgA amounts (black bars) and the reactivities to sperm proteins (white bars) of each fraction were measured by ELISA. Each value represents the average of two independent experiments. IgAs were divided into both the unbound and bound fractions, but the former alone reacted to sperm proteins.

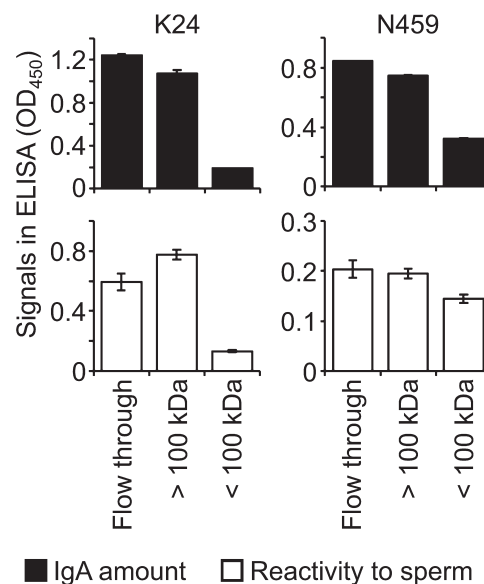


Fig. 3. IgA amounts and reactivities of ultrafiltered fractions. The “Flow through” fractions, prepared by the partial purification with DEAE Sephadex A-50 separation, were further separated by ultrafiltration devices with 100 kDa cutoff. The substances passing through the filter membrane were pooled as “<100 kDa” fraction and those remaining on the filter membrane as “>100 kDa” fraction. The relative IgA amounts (black bars) and the reactivities to sperm proteins (white bars) were measured by ELISA. Each value represents the average \pm standard deviation of three independent experiments. Most of the IgAs were separated into the “>100 kDa” fraction and the fraction was reactive to sperm proteins.

in “Flow through” fractions of K24 and N459 were separated by ultrafiltration with 100 kDa cutoff. Most IgAs in these fractions did not pass through the membrane of ultrafiltration devices, indicating they were bigger than 100 kDa (Fig. 3 upper graphs). They certainly reacted to sperm proteins (Fig. 3 lower graphs). Thus, these active IgAs probably escaped from the severe destruction through the digestive tract and were kept in relatively good state even in feces.

Detection and identification of sperm antigens

The sperm proteins were separated by two-dimensional electrophoresis and the antigens were detected by the immunoblot, in

