

Toll-like receptors (TLR) 2 and 4 on human sperm recognize bacterial endotoxins and mediate apoptosis

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BACKGROUND: Bacterial infections of the genital tract are one of the most serious causes of infertility in males. In some human patients with poor semen quality, leukocytospermia has been observed. Because leukocytes express the bacterial-lipopolysaccharide (LPS) responsive Toll-like receptor (TLR) signaling cascade and secrete tumor necrosis factor- α , secreted cytokines comprise one, but probably not the only, class of factors that can impact sperm motility.

METHODS AND RESULTS: In this study, we documented that bacterial endotoxins, LPS and peptidoglycan, can be detected in human semen. Furthermore, the addition of endotoxins in the absence of leukocytes directly and significantly reduced the motility and increased the apoptotic rate of both human and mouse sperm and suppressed fertilization by mouse sperm both *in vivo* and *in vitro*. The well-known LPS receptor, TLR4, and peptidoglycan receptor, TLR2, were expressed in human and mouse sperm. In *Tlr2/4*^{-/-} double-mutant mice, the negative effects of endotoxins on sperm functions were blocked, suggesting that the bacterial endotoxins mediated activation of TLR-dependent pathways in sperm leading to apoptosis.

CONCLUSIONS: Sperm can recognize bacterial endotoxins by TLRs present in their membranes. The activated TLRs reduce sperm motility, induce sperm apoptosis and significantly impair the potential for fertilization.

Key words: peptidoglycan / lipopolysaccharide / sperm motility / sperm apoptosis / Toll-like receptors

Introduction

Bacterial infection of the genital tract increases the risk of male infertility (Anderson and Hill, 1988; Diemer *et al.*, 2000a; Eley *et al.*, 2005; Ochsendorf, 2008). In bacteria-infected semen specimens, the number of leukocytes is increased concomitantly with a decrease in sperm motility, which is called as leukocytospermia (Saleh *et al.*, 2002). Leukocytes express pattern recognition receptors, including members of the Toll-like receptor (TLR) family, and activated TLRs induce the expression of genes encoding cytokines and chemokines (Takeda and Akira, 2004, 2005). Tumor necrosis factor- α (TNF- α) is one TLR target gene, and secreted TNF- α plays an important role in the induction of apoptosis through binding with the type I TNF- α receptor (TNFR1, Agarwal, 2003). Perdichizzi *et al.* (2007) reported that the addition of TNF- α to human ejaculates decreased sperm motility and induced apoptosis, suggesting that the down-regulation

of sperm motility by bacterial infection was caused primarily by the release of cytokines from the accumulated leukocytes. However, this may be one but not the only mechanism by which sperm mobility is altered in bacteria-infected ejaculates (Diemer *et al.*, 2000b; Fraczek and Kurpisz, 2007).

Recently, several groups have reported that sperm present immune cell-like functions. Members of the chemokine receptor family (CCR and CXCR) are expressed on sperm (Isobe *et al.*, 2002; Cardona-Maya *et al.*, 2006; Shimada *et al.*, 2008; Tamba *et al.*, 2008). When activated by chemokines secreted by cumulus cells, sperm exhibit a chemotactic response to reach the ovulated oocyte during the fertilization process (Shimada *et al.*, 2008; Tamba *et al.*, 2008). Although Palladiano *et al.* (2008) showed the expression of 10 kinds of TLR family members in rat sperm, the role of TLRs in male germ cells in semen has remained unclear. From these reports, we hypothesized that sperm respond to bacteria present in semen not only because of

the production and release of TNF- α from infiltrated leukocytes but also by direct activation of TLRs by bacterial endotoxins. Furthermore, the effects of endotoxins and TLR activation in sperm on IVF including ICSI or conventional IVF in human infertility care have not been reported.

To address the above hypothesis, we identified the kind of bacteria present in infected human semen, the endotoxin levels [lipopolysaccharide (LPS) and peptidoglycan] and the expression of TLRs in human and mouse sperm. In addition, wild-type (WT), *Tlr2* and/or *Tlr4* mutant mice were used to determine the functions of TLR2/4 in sperm in more detail.

Materials and Methods

Materials

LPS was purchased from Sigma Chemical Co. (L 4391, Sigma, St Louis, MO, USA) and Pam3Cys-Sr-(LYS)₄ (Pam3Cys) from Calbiochem (Los Angeles, CA, USA). For western blot analyses, the anti-TLR4 antibody was purchased from IMGENEX (San Diego, CA, USA) and anti-TLR2 antibody from Cell Signaling Technology Inc. (Danvers, MA, USA). Equine chorionic gonadotrophin, eCG, was purchased from Asuka Seiyaku (Tokyo, Japan). hCG was purchased from Asuka Seiyaku. Oligonucleotide poly-(dT) was purchased from GE Healthcare (Buckinghamshire, UK), and AMV reverse transcriptase and Taq polymerase were from Promega (Madison, WI, USA). Bovine serum albumin (BSA, fraction V) was obtained from Sigma (A 7888). Routine chemicals and reagents were obtained from Nakarai Chemical Co (Osaka, Japan) or Sigma.

