

Antimicrobial host defence peptide, LL-37, as a potential vaginal contraceptive

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STUDY QUESTION: Does antimicrobial peptide, LL-37, inhibit sperm fertilizing ability?

SUMMARY ANSWER: Our results indicate that LL-37 inhibits mouse and human sperm fertilizing ability.

WHAT IS KNOWN ALREADY: LL-37, a cationic antimicrobial peptide, exerts its microbicidal effects through the disruption of microbial cytoplasmic membranes following its interaction with microbial surface anionic phospholipids. ALL-38 (an LL-37 close analogue: LL-37 + Ala at the N-terminus) is produced in the vagina 2–6 h post-intercourse from its precursor hCAP-18, a seminal plasma component. At this time, motile sperm have already swum into the uterine cavity, thus unexposed to ALL-38. Since sperm contain a substantial amount of acidic sulfogalactosylglycerolipid (SGG) on their surface, treatment of sperm with LL-37 may cause their membrane disruption in an analogous manner to that occurring on microbial membranes.

STUDY DESIGN, SIZE AND DURATION: Mouse/human sperm treated (2–30 min) with LL-37 in a physiological concentration range (up to 10.8 μ M) were assessed for SGG-dependent LL-37 binding, and parameters relevant to fertilizing ability, namely motility and intactness of the sperm acrosome and plasma membrane. Ability of mouse sperm to fertilize eggs *in vitro* was also evaluated. Each study was performed with greater than or equal to three different sperm samples. The efficacy of LL-37 to inhibit sperm fertilizing ability *in vivo* was determined in female mice ($n = 26$ each for LL-37 treatment and no treatment), using sperm retrieved from 26 males.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human sperm samples were donated by fertile men. LL-37 was chemically synthesized and was biotinylated for sperm binding studies. Sperm motility was assessed by videomicroscopy and the acrosomal status by Coomassie blue staining of acrosome-intact mouse sperm or the exposure of CD46, an inner acrosomal membrane protein, of acrosome reacted human sperm. Sperm membrane permeabilization/disruption was assessed by the loss of hypo-osmotic swelling response, an incorporation of Sytox Green (a membrane impermeable fluorescent DNA dye), and electron microscopy. Mouse IVF was scored by the presence of two

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pronuclei in eggs 6 h post-insemination. Ability of mouse sperm to fertilize eggs *in vivo* was determined by the pregnancy outcome of female mice injected transcervically with sperm with or without LL-37.

MAIN RESULTS AND THE ROLE OF CHANCE: Biotinylated LL-37 bound to both mouse and human sperm and the binding was partially dependent on sperm surface SGG. Mouse and human sperm became immotile and underwent a premature acrosome reaction upon treatment with LL-37 at 3.6 and 10.8 μM , respectively. The initial action of LL-37 on both mouse and human sperm appeared to be through permeabilization/disruption of sperm surface membranes evidenced by the loss of hypo-osmotic swelling response, Sytox Green staining and electron microscopy revealing ultrastructural damage. Mouse sperm treated with 3.6 μM LL-37 lost the ability to fertilize eggs both *in vitro* and *in vivo*. All 26 female mice inseminated with sperm and LL-37 did not become pregnant. No apparent damage to the reproductive tract was observed as revealed by histological characterization in LL-37-inseminated mice and these females resumed fecundity following mating with fertile males.

LIMITATIONS, REASONS FOR CAUTION: Direct demonstration that LL-37 treated human sperm fail to fertilize eggs was limited by legal restrictions on obtaining human eggs for such use.

WIDER IMPLICATIONS OF THE FINDINGS: Our results reveal selective inhibitory effects of LL-37 on sperm fertilizing ability in mice without apparent impairment to the female reproductive tract. LL-37 is therefore a promising candidate to be developed into a vaginal contraceptive with microbicidal activity.