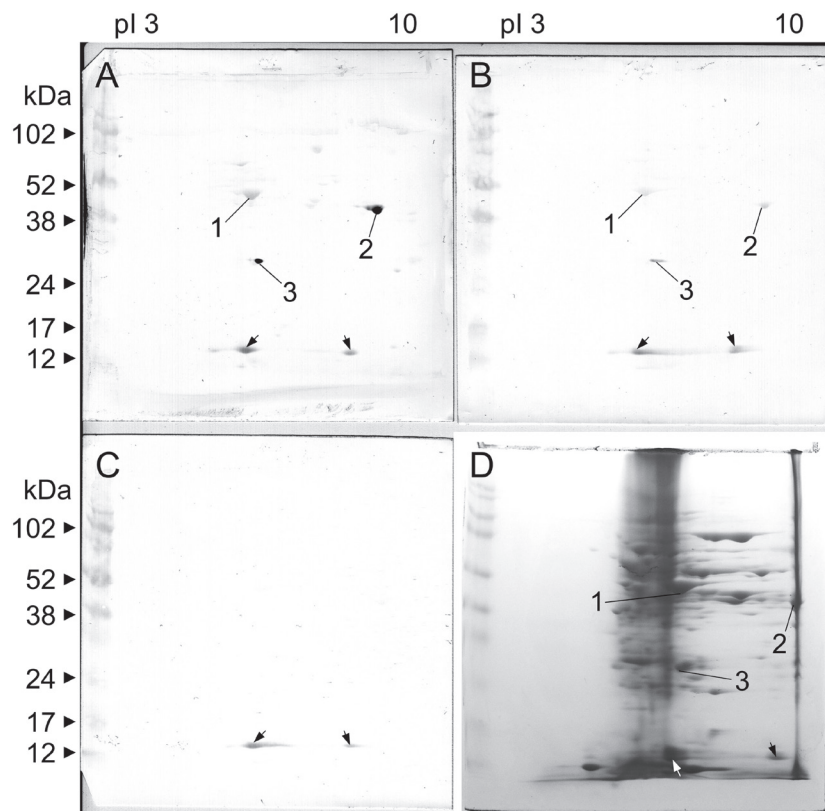


Fig. 4. Detection of the sperm antigens recognized by sperm-reactive antibodies. Sperm proteins were separated by two-dimensional electrophoresis and transferred onto PVDF membrane. On the membrane, the sperm antigens were detected by immunoblot in which the “Flow through” fractions from K24 (A) and N459 (B) were respectively used as primary antibody and HRP-conjugated, anti-IgA antibody was used as secondary antibody. In the negative control, the primary antibody was replaced by PBST (C). Simultaneously, acrylamide gel of two-dimensional electrophoresis was stained by CBB R-250 to ascertain the protein profile (D). Three antigen spots (1–3) and two non-specific spots (arrows) were detected.

which “Flow through” fractions were used for the primary antibodies, and the labeled anti-IgA antibody was used for the secondary antibody. Five major spots were detected in the immunoblot, but two of them were non-specific spots, as they were seen without the primary antibodies (Fig. 4).

The three spots, specifically recognized by “Flow through” fractions of K24 and N459, were excised from the acrylamide gel of two-dimensional electrophoresis and digested by trypsin to determine the sequences by LC-MS/MS and following *de novo* sequence. The determined sequences were searched against Swiss-Prot database to identify each protein. All three proteins were identified as alpha-enolase (ENO1, accession: Q9XSJ4), phosphoglycerate kinase 2 (PGK2, 2Q6RI85) and acrosin-binding protein (ACRBP, Q29016) (Table 1).

Investigation of cross-reactivities of sperm-reactive antibodies to human antigen

In the case of ACRBP identification, the number of matched peptides was relatively small and some other proteins were hit with lower scores (Table 1). Therefore, we examined the reactivities of the antibodies to a commercial recombinant hACRBP. The “Flow through” fractions of K24 and N459 certainly responded to hACRBP and especially that of K24 strongly reacted to it (Fig. 5). This result indicated both the accuracy of protein identification and cross-reactivities of the antibodies to the human sperm antigen.

DISCUSSION

Incidence of the sperm-reactive antibodies

Knowledge about antisperm antibodies has been accumulated by research conducted on humans, domestic animals and experimental animals. The physiology of humans and these animals should be affected by the artificial environment, in which they were born and grown. Their genealogy also influences their physical features. Especially, traits of domestic and experimental

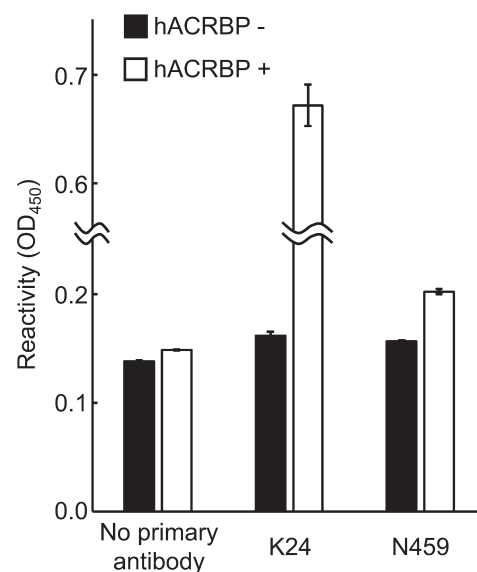


Fig. 5. Reactivities of sperm-reactive antibodies to hACRBP. The affinities of deer sperm-reactive antibodies to hACRBP were investigated by ELISA, in which hACRBP was used as antigen, the “Flow through” fractions from K24 and N459 as primary antibody, and HRP-conjugated anti-IgA antibody as secondary antibody. Each value represented the average \pm standard deviation of four independent experiments. In the negative control, the primary antibody was replaced by PBST. The deer antibodies recognized hACRBP.