Collection of semen from men and analysis of semen motility

Infertile couples visited Women's Clinic Oizumi-Gakuen, Tokyo, Japan, to undergo sperm tests for IVF. A total of 372 infertile men consented to enter this study during the period from February 2009 to December 2010. Semen samples were collected by masturbation after 3–5 days of abstinence, and the sperm parameters were evaluated according to the WHO criteria (World Health Organization, 1999) including the volume of semen, sperm concentration, motility and morphology as well as leukocyte concentration. Semen samples obtained from each individual patient were tested separately. After 30 min liquefaction at 37°C, the semen was diluted 2-fold (~2–5 × 10⁶ sperm/ml) by human follicular fluid (HFF99) medium containing antibiotics, and then was incubated for 3 or 6 h at 37°C under 5% CO₂ atmosphere. At the end of incubation, we checked sperm motility. In some samples, 100 ng/ml of LPS or 10 μg/ml of the synthetic peptidoglycan, Pam3Cys, was added to the culture medium when sperm recovered from bacteria-free semen were used for culture. In other cases, when sperm was recovered from bacteria-infected semen, 100 μg/ml of polymyxin B (PMB) (Pfizer Inc., New York City, NY, USA) was added to the diluted semen. All patients gave written informed consent to participate in this study.

Isolation of bacteria in human seminal plasma

Aerobic cultures (6.9% CO₂) were performed on all samples ($n = 372$) at 37°C for 24 h using Trypticase soy agar with 5% sheep blood or Brom thymol blue lactose agar. Subsequently, respective bacterial colonies were isolated and each colony was further cultured with Trypticase soy agar with 5% sheep blood or Brom thymol blue lactose agar 37°C for room temperature. Identification of the bacterial strain and assays of

drug sensitivity were carried out using Vitek (Sismex Biomerieux, Tokyo, Japan).

Detection of LPS in human semen by limulus test

The level of LPS in seminal plasma was measured by the limulus test (Elin et al., 1975) using limulus ES-II single test WAKO (WAKO Chemical Co., Osaka, Japan). Briefly, samples were diluted with endotoxin-free distilled water, and then heated at 70°C for 10 min (Greenlee and Banks, 1985). After centrifugation, the supernatant was mixed with limulus amebocyte lysate for up to 120 min at 30°C. The reaction was detected by Toxinometer ET-2000 (WAKO Chemical Co.).

Detection of peptidoglycan in human semen

The level of peptidoglycan in seminal plasma was measured by the silkworm larvae plasma (SLP) test that is based on a cascade reaction triggered by peptidoglycan to produce melanin using SLP-HS Single Reagent Set (WAKO Chemical Co.). The semen samples were diluted with endotoxin-free distilled water, and then heated at 90°C for 10 min. After centrifugation, the supernatant was mixed with SLP reagent derived from the hemolymph of the silkworm, *Bombyx mori*, and 3,4-dihydroxyphenylalanine. The level of melanin produced by above reaction was measured by Toxinometer ET-2000.

Animals

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 (i.p.) 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 IVF (Shimada et al., 2008). For artificial insemination study, the adult female ICR mice were injected i.p. with 5 IU of eCG to stimulate follicular growth followed 48 h later with 5 IU hCG. These mice were housed under a 16 h light/8 h dark schedule in Experiment Animal Center at Hiroshima University.

TLR2- and TLR4-deficient mice have been described (Takeuchi et al., 1999; Jiang et al., 2005). We crossed *Tlr2*^{-/-} and *Tlr4*^{-/-} mice for six generations before use. C57Bl/6 mice were obtained from Jackson Laboratories. The mutant mice were housed in a pathogen-free facility under a 12 h light/12 h dark schedule at Baylor College of Medicine, Houston, TX, USA. All 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 Baylor College of Medicine or Hiroshima University.

IVF of mouse

COCs were collected from mouse oviductal ampullae 16 h after the hCG injections and placed in 150 μl of human tubal fluid (HTF) medium. Spermatozoa were collected from the cauda epididymi of adult (4-month old) ICR strain mice into 500 μl of HTF medium. After 60 min incubation with or without 10 μg/ml of Pam3Cys, the spermatozoa were introduced into the fertilization medium at a final concentration of 1000 spermatozoa/μl. After 12 h after insemination, oocytes were washed thoroughly five times, and then checked the formation of pronuclei under a phase-contrast microscopy.

Artificial insemination of mouse

The sperm recovered from cauda epididymi were incubated for 60 min in HTF medium with or without bacteria. Then, 50 μl of sperm were

surgically injected into the uterus of eCG-primed female mice 12 h after hCG injection (Kile, 1951).

Evaluation of apoptosis

To detect apoptotic sperm, we used the terminal deoxynucleotidyl transferase (Tdt)-mediated biotinylated deoxyuridine triphosphates (dUTP) nick end-labeling (TUNEL) method (In-Situ Cell Death Detection Kit, Roche Diagnostics GmbH, Mannheim, Germany). The fluorescent staining of spermatozoa was monitored and counted by an epifluorescence microscope, and apoptotic cells (containing fragmented DNA) were also detected by this method. Briefly, the slides were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), and then incubated with TUNEL reaction mixture in a humidified chamber at 37°C for 60 min. The presence of TUNEL-positive signal in each cell was determined by fluorescent microscopy.

Western blot analyses

Protein samples from human or mouse sperm were prepared by homogenization in Laemmli sample buffer. The samples were heated at 100°C for 5 min, and 20 µl of each sperm extract was loaded in 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 with primary antibody (as listed above) overnight at 4°C. After washing in TBST, enhanced chemiluminescence (ECL) detection was performed by using ECL 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). Human leukocytes or mouse spleen were used as the positive control for TLR4 and TLR2.

