Murine Sperm Expresses Toll-like Receptor (TLR) Family that Responds to the Pathogens Released from Virus, and Decreases Fertilization Ability by the Stimuli

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Abstract: *Sperm express the Toll-like receptor (TLR) family and have an innate immune function defending against bacterial infection in semen. It has been known that virus infection is also observed in semen with low motility of sperm, suggesting that sperm are also able to respond to virus infection. However, there is little information about the expression and roles of TLRs that recognize virus-released pathogens in semen. The present study clearly showed that sperm express functional TLR9 that recognizes the unmethylated CpG DNA sequence. Using a specific antibody, we detected the expression of TLR9 in sperm. However, the positive band was disappeared by progesterone treatment that induced the acrosome reaction. When sperm were cultured with the TLR9 ligand, sperm motility significantly decreased in a time-dependent manner. The treatment of sperm with the TLR9 ligand affected the acrosomal status and suppressed the BSA-induced capacitation of sperm. Additionally, sperm were used for in vitro fertilization and injection into the uterus of super-ovulated female mice following pre-culture with the TLR9 ligand. The fertilization rates were significantly suppressed both in vitro and in vivo. From these results, we conclude that sperm express and have functional TLR9 that potentially recognizes virus infection in semen.*

Key words: *Toll-like receptor, Sperm, Virus infection, Fertilization, Innate immune function*

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Introduction

In the innate immune system, pathogens (known as pathogen-associated molecular patterns: PAMPs) are recognized by the Toll-like receptor (TLR) family, which is one of the pattern-recognition receptors (PPRs) [1]. Although the innate immune function has been known to be dependent on immune cells, we have recently shown that TLR4 is expressed on sperm, and recognizes lipopolysaccharide (LPS), a Gram negative bacterial factor, which results in the loss of sperm ability (motility, apoptosis, acrosome reaction, fertilization rate) [2]. Additionally, sperm also express TLR2 that recognizes Gram positive bacterial factor, a lipopeptide [2]. Similar results were shown when mice sperm were cultured with a TLR2 ligand, Pam3Cys-Ser-(lys)4 (Pam3Cys) as well as LPS [2]. A significant negative correlation was observed between the number of bacteria and sperm motility, suggesting that TLR2 and TLR4 expressed on sperm might play important roles in responses to bacterial infection in semen.

The impact on male infertility of virus infection as well as of bacterial infection has been reported. Virus DNA or RNA are detected in semen with low sperm quality [3, 4]. The unmethylated CpG DNA sequence, single strand RNA (ssRNA) and double stranded RNA (ds RNA) are components of viruses that are recognized as pathogens by immune cells [5]. In immune cells such as macrophages, dendritic cells and natural killer cells, virus infection is recognized by TLR3, TLR7, TLR8 and TLR9 [6–8]. TLR3 recognizes dsRNA to induce the

secretion of pro-inflammatory cytokines [6]. When TLR7, TLR8 and TLR9 are stimulated by ssRNA or the unmethylated CpG DNA sequence, the myeloid differentiation factor 88 (MyD88) pathway is activated and MyD88 interacts with IL-1-receptor-associated kinase (IRAK)-1, IRAK-4 and TNF receptor-associated factor (TRAF) 6 [9]. This complex induces phosphorylation and degradation of IκB, then NF-κB is transferred to nuclei, or activates interferon-regulatory factor (IRF) 7 to induce target gene expressions such as *Infa* or *Infb* [9–11]. The MyD88 dependent pathway is also involved in the progression of apoptosis in endothelial cells [12]. Based on these reports, we hypothesized that sperm directly recognizes virus as well as bacerial infections in semen via TLR3, TLR7, TLR8 and TLR9, resulting in the reduction of sperm functions, including fertilization ability. Although Palladino *et al.* [13] reported the expression of the TLR family that recognize virus pathogens in rat testes, epididymidi and sperm, the relationship between TLRs in sperm and the decrease of sperm fertilization ability due to virus infection has remained unclear.