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Key words: LL-37 / sperm fertilizing ability / sperm plasma membrane / vaginal contraceptive

Introduction

LL-37 is an antimicrobial host defence peptide of the cathelicidin family. It is proteolytically processed from its propeptide, hCAP-18 (Sorensen et al., 2001; Morizane et al., 2010), which is produced by human immune cells and epithelial cells (Sorensen et al., 2008; Pasupuleti et al., 2012). LL-37 is a multifunctional peptide that provides host defence against microbial attacks through various mechanisms, including moderate direct antimicrobial activity, good activity against bacterial biofilm production, anti-infective activity including recruitment of phagocytic and other immune cells, promotion of wound healing and anti-inflammatory activity in suppressing proinflammatory cytokines in response to various agonists including lipopolysaccharides (Brogden, 2005; Overhage et al., 2008; Nijnik and Hancock, 2009). Of relevance here are the antimicrobial activities. LL-37 initially interacts with polyanionic lipopolysaccharides on the surface of the outer membrane, disrupts this membrane and then translocates via self-promoted uptake (Brogden, 2005). Upon encountering the cytoplasmic membrane, the cationic amino acids lying on one side of the α -helical structure of LL-37 electrostatically interact with the anionic head groups of phosphatidylglycerol and cardiolipin exposed from the outer leaflet of the cytoplasmic membrane. This is followed by the peptide insertion into the membrane core via its hydrophobic interaction with the lipid hydrocarbon chains (Wang, 2008). When the surface bound LL-37 supersedes a threshold concentration, a cluster of peptide molecules intercalate perpendicularly into the membrane bilayer, resulting in membrane perforation and disruption of membrane function (Sood et al., 2008; Bucki et al., 2010). The cause of cell death is not clear, but may be due to a loss of metabolic homeostasis or a disruption of membrane linked cell wall synthesis. The microbicidal effects of LL-37 have been shown with a number of pathogens that cause sexually transmitted infection (*Escherichia coli*, *Treponema pallidum*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*; Smeianov et al., 2000; Cox et al., 2003; Bergman et al., 2005; Donati et al., 2005). LL-37 has also been documented for its anti-fungal and

anti-human immunodeficiency virus-1 effects (den Hertog et al., 2005; Bergman et al., 2007).

The LL-37 precursor, hCAP-18, is secreted by epithelial cells of both the female and male reproductive tracts. The amount of hCAP-18 in the female reproductive tract is minimal (0.014–0.22 μM ; Valore et al., 2002), when compared with that present in the seminal plasma (3–10 μM), where it is secreted from epididymal epithelial cells (Malm et al., 2000). Gastricsin processes seminal plasma hCAP-18 to generate ALL-38 (LL-37 with an additional Ala at the N-terminus). However, ALL-38 is not produced in the semen prior to intercourse despite the presence of gastricsin in the seminal plasma because gastricsin cleaves hCAP-18 at the pH optimum of ~ 4 (Sorensen et al., 2003). Uniquely, the seminal plasma has a high buffering capacity at neutral pH, and once it bathes the vaginal lumen following sexual intercourse, it takes 2–6 h post-ejaculation for the vagina to resume its acidic pH (~ 4), and it is only at this time that ALL-38 is produced in the vaginal lumen (Sorensen et al., 2003). However, motile sperm swim out from the liquefied seminal plasma into the uterine cavity within 30 min after ejaculation. Thus, sperm are not exposed to ALL-38, which likely functions to protect the vaginal epithelium from infections by microbes usually present in the seminal plasma (Cottell et al., 2000), and to dampen potential inflammation and to heal minor tissue damage after intercourse.

Since motile sperm do not encounter ALL-38 under physiological conditions, we considered the possibility that ALL-38/LL-37 might have adverse effects on them. Anionic sulfogalactosylglycerolipid (SGG, structure shown in Supplementary data, Fig. S1) is selectively present on sperm at 10 mol% of total lipids (Tanphaichitr et al., 2003). It is localized on the sperm head mainly in lipid rafts, and is involved in sperm–egg interaction (White et al., 2000; Weerachatanukul et al., 2001). We proposed here that, as with anionic molecules on the microbial surface, SGG might bind electrostatically to exogenous LL-37, leading to sperm plasma membrane disruption and subsequent impairment to sperm parameters essential for fertilization (e.g. motility and acrosomal intactness; Florman

and Ducibella, 2006). Results reported herein support this postulate; mouse and human sperm treated with LL-37 became immotile and underwent a premature acrosomal exocytosis, therefore losing their fertilizing ability. Our results point to the possibility of using LL-37 as a vaginal contraceptive.