Immunofluorescence

Collected spermatozoa were washed in PBS to remove seminal plasma, and diluted 1:10 (v/v) with 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 4°C. Following a rinse with PBS, the slides were incubated with Mouse Ig blocking reagent of Vector M.O.M. Immunodetection kit (Vector Laboratories Inc., Burlingame, CA, USA) for 1 h. The slides were then sequentially probed with primary anti-TLR4 or anti-TLR2 antibodies (diluted 1:500, In VivoGen, San Diego, CA, USA) and secondary Cy3 or FITC-conjugated goat anti-rat (mouse) IgG antibody (1:250, Sigma). Slides were mounted using VectaShield with 4',6-diamidino-2-phenylindole (Vector Laboratories). The negative controls for TLR2 and TLR4 were human sperm cultured without primary antibody.

RT–PCR analyses

Total RNA was obtained from human sperm using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions and RT–PCR analyses were performed as previously described (Shimada *et al.*, 2008). Briefly, total RNA was reverse transcribed using 500 ng poly-dT (Amersham Pharmacia Biotech, Newark, NJ, USA) and 0.25 U avian myeloblastosis virus-reverse transcriptase (Promega Corp., Madison, WI, USA) at 42°C for 75 min and 95°C for 5 min. For RT–PCR analysis, specific primers pairs (TLR2; F: 5'-GAGCGAGTGGTGCAAGTACG-3', R: 5'-ACCAGCATCGGACCAAGACT-3', TLR4; F: 5'-TTGGTCAGCCTCCAAACCTT-3', R: 5'-CCA GAATCCTGAGGGAGTGG-3'), dNTP (Promega), Taq polymerase and Thermocycle buffer (Promega) were added to PCR mixture. cDNA products were resolved on 2% (w/v) agarose gels. The PCR primers were

designed in deferent exon regions, and the amplified cDNAs were directly sequenced according to our previous study to verify their authenticity.

Statistical analysis

Data from three ejaculate trials in each patient are presented as mean \pm SEM. Data were analyzed using the Statistical Analysis System Package (SAS Institute Inc., Cary, NC, USA). All percentage data were subjected to arcsine transformation before analysis of variance (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

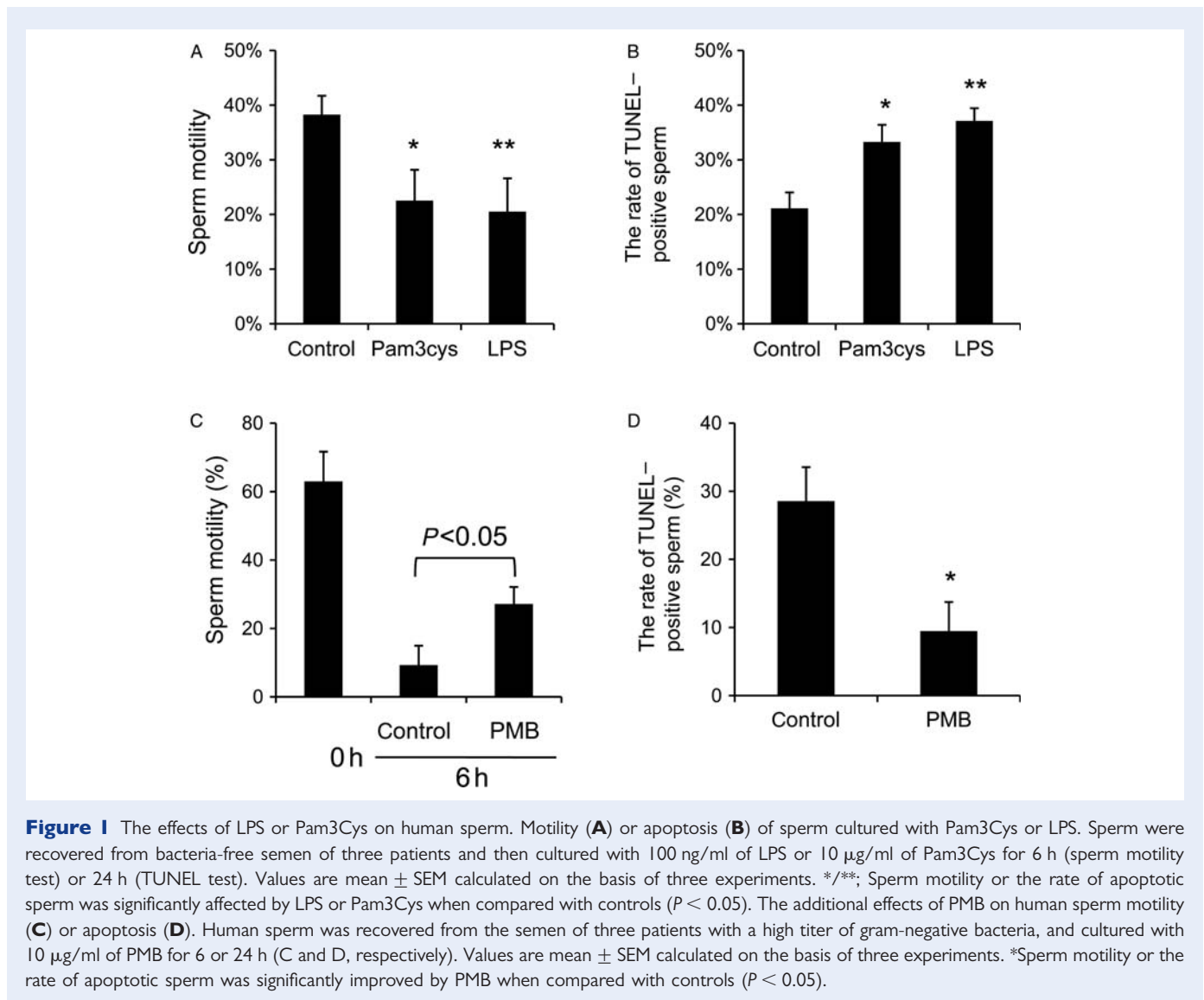
Bacteria present in seminal plasma and release endotoxins (LPS and Peptidoglycan) suppress sperm motility