In this report, we examined whether TLR3, TLR7, TLR8 and TLR9 were expressed in mouse sperm and played functional roles in sperm motility and fertilization ability. *Tlr3*, *Tlr7*, *Tlr8* and *Tlr9* expression at the mRNA level were detected by RT-PCR, and the protein level was analyzed using a specific antibody. Additionally, to clarify the functional role of these receptors, we cultured sperm with specific ligands and then analyzed the motility, acrosomal status and induction of capacitation. Finally, we also investigated whether the virus pathogens inhibited sperm penetration to the oocyte both *in vivo* and *in vitro*.

Materials and Methods

Materials

The TLR7 ligand, single strand RNA (ssRNA40) and TLR9 ligand, unmethylated CpG DNA (ODN1826), were obtained from InvivoGen (San Diego, CA, USA). For western blot analyses, anti-TLR9 antibody was purchased from IMGENEX (IMG-305A, San Diego, CA, USA), anti-phospho-Tyr (P-Tyr-100, 9411), from Cell Signaling Technology Inc. (Danvers, MA, USA), and anti-β-actin antibody from Sigma Chemical Co (Sigma, St. Louis, MO). Equine chorionic gonadotropin, eCG was purchased from Asuka Seiyaku (Tokyo, Japan). Human chorionic gonadotropin, hCG was purchased from Asuka Seiyaku. Oligonucleotide poly-(dT) was purchased from GE Healthcare (Buckinghamshire, UK), and AMV reverse transcriptase and Taq polymerase were purchased from Promega (Madison, WI, USA). Bovine serum albumin (BSA, fraction V) was obtained from Sigma (A 7888), and routine chemicals and reagents were obtained from Nacalai Tesque, Inc. (Kyoto, Japan) or Sigma.

Animals

Specific pathogen free (SPF) immature female C57BL/6 mice, adult (8-week-old) female ICR mice and 4-month-old male ICR mice were obtained from Clea Japan (Tokyo, Japan). On day 23 of age, immature female mice were injected intraperitoneally (IP) with 4 IU of eCG to stimulate follicular growth followed 48 h later with 5 IU hCG to collect the ovulated cumulus oocyte complexes (COCs) for *in vitro* fertilization [14]. For the artificial insemination study, adult female ICR mice were injected intraperitoneally (IP) with 5 IU of eCG to stimulate follicular growth followed 48 h later with an injection of 5 IU hCG. These mice were housed under a 16-hour light/8-hour dark schedule at the Experiment Animal Center at Hiroshima University, and provided food and water *ad libitum*.

Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee at Hiroshima University.

Collection and culture of sperm

Spermatozoa were collected from the cauda epididymidi of 4-month-old ICR strain mice into 500 μl of human tubal fluid (HTF) medium containing 0.4% (w/v) BSA [14]. The sperm was cultured with 5 or 10 μg/ml of ssRNA40 or 1 or 5 μM of ODN1826 for 6 h, before analyzing the motility. To induce the acrosome reaction, the sperm were treated with 100 ng/ml of progesterone (Sigma) for 60 min following pre-cultivation with BSA for 60 min.

The detection of acrosome reaction

The acrosomal status of spermatozoa was measured using FITC-labeled peanut agglutinin (FITC-PNA, Sigma) and propidium iodide (PI) staining [15]. The sperm were treated with progesterone following the culture with BSA. The mixture was spread over slides and air-dried at room temperature. Samples were then fixed with absolute methanol for 10 min at room temperature, and 30 μl of FITC-PNA solution (100 μg/ml) in PBS was spread over each slide. The slides were then incubated in a dark, moist chamber for 20 min at 37ºC. Following a rinse with PBS and air-drying, the

Table 1. List of primers employed for RT-PCR and the expected size of amplification products

gene	Forward Primer	Reverse Primer	Size (bp)	annealing temperature (°C)	Cycle
Tlr3	5'-TTCATGCCCATAAAGACAGA-3'	5'-CTGAATTCCGAGATCCAAGT-3'	426	62	37
Tlr7	5'-GGAAATTGCCCTCGATGTTA-3'	5'-CAAAAATTTGGCCTCCTCAA-3'	237	60	37
Tlr8	5'-GAAGCATTTCGAGCATCTCC-3'	5'-GAAGACGATTTCGCCAAGAG-3'	188	60	37
Tlr9	5'-TACGCTTGTGTCTGGAGGAC-3'	5'-GATCACCAACACCACCACGT-3'	200	60	37

slides were mounted using VectaShield with PI (Vector Laboratories Inc., Burlingame, CA, USA). More than 200 spermatozoa were evaluated in each sample.