Table 1. Proteins specifically reacted to the sperm-reactive antibodies

Spot no.	Matched peptide ^{a)}	Protein name (Accession)	Species	Bit-score/E-value	Protein function (Subcellular location)
1	PTVEVDLFTAK PSGASTGLYEALRL AVEHLNK TLAPALVSK KLNVEVEQEK LMLEMDGTENK NALGVSILAVCK GAEVYHNLK GDEGGFAPNLENK EAELELK LGMDVAASEFYR SGKYDLDFK YLTPELANLYK EDPFDQDDWEAWQK AGLQVVGDDLTVTN QLGSVTESLQACK YNQLLR LEEELGSK	Alpha-enolase (Q9XSJ4)	<i>Bos taurus</i> (Cattle)	596.4/0	Glycolysis (Cytoplasm) Plasminogen activation (Cell membrane)
2	KLTLDKVDVK VDFNVPMK NQLTNNQRTNNQR LKASLPSL (K) YCLDNGA (K) SLLGKDVFLK A (D) P (A) AGSVLLE AEPDKLEAFR YVNDAFGTAHR AHSSMVGVNLPQK ASGFLMKK ELDYFAK LALLGGAK DKLQLLK LVKELMAK PVDFV (T) ADKFEEN YADVVG ALMDEVVKATSK (STNQ) SLELLEGK	Phosphoglycerate kinase 2 (Q6RI85)	<i>Sus scrofa</i> (Pig)	432.5/0	Glycolysis (Cytoplasm)
3	FYGLDLYGGLR QTEFLSFQDGDF SQQCMMR SQEFSTLTL	Acrosin-binding protein (Q29016)	<i>Sus scrofa</i> (Pig)	129.1/4.4E-29	Packaging and condensation of acrosin zymogen (Acrosome, secreted)

a) The amino acids underlined were replaced by the structurally similar amino acids in the homologous protein in Swiss-Prot database. The amino acids in parentheses were unmatched.

animals have been manufactured by selective breeding and/or gene manipulation. Therefore, it is possible that the previous results may have contained artifacts and/or may have concealed what happens in nature. From this point of view, we tried to detect the sperm-reactive antibodies, which were spontaneously produced in wildlife.

In our research, 2 of 529 samples were highly reactive to sperm proteins and 8 more samples were judged positive if the cutoff value of relative reactivity was 3.0 (Fig. 1). The incidence calculated with these positive samples was 1.9%. A previous research showed that antisperm antibodies were detected in 14% of infertile and 2.5% of fertile men [9]. In other research, 18.8% of men and 17.6% of women in infertile couples were positive, while 4% of fertile women were positive [19]. Research in cattle, which is a relatively close species to deer, demonstrated that the incidences were much higher. Five point eight eight % of heifers, 25.00% of normal breeders, 18.75% of pregnant cows and 59.00% of repeat-breeders were positive in the sperm agglutination test [23]. Although we did not distinguish the gender and reproductive potential of individual deer, the prevalence was lower than that of fertile humans and domestic animals.

Our results are still too rough to be precisely compared with previous research because we detected the antibodies extracted from feces. Use of blood or mucous of wild deer will give us indubitable results, but it is too difficult to collect many samples.

In fecal samples, there are some factors which may affect the incidence of sperm-reactive antibodies. The freshness of the feces may affect because the feces contain digestive enzymes, microbes, and other materials, destroying the antibodies. The partial purification of extracts showed that they contained non-reactive IgA populations (Fig. 2). N459 extract gradually lost its reactivity through the experiments. Also, we did not pick up N381, the reactivity of which was the third highest, due to rapid deterioration of its reactivity. These facts may have been due to the digestion and/or denaturation of antibodies. Meanwhile, we collected fecal masses without individual identification and some of them may have been from same deer. It is another factor possibly affecting the incidence of sperm-reactive antibodies in our research. More effort for a direct investigation with blood or mucous is required to strictly compare the incidences of the antibodies.