The presence of bacteria in human semen was determined first by collecting semen samples from 372 patients with a mean age of 37.78 years (range, 25–54) and then culturing the seminal plasma in agar without antibiotics. Bacterial colonies were observed in 119 (9 gram-negative; 110 gram-positive; double positive 0) of the 372 samples but were absent in 253 samples (Table I). Gram-negative bacteria release the endotoxin LPS, whereas gram-positive bacteria release peptidoglycan. Both endotoxins were detected in human semen (LPS; $1.275 \pm 0.931 \mu\text{g/ml}$, peptidoglycan; $3.265 \pm 1.694 \mu\text{g/ml}$); however, the variation in concentrations among the samples was large.

To determine the effects of LPS or of the synthetic gram-positive bacterial peptidoglycan, Pam3Cys, on sperm motility, sperm was recovered from bacteria-free semen samples and cultured with LPS or Pam3Cys for 6 h. Sperm motility was significantly decreased by either LPS or Pam3Cys (Fig. 1A). The rate of apoptosis (TUNEL-positive sperm) was significantly higher in the treatment groups than in the controls (Fig. 1B). Because PMB binds directly to LPS and thereby neutralizes

Table I Isolation of bacterial infections in male patients.

Non-infected patients	253
Infected patients	119
Gram-positive bacteria	110
<i>Staphylococcus epidermidis</i>	39
<i>Streptococcus agalactiae</i>	50
<i>Enterococcus</i> sp.	29
<i>Corynebacterium</i> sp.	4
Gram-negative bacteria	9
<i>Proteus mirabilis</i>	1
<i>Acinetobacter</i> sp.	2
<i>Escherichia coli</i>	3
<i>Chryseobacterium</i> sp.	2
<i>Neisseria</i> sp.	1
Total	372



its endotoxic activity (Greenlee and Banks, 1985), PMB (100 µg/ml) was added to human semen samples infected with gram-negative bacteria. Strikingly, PMB significantly increased the motility of sperm exposed to infected serum for 6 h even when the semen samples contained high titers of gram-negative bacteria (Fig. 1C), while the rate of apoptotic sperm was decreased by PMB treatment (Fig. 1D).

TLR2 and TLR4 are expressed in human sperm

The gram-negative bacteria factor, LPS, binds directly to TLR4–CD14 complexes present on macrophage cell membranes (Poltorak et al., 1998; Hoshino et al., 1999; Means et al., 1999; Qureshi et al., 1999) and also on cumulus cells of ovulated COCs (Shimada et al., 2006). TLR2 is also expressed on macrophages and recognizes gram-positive bacterial factors (Müller et al., 2001). Therefore, the expression and localization of TLR2 and TLR4 were analyzed in human sperm. As shown by RT–PCR and western blots (Fig. 2A and B), both TLR2 and TLR4 were detected in human sperm as well as in leukocyte controls (Fig. 2B). Immunofluorescence analyses showed further that

TLR2 and TLR4 were localized to the acrosomal and tail regions of human sperm (Fig. 2C).

Effects of LPS or Pam3Cys on sperm functions in TLR4 and/or TLR2 knockout mice

To determine the function(s) of TLRs in sperm in more detail, we analyzed the effects of LPS or Pam3Cys on sperm in specific mutant mouse models. *Tlr2*^{−/−}, *Tlr4*^{−/−} and *Tlr2*^{−/−}; *Tlr4*^{−/−} male mice were used for this study. Mouse sperm recovered from WT mice expressed both TLR2 and TLR4 (Fig. 3A). Sperm motility decreased significantly in WT mice exposed to 100 ng/ml of LPS or 10 µg/ml of Pam3Cys for 6 h (Fig. 3B), whereas LPS had little or no effect on sperm motility in *Tlr4*^{−/−} mice or *Tlr2/4* double knockout mice (Fig. 3B). Pam3Cys significantly suppressed the motility of sperm from *Tlr4*^{−/−} mice, whereas the negative effect was not observed in *Tlr2*^{−/−} mice or *Tlr2/4* double knockout mice. Increased numbers of TUNEL-positive sperm were observed in WT mice