Western blot analyses

Protein samples from spermatozoa were prepared by homogenization in Laemmli sample buffer. The samples were heated at 100ºC for 5 min, and 20 μl of each sperm extract was loaded onto each lane (1×10^6 spermatozoa/lane) of a 10% SDS-polyacrylamide gel. Membranes were blocked in Tris-buffered saline and Tween 20 (TBST; 10 mM Tris (pH 7.5), 150 mM NaCl and 0.05% Tween 20) containing 5% (w/v) BSA (Sigma). Blots were incubated in primary antibody (1:1,000 dilution of anti-TLR9 antibody, 1:2,000 of anti-phospho-Tyr antibody, or 1:10,000 of anti-β-actin antibody) at 4ºC. After washing in TBST, enhanced chemiluminescence (ECL) detection was performed using ECL Plus western blot detection reagents (GE Healthcare) and appropriate exposure of the blots to X-ray films (Fuji Medical X-Ray Film, FUJI FILM, Tokyo, Japan). Mouse spleen was used for positive control cells for TLR9; 10 mg/ml of control tissue sample was loaded onto each lane.

Immunofluorescence

Collected spermatozoa were washed in PBS, after which the mixture was spread over the slides and air-dried at room temperature. Samples were then fixed with PBS-buffered 4% (w/v) paraformaldehyde for 30 min at 4ºC. Following a rinse with PBS, the slides were incubated with Mouse Ig blocking reagent of Vector M.O.M. immunodetection kit for 1 h. The slides were then sequentially probed with primary anti-TLR9 antibody (diluted 1:100) and secondary anti-mouse IgG (whole molecule) F(ab')2 Fragment-Cy3 (1:100, Sigma). Slides were mounted using VectaShield with DAPI (Vector Laboratories Inc.). The sperm samples were incubated without primary antibody as a negative control.

RT-PCR analysis

Total RNA was obtained from mouse brain, spleen or sperm using the RNAeasy Mini Kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions, and RT-PCR analyses were performed as previously described [14]. Briefly, total RNA was reverse transcribed using 500 ng poly-dT and 0.25 U of avian myeloblastosis virus-reverse transcriptase at 42ºC for 75 min and 95ºC for 5 min. For RT-PCR analysis, specific primers pairs (Table 1), dNTP (Promega), *Taq* polymerase and Thermocycle buffer (Promega) were added to the PCR mixture. cDNA products were resolved on 2% (w/v) agarose gels and the cDNA reverse-transcribed from spleen mRNA was used as a positive control.

In vitro fertilization

In vitro fertilization was performed as in our previous study [14]. Briefly, ovulated COCs were collected from mouse oviductal ampullae 16 h after an hCG injection and placed in 150 μl of the HTF medium containing 0.4% (w/v) BSA. Spermatozoa were collected from the cauda epididymidi of adult (4 months old) ICR strain mice and put in 500 μl of the HTF medium. After 60 min, the spermatozoa were introduced into the HTF medium at a final concentration of 1,000 spermatozoa/μl. Twelve hours after insemination, oocytes were washed thoroughly five times, then checked ofr the formation of pronuclei under a phase-contrast microscope.

Artificial insemination of mice

The sperm recovered from cauda epididymidi were incubated for 60 min in 0.4% (w/v) BSA-supplemented HTF medium with or without unmethylated CpG DNA (ODN1826). Then, 50 μl of sperm were surgically injected into uteri of eCG-primed female mice 12 h after hCG injection [2, 16]. Twenty-four after artificial insemination, putative zygotes were collected from oviducts and checked for the formation of pronuclei or cleavage under a phase-contrast microscope.

Statistical analysis

Data from three experimental trials are presented as mean ± S.E.M. Data were analyzed using the Statistical Analysis System Package (SAS Institute Inc., Cary, NC, USA). All percentage data were subjected to arcsine transformation before ANOVA. When ANOVA revealed a significant effect, means were compared using Fisher's protected least significant difference post-hoc test, and were considered to be significant when $P < 0.05$.