Materials and Methods

LL-37 and antibodies

LL-37 peptide was chemically synthesized by CPC scientific (San Jose, CA, USA) with >98% purity as shown by HPLC. LL-37 biotinylated (LL-37B) at its terminus was generated as described (Lau *et al.*, 2005). Anti-LL-37 antiserum was raised in a rabbit using aggregated, full-length LL-37 and the antibody was affinity purified using an LL-37 solid-phase matrix (see Supplementary data). Purified monoclonal antibody O4 immunoglobulin (IgM) specifically recognizing sperm SGG (Gadella *et al.*, 1994) was from Neuromics (Edina, MN, USA).

Mouse and human sperm preparation

The use and handling of mice followed the protocols approved by the Animal Care Facility, Ottawa Hospital Research Institute, and the accessibility to sperm donors was authorized by the Ottawa Hospital Research Ethics Board following informed consent of participants (approval number: 2005256-01H). Mouse sperm, collected from the epididymis and vas deferens of CD-1 males (8–10 weeks old), were subjected to centrifugation through a two-step Percoll gradient (45%/90%) to select for the motile population, which sedimented as a pellet (Tanphaichitr *et al.*, 1988). These Percoll gradient centrifuged (PGC) sperm were washed once (300g, 5 min) and resuspended to 1×10^7 /ml in HEPES (21 mM)-buffered KSOM medium. Because of the presence of calcium and bicarbonate in KSOM, these sperm were partially capacitated (P-CAP; Visconti *et al.*, 1999). To prepare fully capacitated (F-CAP) sperm samples, PGC sperm were incubated (1 h, 37°C, 5% CO₂) in KSOM supplemented with 0.3% bovine serum albumin (BSA; Visconti *et al.*, 1999). In alternate experiments, non-capacitated (N-CAP) sperm were prepared by resuspending PGC sperm in KSOM–HEPES medium made without calcium and bicarbonate.

Human semen samples with normal semen parameters (WHO, 2010) were donated from healthy donors (recruited by informal and formal announcement) after 2–3 days of abstinence. P-CAP and F-CAP PGC sperm were prepared following the same procedure as described for mouse sperm except that human tubal fluid (HTF) medium was used in place of KSOM, and capacitation was for 4 h. In an alternate experiment, motile sperm were selected by a swim-up procedure (WHO, 2010). Briefly, semen (1 ml) was placed in a round bottom tube tilted at a 45° angle, and was over layered with 1.2 ml HTF medium. The tube was incubated at 37°C under 5% CO₂ for 60 min. Motile sperm swam up into the medium carrying residual amounts of seminal plasma. Swim-up sperm from the top 1 ml of HTF were collected and placed into another tube for treatment with LL-37.

Treatment of sperm with LL-37

Mouse and human sperm (10^7 /ml) of various capacitation states were treated (30 min, 37°C) with 0–10.8 μM LL-37. These LL-37 concentrations are within a physiological range (Sorensen *et al.*, 2003) and the 3.6 μM value is the equivalent molar amount of SGG on mouse sperm at 10^7 /ml density (Furimsky *et al.*, 2005).

Binding of LL-37 to mouse and human sperm

Both P-CAP and F-CAP mouse and human sperm resuspended in phosphate-buffered saline (PBS) were treated (30 min, 37°C) with 0, 0.36 or 3.6 μM LL-37B. Unbound LL-37B was removed by washing sperm twice (300g, 5 min) in PBS + 0.1% Tween-20 (PBST). Sperm were incubated (30 min, 37°C) with 0.125 μg/ml Alexa Fluor 488-avidin (Life Technologies, Burlington, ON, Canada), washed twice with PBST (300g, 5 min) and viewed under an epifluorescence microscope. In alternate experiments, sperm were pretreated (30 min, 37°C) with 50 μg/ml anti-SGG O4 IgM or non-immune mouse IgM (Sigma, Mississauga, ON, Canada), or untreated prior to 0.09 μM LL-37B incubation. Following incubation with Alexa Fluor 488-avidin, levels of sperm bound LL-37B in the three different sperm samples were assessed by flow cytometry. In all experiments including this one, each data point within each sperm sample was an average of duplicates.