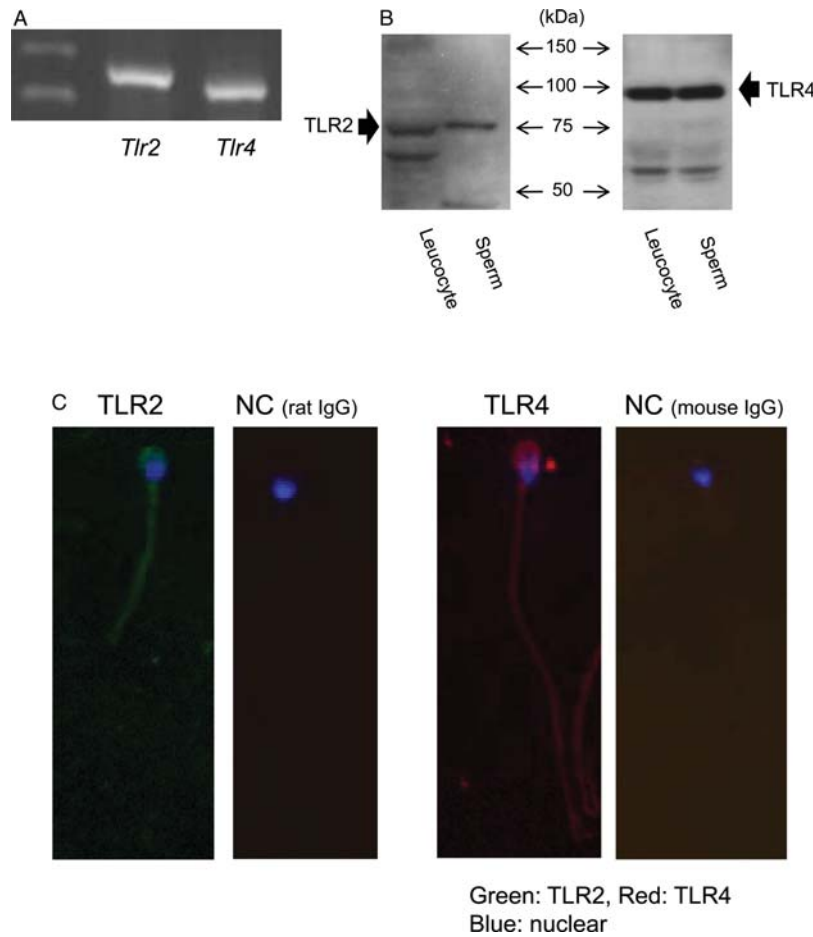


Figure 2 The expression of TLR2 and TLR 4 in human sperm. **(A)** The expression of *TLR2* and *TLR4* mRNA in human sperm was detected by RT-PCR. **(B)** The expression of TLR2 and TLR4 protein was detected by western blot analyses in human sperm. Human leukocytes were used as positive control cells for TLRs. **(C)** The TLR2 and TLR4 were localized on human sperm using anti-rat monoclonal TLR2 antibody or anti-mouse monoclonal TLR4 antibody. The negative controls (NC) for TLR2 and TLR4 were human sperm cultured without primary antibody. Results in each study are representative of three separate experiments.

exposed to LPS or Pam3Cys but not in each *Tlr* mutant mice (Fig. 3C). These results provide strong evidence that the bacterial endotoxins LPS and Pam3Cys can exert apoptotic effects in sperm by activating the TLR2/4 signaling pathways.

Negative effects of bacterial endotoxins on sperm fertilization activity

When sperm were pre-cultured with either LPS or Pam3Cys prior to IVF protocols, the subsequent rate of fertilization was significantly lower in WT mice compared with *Tlr2/4* double-mutant mice (Fig. 4A). Sperm recovered from the epididymis of mice were cultured with gram-negative bacteria with or without PMB in the presence of penicillin G and streptomycin (to suppress bacterial proliferation). The sperm samples were then injected into uteri of female mice as reported previously (Kile, 1951). Following this artificial insemination protocol, the fertilization rate in control mice was ~60%, whereas the fertilization rate in mice injected with sperm that had been exposed to the higher titer of bacteria was <20% (Fig. 4B).

However, the presence of PMB reversed the negative effects of bacteria and increased fertilization rates to ~50%, suggesting that it was LPS secreted by the bacteria which reduced the *in vivo* fertilization ability of mouse sperm. We did not observe altered uterine or oviductal functions, regarding expression levels of cytokine families, as a consequence of the insemination protocols (data not shown).

Discussion

Bacterial infections of male accessory sex glands are associated with known causes of infertility in men (Eley *et al.*, 2005; Ochsendorf, 2008; Moretti *et al.*, 2009). In this study, bacterial infections were detected in seminal plasma from 119 of 372 men. Additionally, we detected the gram-negative bacteria factor, LPS, and gram-positive bacteria factor, peptidoglycan, in human semen. The addition of PMB, an LPS antagonist, to sperm culture media preserved sperm motility and viability in semen infected with a high titer of gram-negative bacteria. In the absence of infection, both LPS and the synthetic gram-positive bacterial endotoxin, Pam3Cys, significantly