Results

Expression of the TLR family that recognizes virus pathogens in mouse sperm

To detect the expression of *Tlr3*, *Tlr7*, *Tlr8* and *Tlr9* mRNA in sperm, total RNA was purified from sperm and then used in a RT-PCR study. The expression of *Tlr3* *Tlr7*, *Tlr8* and *Tlr9* mRNA was detected by each specific primer set in the spleen and brain, but *Tlr7*, *Tlr8* and *Tlr9* were expressed in sperm (Fig. 1A).

ssRNA binds to TLR7/TLR8 present in human macrophage, whereas in mice TLR8 is expressed but has no functional activity [1, 17]. TLR9 is also expressed in macrophages and recognizes unmethylated CpG DNA sequences [18]. Therefore, to examine whether or not the expressed TLR7 or TLR9 had functional activity, the sperm were cultured with ssRNA or unmethylated CpG DNA for up to 6 h. The results show that unmethylated CpG DNA but not ssRNA significantly decreased the sperm motility as compared with that of sperm cultured without any agonists (Fig. 1B). In western blotting, the immunoreactive band at about 90 kDa corresponding to TLR9 was present in the spleen used as a positive control (Fig. 2A). In sperm samples, a 90-kDa band as well as an approximately 120-kDa band were detected by the anti-TLR9 antibody (Fig. 2A). When the sperm was treated with progesterone to dissolve the acrosomal cap, the intensity of the 90-kDa band but not the 120-kDa band (non-specific band, N.S.) was decreased in the acrosome reacted sperm, suggesting that TLR9 is localized in sperm acrosomal region. To clarify the localization in detail, an immunofluorescence study was performed. Whole mount preparations of spermatozoa provided additional evidence that TLR9 is present in the acrosomal region and the midpiece of sperm. (Fig. 2Ba). Signals for TLR9 were also detected at very weak levels in the sperm tail region in the negative control (without primary antibody) (Fig. 2Bb).

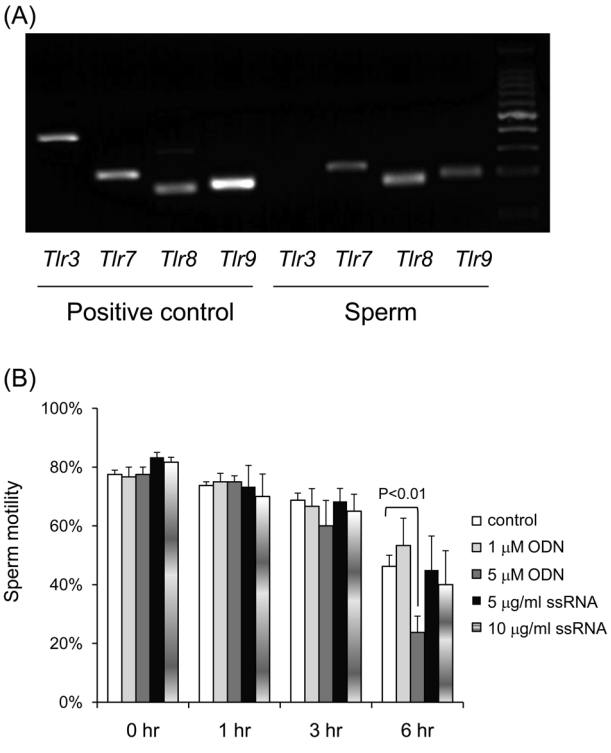

Fig. 1. The expression and function of TLR3, TLR7, TLR8 and TLR9 in sperm; the spleen and brain were used as positive controls. (A) Total RNA was recovered from the spleen or sperm, and then used in the RT-PCR study. PCRs for detection of *Tlr3*, *Tlr7* and *Tlr9* were performed using spleen as a positive control. Brain was used as a positive control for *Tlr8* PCR. (B) The motility of sperm when sperm were cultured with 5 or 10 μg/ml of single strand RNA (ssRNA) or 1 or 5 μM of unmethylated CpG DNA (ODN) for 6 h. A significant difference between control and 5 μM of unmethylated CpG DNA treatment group was observed at 6 h of culture ($P < 0.01$). Control: the sperm were cultured in HTF containing 0.4% (w/v) BSA. Values are mean ± SEM calculated on the basis of 3 experiments.