Similarity of sperm antigens in human and wild deer

Three sperm antigens, ENO1, PGK2 and ACRBP, were identified by two-dimensional electrophoresis and following immunoblot. They were similarly reactive to the fecal extracts from two distant places, Kushiro and Nara. It means that the antigens are common in this deer species. Furthermore, similar attempts already showed their antigenicities in human sperm. In a research, ENO1 was recognized by immunoinfertile sera, which were derived from infertile women and were positive in the sperm agglutination test, the sperm immobilization test and/or the indirect immunobead test [4]. Another research showed that ENO1 reacted with sera and seminal plasma of immunoinfertile men [6]. Simultaneously, PGK2 and ACRBP were found to react to seminal plasma of immunoinfertile men and sera of vasectomized patients respectively. The other research also indicated that ENO1 reacted to both antisperm antibody-positive and -negative sera, whereas ACRBP did only to the positive serum, but not to the negative [16].

Consequently, we detected and identified well known sperm antigens. This resemblance was supported by the cross-reactivity of ACRBP (Fig. 5). An epitope of hACRBP was already determined as KTPFVSPLL, which was recognized by CD8 T-cells [18]. This amino acid sequence is completely conserved in the ACRBP of Hungarian red deer, *Cervus elaphus hippelaphus*, although the homology of whole sequence is about 80% between human and the deer. These facts clearly show that both human and deer potentially develop the immune response to common sperm antigens, maybe recognizing same epitope. This is the point of our research, which suggests that sperm-reactive antibodies are not “unnatural” products limited to humans and the animals under human influences, but “natural” products similarly found in wildlife.

We guess that the common antigens might be the key molecules for “the trade-off between immunity and fertility”. According to a research in a wild population of Soay sheep, individuals with strong immunity were able to survive the harsh winters, but they reproduced less during moderate winters [8].

Putative action of the sperm-reactive antibodies on fertility

The sperm antigens must be exposed on sperm surface to make the antibodies exhibit inhibitory effects. Although all antigens identified in this research are generally thought to be inside the sperm, there are some reports about their localization on sperm surface and their functions there.

ENO1 and PGK2 are generally categorized into glycolytic enzymes localized in cytoplasm. However, the proteomic analyses of surface proteins in boar and bull sperm showed that both ENO1 and PGK2 localized on sperm surface [3, 5]. As ENO1 expressing on the surface of leukocytes promotes the plasminogen activation, it is possible that it works on the sperm surface [11]. Supporting this idea, ENO1 in human sperm was shown to have affinity to recombinant zona pellucida glycoproteins [21]. Surface PGK2 also seems to be important for sperm motility in humans, as incubating sperm with anti-PGK2 antibody decreases the sperm motility [10].

ACRBP is regarded as an acrosomal protein probably involved in sperm capacitation, assembling acrosin zymogen into acrosomal matrix [2]. In mice, knockout of the proprotein convertase 4 caused the loss of maturation of ACRBP and simultaneously causing head/acrosome malformations [25]. The function in acrosome formation and capacitation must be exhibited in acrosome, but instead it sorted on the surface proteins in proteomic research in mice and bull sperm [5, 24]. Additionally, another research showed an affinity of ACRBP to zona pellucida in boar sperm [27]. Moreover, boar ACRBP was found in lipid raft, which is defined as sphingolipid and cholesterol-rich micro domains in the plasma membrane concentrating signaling molecules [28].

Thus, these proteins have the potential to work on sperm surface. Deer sperm-reactive antibodies may inhibit sperm functions, especially the sperm-zona pellucida interaction and sperm motility. Unfortunately, it is unclear that the sperm-reactive antibodies really affected deer fertility, because we just collected most feces on the ground without watching their evacuations and did not checked the reproductive status of the deer from which the positive feces were derived.

To know the effect of the antibodies on deer fertility, we need further experiments to get the direct evidence. However, if the antibodies are proved to reduce deer fertility, the antigens could be utilized as the component of contraceptive vaccine. Overabundant deer population is serious issue in Japan. Hence, this research might contribute to solve the social problem.

Conclusion

We extracted the mucosal antibodies from the feces of sika deer and showed that the antibodies could react to sperm antigens, ENO1, PGK2 and ACRBP. In addition, it was demonstrated that the antibodies cross-reacted to a human antigen, hACRBP. This research firstly illustrates the spontaneous emergence of sperm-reactive antibody in wildlife and the commonness of the sperm antigens between wildlife and humans.

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