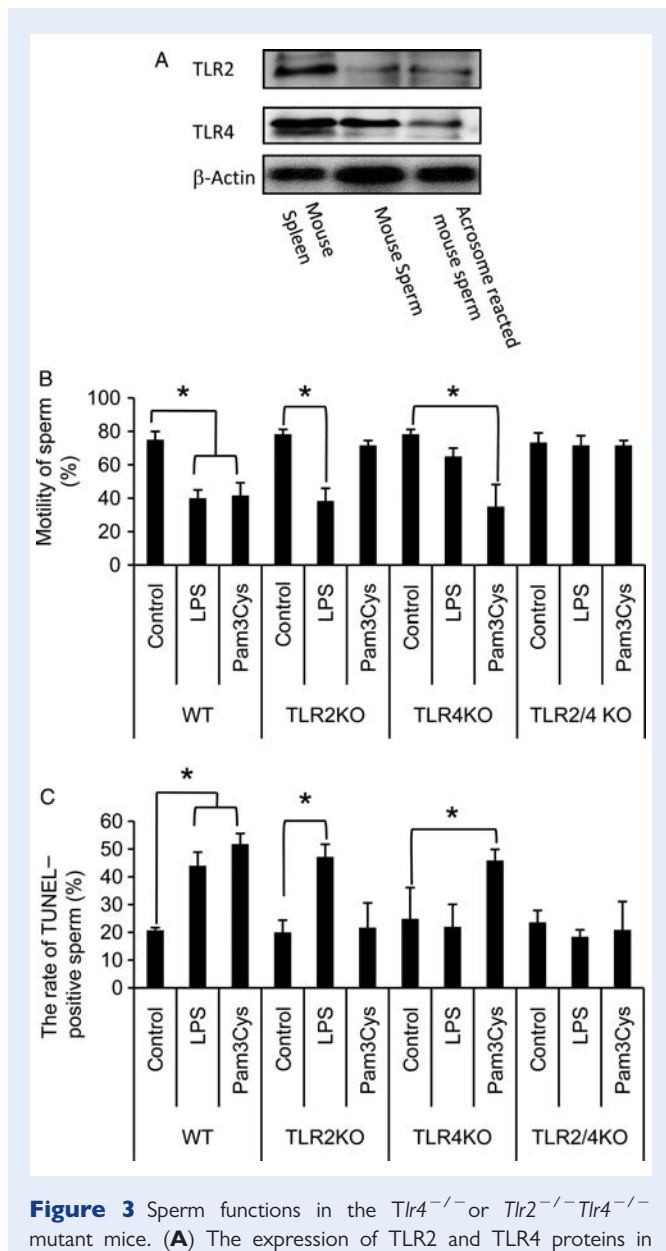


Figure 3 Sperm functions in the $Tlr4^{-/-}$ or $Tlr2^{-/-}Tlr4^{-/-}$ mutant mice. **(A)** The expression of TLR2 and TLR4 proteins in mouse sperm. Mouse spleen was used as the positive control cells for TLRs. **(B)** The effect of bacterial endotoxins on motility of sperm was examined using WT, $Tlr2^{-/-}$, $Tlr4^{-/-}$ and $Tlr2^{-/-}Tlr4^{-/-}$ mutant mice. Mouse sperm were cultured with 100 ng/ml of LPS or 10 μ g/ml of Pam3Cys for 3 h. Values are mean \pm SEM calculated on the basis of three experiments. *Sperm motility was significantly suppressed by LPS or Pam3Cys when compared with controls ($P < 0.05$). **(C)** Mouse sperm were cultured with 100 ng/ml of LPS or 10 μ g/ml of Pam3Cys for 6 h. Apoptosis of mouse sperm was detected by the TUNEL method. Values are mean \pm SEM calculated on the basis of three experiments. *The apoptotic rate was significantly increased by LPS or Pam3Cys when compared with controls ($P < 0.05$). More than 200 spermatozoa were evaluated for every sample/each experiment.

decreased sperm motility and increased the number of apoptotic sperm. Therefore, it is likely that the reduced sperm motility and enhanced rate of apoptosis caused by bacterial infection is mediated

primarily, or at least in part, by bacterial production and release of LPS and peptidoglycan.

Using mouse sperm recovered from the epididymis of pathogen-free knockout mice, we showed that LPS and Pam3Cys (in the absence of immune cells and immune cell-secreted cytokines and chemokines) suppressed sperm motility by binding to and activating TLR2/4. Eley et al. (2005) investigated how human sperm function was suppressed by *Chlamydia trachomatis* infection and showed that co-culture of sperm with LPS-induced apoptosis. Herein we show that TLR2 and TLR4 mRNAs and protein are expressed in human and mouse sperm and that the receptors are localized to the acrosomal and tail regions of the sperm. In $Tlr2/4^{-/-}$ mice that lack receptors for endotoxins, neither LPS nor Pam3Cys increased the number of TUNEL-positive cells. During LPS activation of apoptosis in immune cells, TNFR1-associated death domain protein (TRADD) was critical in both the TNFR1- or TLR4-dependent mechanisms (Hsu et al., 1996; Chen et al., 2008). In TRADD-deficient mice, the phosphorylation of nuclear factor κ B and activation of the caspase pathway were not induced by either TNF- α or LPS treatment (Ermolaeva et al., 2008). Aliprantis et al. (2000) reported that activated TLR2 mediates apoptosis through MyD88 and by a pathway involving Fas-associated death domain protein that directly binds TRADD in a manner similar to that in the TLR4-dependent pathway. Additionally, it is known that TNF- α secreted from leukocytes in semen induces sperm apoptosis (Perdichizzi et al., 2007; Allam et al., 2008). Therefore, because apoptosis can be activated in sperm by both the TNF- α -TNFR1 and TLRs pathways, we suggest that sperm respond to bacterial infection in semen by direct TLR-regulated mechanisms and to leukocytes via a TNF- α -dependent pathway.

Fertilization competence of sperm from mice was also impaired by bacterial-endotoxins both *in vivo* and *in vitro* in a TLR-dependent manner. Specifically, the rate of fertilization *in vivo* was $<20\%$ when the sperm were exposed to LPS, suggesting that the apoptotic response may be required to ensure the removal of poor quality sperm. Studies have shown that a high percentage apoptotic sperm predicts low developmental competence following IVF, and even increases the rate of pregnancy loss after embryo transfer in assisted reproductive technology for infertility patients (Borini et al., 2006).

In 25% of infertile couples, sperm motility was over 50% immediately after collection but dramatically decreased within 3 h. The addition of PMB to human semen samples immediately after collection of sperm improved the sperm motility during culture in gram-negative bacteria-infected samples. Based on the studies presented herein, we conclude that bacterial infections may be an underlying cause of poor sperm quality, at least in some men. Thus, treatment of sperm with PMB is considered to improve assisted reproductive technology protocols not only for humans but also for domestic animals such as the pig where bacterial infections are also common. Although only a few papers have documented the positive effects of PMB on sperm motility during long storage (Sullivan et al., 1966; Vaillancourt et al., 1993), we clearly showed in the recent study that the combined treatment of pig ejaculates with PMB and Penicillin G provide an improved method by which to maintain *in vivo* fertilization competence in semen samples that are contaminated with bacteria (Okazaki et al., 2010). However, a major problem remains because there is no beneficial antibiotic to neutralize the endotoxic activity released by gram-positive bacteria that are the most prevalent type of bacteria in human semen.