The negative effects of TLR9 ligand on sperm fertilization activity

The tyrosine phosphorylation of sperm protein was detected as a marker of capacitation [19] by western blotting using pan anti-Tyr phosphorylation antibody. Positive immuno-reactive bands were detected at ~110 kDa, ~75 kDa, 65 kDa and 50 kDa when sperm were cultured with BSA for 60 min (Fig. 3A). The level of phosphorylation of the ~110-kDa band was not dramatically changed by BSA within 30 min of culture whereas other bands were increased (Fig. 3A). However, the intensity of ~75-kDa, 65-kDa and 50-kDa

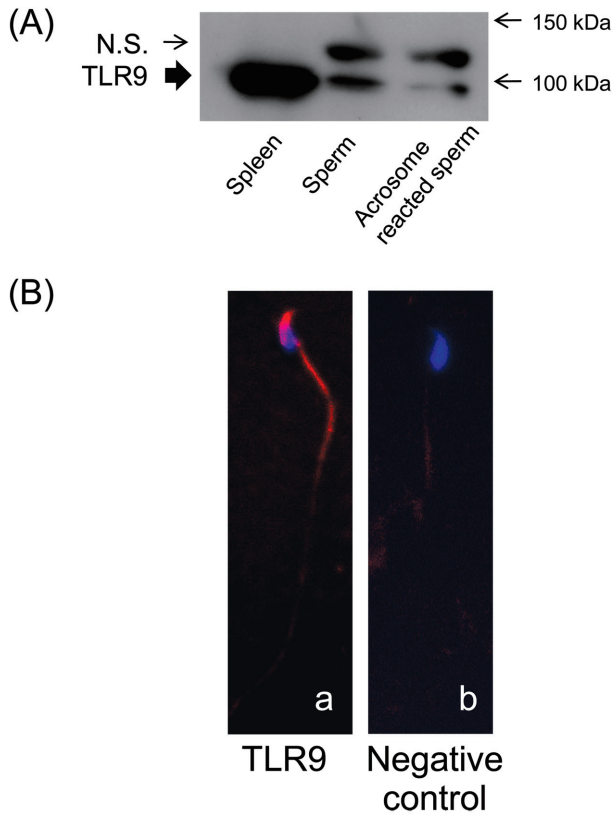

Fig. 2. The localization of TLR9 in sperm. (A) The expression of TLR9 at the protein levels in sperm. Sperm were cultured for 60 min in HTF containing 0.4% (w/v) BSA and then treated with or without progesterone for 1 h. Mouse spleen was used as a positive control for TLR9. N.S.: a non-specific band of about 120 kDa was detected only in sperm, not in spleen, the positive control. (B) TLR9 was localized on mice sperm using anti-mouse monoclonal TLR9 antibody. a) TLR9 in sperm, b) the negative control for TLR9 of sperm without primary antibody. Red is the Cys3 signal conjugating anti-mouse IgG antibody and blue is DAPI staining. The sperm was fixed just after collection and then stained by the antibody.

bands was dramatically decreased in sperm cultured with unmethylated CpG DNA (Fig. 3A).

Loss of the acrosomal cap (acrosome reaction) was detected by FITC-PNA. The rate of FITC-PNA-positive sperm after the treatment with progesterone was significantly decreased as compared with that for control sperm (without progesterone treatment) (Fig. 3B). When the TLR9 ligand, unmethylated CpG DNA, was added to progesterone-containing medium, the rate of FITC-positive sperm was not significantly changed as compared with that for progesterone-treated sperm (Fig. 3B). However, when the sperm were cultured with unmethylated CpG DNA alone, the rate of FITC-positive sperm was significantly decreased as compared with that in sperm without any agonists, and was comparable with that for progesterone-treated sperm (Fig. 3C).

The effects of unmethylated CpG DNA on sperm penetration to oocytes both in vitro and in vivo

Sperm recovered from the epididymidi of SPF mice were cultured with or without unmethylated CpG DNA. The sperm samples were then used in an *in vitro* fertilization protocol or injected into the uteri of female mice as reported previously [2]. The treatment of sperm with unmethylated CpG DNA prior to *in vitro* fertilization significantly decreased the subsequent rate of fertilization as compared with that for sperm pre-cultured with BSA alone (control) (Table 2).

Following this artificial insemination protocol, the fertilization rate in control mice (without ligand treatment) was about 60% whereas the fertilization rate in mice injected with sperm exposed to unmethylated CpG DNA was about 30% (Table 2).

Discussion

Sexually transmitted virus infections interfere with male fertility. In humans, the hepatitis B and C viruses, human immunodeficiency virus (HIV), papillomavirus and herpes simplex virus have been detected in semen, and infection increases the risk of infertility due to a decrease in sperm motility [20–22]. In pig, porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszky's disease virus and porcine parvovirus (PPV) decrease sperm fertilization activity, which results in economic losses in domestic animals [23–25]. The reduction of sperm functions due to virus infections has been thought to be caused by the immune function accumulating leukocytes in semen [26]. Leukocytes express TLR3, TLR7, TLR8 and TLR9 that recognize virus-released pathogens, ssRNA, double strand RNA or unmethylated CpG DNA, then secrete cytokines or chemokine families [27]. However, in this study we clearly showed that sperm also had immune functions that recognize virus infection which decreases their own fertilization activity.

The present study revealed that TLR9 was expressed in sperm. Although details of the mechanism by which sperm fertilization activity is reduced are unknown, a TLR9 specific ligand strongly suppressed BSA-induced tyrosine phosphorylation of sperm protein. The tyrosine phosphorylation of proteins in sperm is thought to be

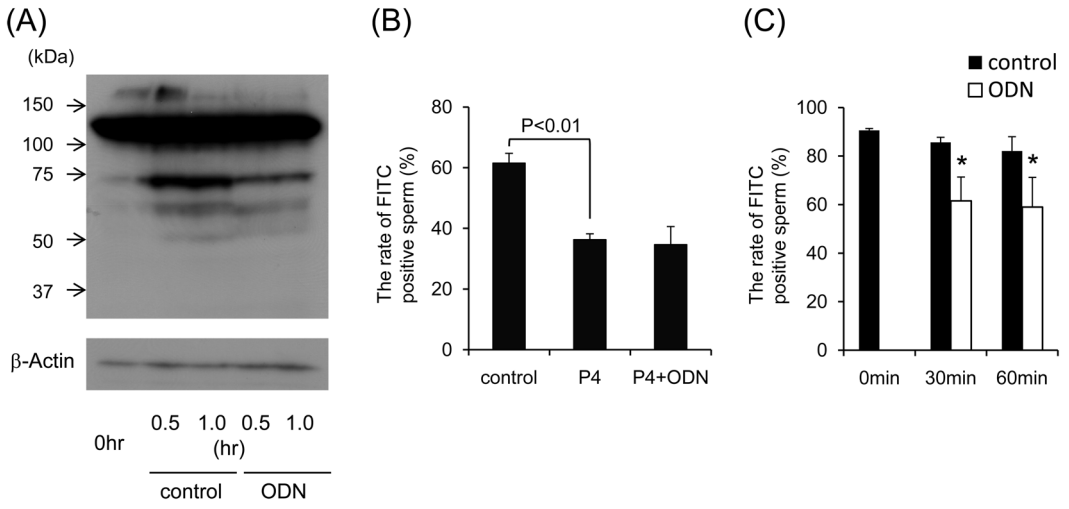

Fig. 3. The effects of TLR9 ligand, unmethylated CpG DNA (ODN), on sperm capacitation and acrosome status. (A) The induction of protein tyrosine phosphorylation in sperm cultured with or without 10 μM of ODN. Tyrosine phosphorylation (P-Tyr) was detected by an anti-phospho-Tyr antibody. Control: sperm were cultured without ODN in HTF medium containing 0.4% (w/v) BSA. (B) The acrosome status was measured using FITC-labeled peanut agglutinin. Sperm were cultured in HTF medium containing 0.4% (w/v) BSA for 60 min and then further cultured with 100 nM of progesterone and/or 10 μM of ODN. Values are mean ± SEM calculated on the basis of 3 experiments. Control: sperm were further cultured in HTF medium after 60-min precultivation. (C) The acrosome status was detected using FITC-labeled peanut agglutinin. Just after collection of sperm, the sperm were cultured with or without 10 μM of ODN in HTF medium containing 0.4% (w/v) BSA. Values are mean ± SEM calculated on the basis of 3 experiments. Control: sperm were cultured in HTF medium containing 0.4% (w/v) BSA. *, The rate of FITC-negative sperm was significantly decreased by the culture with 10 μM of ODN at both 30 and 60 min of culture ($P < 0.05$).