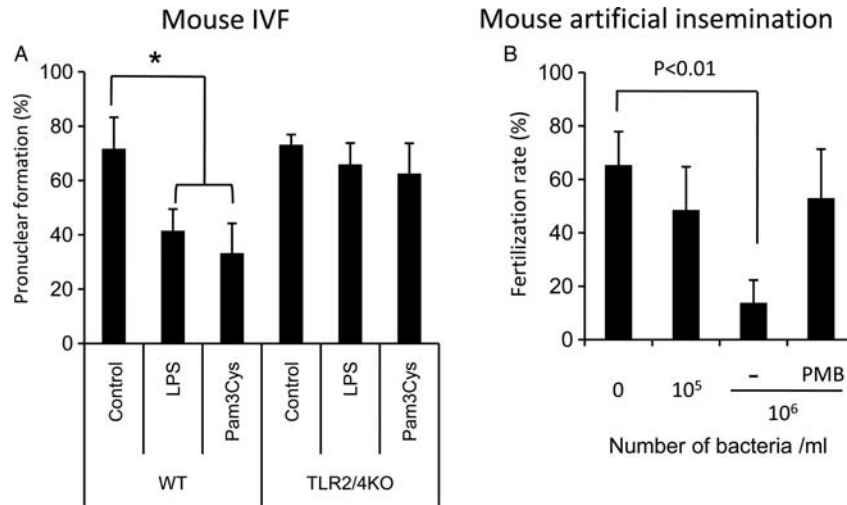


Figure 4 The effects of bacterial endotoxins on mouse sperm fertilization ability both *in vivo* and *in vitro*. **(A)** The effects of Pam3Cys or LPS on IVF activity of mouse sperm. Sperm were collected from cauda epididymi of three WT mice or three *Tlr2*^{-/-}*Tlr4*^{-/-} mutant mice. After sperm were cultured for 60 min with medium alone or with Pam3Cys or LPS, the sperm were introduced into the fertilization medium at a final concentration of 1000 spermatozoa/ μ l. At 12 h after insemination, oocytes were checked for the formation of pronuclei under phase-contrast microscopy. Values are mean \pm SEM calculated on the basis of three experiments. *Pronuclear formation rate was significantly suppressed by LPS or Pam3Cys when compared with controls ($P < 0.05$). **(B)** The *in vivo* fertilization activity of mouse sperm pre-cultured with bacteria for 1 h was reduced. Mouse sperm (10^8 sperm/ml) were incubated for 60 min with bacteria with or without 100 μ g/ml of PMB, and then 50 μ l of sperm samples were surgically injected into the uterus of hormonally primed (eCG/hCG) female mice (two mice per treatment group/each experiment) 12 h post-hCG. Twelve hours after insemination, oocytes were checked for the formation of pronuclei under a phase-contrast microscopy. Data are presented as the percent of oocytes fertilized. Values are mean \pm SEM calculated on the basis of five experiments.

For pig sperm, rapid removal of seminal plasma is the most useful technique for maintaining high quality sperm functions (Kawano *et al.*, 2004; Okazaki *et al.*, 2009). Thus, to reduce the risk of decreasing sperm quality during sperm handling in human assisted reproductive technology, we propose that semen be immediately treated with PMB, and then centrifuged to remove the seminal plasma just after liquefaction.

In conclusion, sperm possess some innate immune cell functions; they possess TLRs and respond to the bacterial endotoxins LPS and peptidoglycan by exhibiting reduced motility and fertilization competence. Thus, to enhance sperm quality for reproductive technology procedures, the addition of the LPS antagonist PMB and removal of seminal plasma could provide safeguards for maintaining sperm viability and function.

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References

- Agarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003;**3**:745–756.
- Aliprantis AO, Yang RB, Weiss DS, Godowski P, Zychlinsky A. The apoptotic signaling pathway activated by Toll-like receptor-2. *EMBO J* 2000;**19**:3325–3336.
- Allam JP, Fronhoffs F, Fathy A, Novak N, Oltermann I, Bieber T, Schuppe HC, Haidl G. High percentage of apoptotic spermatozoa in ejaculates from men with chronic genital tract inflammation. *Andrologia* 2008;**40**:329–334.
- Anderson DJ, Hill JA. Cell-mediated immunity in infertility. *Am J Reprod Immunol Microbiol* 1988;**17**:22–30.
- Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, Coticchio G. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Hum Reprod* 2006;**21**:2876–2881.
- Cardona-Maya W, López-Herrera A, Velilla-Hernández P, Rugeles MT, Cadavid AP. The role of mannose receptor on HIV-1 entry into human spermatozoa. *Am J Reprod Immunol* 2006;**55**:241–245.
- Chen NJ, Chio II, Lin WJ, Duncan G, Chau H, Katz D, Huang HL, Pike KA, Hao Z, Su YW *et al.* Beyond tumor necrosis factor receptor: TRADD signaling in toll-like receptors. *Proc Natl Acad Sci USA* 2008;**105**:12429–12434.

- Diemer T, Ludwig M, Huwe P, Hales DB, Weidner W. Influence of urogenital infection on sperm function. *Curr Opin Urol* 2000a;**10**:39–44.
- Diemer T, Huwe P, Michelmann HW, Mayer F, Schiefer HG, Weidner W. *Escherichia coli*-induced alterations of human spermatozoa. An electron microscopy analysis. *Int J Androl* 2000b;**23**:178–186.
- Eley A, Pacey AA, Galdiero M, Galdiero M, Galdiero F. Can *Chlamydia trachomatis* directly damage your sperm? *Lancet Infect Dis* 2005;**5**:53–57.
- Elin RJ, Robinson RA, Levine AS, Wolff SM. Lack of clinical usefulness of the limulus test in the diagnosis of endotoxemia. *N Engl J Med* 1975;**293**:521–524.
- Ermolaeva MA, Michallet MC, Papadopoulou N, Utermöhlen O, Kranidioti K, Kollias G, Tschopp J, Pasparkis M. Function of TRADD in tumor necrosis factor receptor I signaling and in TRIF-dependent inflammatory responses. *Nat Immunol* 2008;**9**:1037–1046.
- Fraczek M, Kurpisz M. Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa. *J Androl* 2007;**28**:325–333.
- Greenlee A, Banks KL. Cellular and lipopolysaccharide subunit requirements for the caprine lymphoblastogenic response to endotoxin. *Am J Vet Res* 1985;**46**:75–79.
- Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999;**162**:3749–3752.
- Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-I signaling complex. *Immunity* 1996;**4**:387–396.
- Isobe T, Minoura H, Tanaka K, Shibahara T, Hayashi N, Toyoda N. The effect of RANTES on human sperm chemotaxis. *Hum Reprod* 2002;**17**:1441–1446.
- Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, Garg HG, Quinn DA et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat Med* 2005;**11**:1173–1179.
- Kawano N, Shimada M, Terada T. Motility and penetration competence of frozen-thawed miniature pig spermatozoa are substantially altered by exposure to seminal plasma before freezing. *Theriogenology* 2004;**61**:351–364.
- Kile JC Jr. An improved method for the artificial insemination of mice. *Anat Rec* 1951;**109**:109–117.
- Means TK, Lien E, Yoshimura A, Wang S, Golenbock DT, Fenton MJ. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J Immunol* 1999;**163**:6748–6755.
- Moretti E, Capitani S, Figura N, Pammolli A, Federico MG, Giannerini V, Collodel G. The presence of bacteria species in semen and sperm quality. *J Assist Reprod Genet* 2009;**26**:47–56.
- Müller MR, Pfannes SD, Ayoub M, Hoffmann P, Bessler WG, Mittenbühler K. Immunostimulation by the synthetic lipopeptide P3CSK4: TLR4-independent activation of the ERK1/2 signal transduction pathway in macrophages. *Immunology* 2001;**103**:49–60.
- Ochsendorf FR. Sexually transmitted infections: impact on male fertility. *Andrologia* 2008;**40**:72–75.
- Okazaki T, Abe S, Yoshida S, Shimada M. Seminal plasma damages sperm during cryopreservation, but its presence during thawing improves semen quality and conception rates in boars with poor post-thaw semen quality. *Theriogenology* 2009;**71**:491–498.
- Okazaki T, Mihara T, Fujita Y, Yoshida S, Teshima H, Shimada M. Polymyxin B neutralizes bacteria-released endotoxin and improves the quality of boar sperm during liquid storage and cryopreservation. *Theriogenology* 2010;**74**:1691–1700.
- Palladiano MA, Savarese MA, Chapman JL, Dughi MK, Plaska D. Localization of toll-like receptors on epididymal epithelial cells and spermatozoa. *Am J Reprod Immunol* 2008;**60**:541–555.
- Perdichizzi A, Nicoletti F, La Vignera S, Barone N, D'Agata R, Vicari E, Calogero AE. Effects of tumour necrosis factor-alpha on human sperm motility and apoptosis. *J Clin Immunol* 2007;**27**:152–162.
- Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;**282**:2085–2088.
- Qureshi ST, Larivière L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 1999;**189**:615–625.
- Saleh RA, Agarwal A, Kandirali E, Sharma RK, Thomas AJ, Nada EA, Evenson DP, Alvarez JG. Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fertil Steril* 2002;**78**:1215–1224.
- Shimada M, Hernandez-Gonzalez I, Gonzalez-Robanya I, Richards JS. Induced expression of pattern recognition receptors in cumulus oocyte complexes: novel evidence for innate immune-like functions during ovulation. *Mol Endocrinol* 2006;**20**:3228–3239.
- Shimada M, Yanai Y, Okazaki T, Noma N, Kawashima I, Mori T, Richards JS. Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. *Development* 2008;**135**:2001–2011.
- Sullivan JJ, Elliott FI, Bartlett DE, Murphy DM, Kuzdas CD. Further studies on use of polymyxin B sulfate with dihydrostreptomycin and penicillin for control of *Vibrio fetus* in a frozen semen process. *J Dairy Sci* 1966;**49**:1569–1571.
- Takeda K, Akira S. TLR signaling pathways. *Semin Immunol* 2004;**16**:3–9.
- Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005;**17**:1–14.
- Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999;**11**:443–451.
- Tamba S, Yodoi R, Segi-Nishida E, Ichikawa A, Narumiya S, Sugimoto Y. Timely interaction between prostaglandin and chemokine signaling is a prerequisite for successful fertilization. *Proc Natl Acad Sci USA* 2008;**105**:14539–14544.
- Vaillancourt D, Guay P, Higgins R. The effectiveness of gentamicin or polymyxin B for the control of bacterial growth in equine semen stored at 20°C or 5°C for up to forty-eight hours. *Can J Vet Res* 1993;**57**:277–280.
- World Health Organization. *WHO Laboratory Manual of the Examination of Human Semen and Sperm-Cervical Mucus Interaction*, 4th edn. Cambridge, UK: Cambridge University Press, 1999