Table 2. *In vitro* and *in vivo* fertilization rates

	Treatment	Number of total oocyte	Number of matured oocyte (%)	Number of fertilized oocyte (%)
in vitro fertilization	Control	52	40 (76.9)	30 (75)[a]
	ODN	50	36 (72.0)	6 (16.7)[b]
in vivo fertilization	Control	58	44 (75.8)	27 (61.4)[c]
	ODN	62	43 (69.4)	13 (30.0)[d]

a, b, c, d: the significant differences were observed between the control and ODN treated group. ODN: the sperm were pre-cultured with 10 μM of ODN for 60 min and then used for fertilization.

critical for the acquisition of a capacitated state [19], suggesting that TLR9 negatively regulates sperm capacitation. However, the rate of FITC-PNA-positive sperm was decreased by the TLR9 ligand as well as by progesterone treatment. It is possible that the increase of FITC-negative sperm was caused by damage to the acrosomal membrane, because sperm motility was significantly decreased by the TLR9 ligand in the present study, and because we showed that a significant positive correlation was observed between the high level of sperm motility and acrosome integrity [28]. It is known that TLR9 resides in the endosome, which is a site of virus replication after infection [29]. This localization of TLR9 is necessary to prevent to insert the virus DNA to host DNA and to identify the virus DNA uniquely [30]. On the other hand, Fischer *et al.* [31] reported that the TLR9 signaling pathway was involved in apoptotic induction in 293 human embryonic kidney fibroblasts. In the present study, we did not detect the apoptosis of sperm in the TUNEL assay

because the ligand, unmethylated CpG DNA, has numerous terminal regions that bind to the TUNEL probe. However, we hypothesize that the TLR9 pathway induces loss of acrosomal integrity and the apoptotic process, suppressing the capacitation of sperm. Our previous [2] study showing the increase of apoptosis by the TLR2 and TLR4 pathways in sperm supports the above hypothesis. Further study is required to clarify the mechanisms by which the TLR9 pathway downregulates sperm fertilization ability.

The expression and function of TLR9 in the acrosomal region of sperm were revealed in this study. The innate immune function reducing sperm fertilization activity, reduces the risk of infecting the fetus with a virus. If semen were infected by a virus, the risk of virus infection would increase in the female reproductive tract. Wira and Fahey [32] in their review presented that in a normal menstrual cycle, there is a window of vulnerability (7–10 days following ovulation) in which the potential for viral infection in the female reproductive tract is enhanced. To reduce the infection risk, especially the HIV risk, a sperm-washing technique to remove seminal plasma that contains the virus was reported in 1992 [33]. Sperm-washing techniques have substantially changed the paradigm of fathering children in serodiscordant couples with male human immunodeficiency virus (HIV) infection, because removing virus from the semen is beneficial for both the fetus and mother [34]. However, the results of the present study appear to support the possibility that not only semen but also sperm is infected, because TLR9 is known to localize within the cytoplasm. Thus, before using sperm from virus-infected semen for artificial insemination or *in vitro* fertilization, a highly sensitive analysis of virus DNA purified from the washed sperm is essential to reduce the fetal infection risk. Savasi *et al.* [35] reported that the sperm from an HIV-positive men was washed and then checked for virus contamination by real-time PCR following artificial insemination (AI), *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). They showed that the overall pregnancy rate (70.3%), independent of the procedure used (AI or IVF/ICSI), justifies the efforts of the individual patient to have a safe pregnancy [35].

In conclusion, sperm expresses and has a functional TLR9 that potentially recognizes virus infection in semen. The ligand for TLR9 reduces the fertilization ability both *in vitro* and *in vivo* due to the suppression of capacitation and a decrease in acrosome integrity. Because TLR9 is known to localize within the cytoplasm, and because it is known that the receptors involved in virus uptake are expressed in sperm, sperm is infected by viruses. To reduce the infection risk, the sperm-washing technique and analysis of the contamination by a high sensitivity method is required for not only the human HIV problem, but also large animal reproductive performance.

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