Binding of LL-37 to mouse and human sperm was also detected by immunoblotting. Untreated and LL-37-treated sperm were subjected to tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis (16.5% acrylamide gel; Schagger, 2006) followed by western blotting (Towbin and Gordon, 1984). The nitrocellulose membrane containing the sperm extracts was first blocked for non-specific binding with 5% skim milk in Tris-buffered saline (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) + 0.1% Tween (TBST) (1 h, room temperature). The presence of LL-37 in sperm extracts was detected by its reactivity to anti-LL-37 antibody (0.5 μg/ml, 4°C, overnight). The secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad, Mississauga, ON, Canada, 1:10 000 dilution, 1 h, room temperature). Antibody-antigen recognition was detected by enhanced chemiluminescence using an ECL kit from Pierce (Rockford, IL, USA).

Assessment of LL-37 effects on sperm parameters

Sperm fertilizing ability

F-CAP mouse sperm, treated with LL-37, were subjected to low-speed centrifugation to remove the unbound peptide. Control and LL-37 treated sperm were then added to mature mouse eggs in 500 μl of KSOM–BSA. After 6 h of gamete co-incubation, eggs were microscopically scored for 2-pronuclei as evidence of fertilization (Tantibhedhyangkul *et al.*, 2002).

Sperm motility

A small aliquot of P-CAP and F-CAP mouse and human sperm, untreated and LL-37 treated, was applied into a pre-warmed haemocytometer for video-recording under a microscope. Motile sperm showing progressive motility and immotile sperm (>300 in total) were counted from the recorded video, and the percentage of immotile population was calculated.

Sperm acrosomal status

This was assessed on control and LL-37 treated mouse sperm with all capacitation states and human sperm P-CAP and F-CAP. Coomassie Brilliant Blue staining distinguished acrosome intact and reacted mouse sperm from each other (Tantibhedhyangkul *et al.*, 2002). The exposure of an inner acrosomal membrane protein CD46 on live human sperm, as detected by phycoerythrin-conjugated anti-CD46 antibody (Abcam, Toronto, Canada) through flow cytometry, was used as an indication of human sperm acrosome reaction (Harper *et al.*, 2008).

Hypo-osmotic Swelling (HOS) assay

The HOS assay in human (Jeyendran *et al.*, 1984) and mouse (Netzel-Arnett *et al.*, 2009) sperm is based on the observation that intact sperm membranes swell upon incubation of the gametes in a hypo-osmotic buffer and, as the membranes do not return to their original state, this results in the curling

of the tail towards the head. Untreated and LL-37-treated mouse and human sperm (P-CAP and F-CAP) were removed from iso-osmotic medium (KSOM and HTF, respectively) to resuspend in a hypo-osmotic solution (25 mM sodium citrate dihydrate and 75 mM D-fructose) for 30 min (Jeyendran et al., 1984). At least, 300 sperm were viewed in a hang-drop under a microscope and scored for the curling of the sperm tail towards the sperm head as an indication of the intactness of their plasma membrane in response to hypo-osmotic conditions. Since the mouse sperm head is sizable, detection of its swelling in a hypo-osmotic buffer using forward scatter/side scatter flow cytometry is possible (Netzel-Arnett et al., 2009). Therefore, in a parallel experiment, control and LL-37 treated mouse sperm were also assessed by flow cytometry for the volume changes of their heads upon incubation in the hypo-osmotic solution, through the forward scatter profiling (Netzel-Arnett et al., 2009). The forward scatter pattern of control sperm in iso-osmotic medium served as a baseline for the normal forward scatter distribution.

Sperm membrane permeabilization

Sytox Green (Life Technologies), a membrane impermeable fluorescent DNA dye, was used for this assessment. P-CAP and F-CAP mouse and human sperm, as well as N-CAP mouse sperm, control and LL-37 treated, were incubated (5 min, 37°C) with Sytox Green (3 µM) and the dye staining was analysed by flow cytometry.

Transmission electron microscopy of LL-37 treated sperm

Two sets of transmission electron microscopy (TEM) experiments were performed. In the first experiment, where two mice were used, sperm were collected from two epididymis + vas deferens (from one side of the organs of each mouse) directly into 1 ml of KSOM (control) or into 1 ml of 3.6 µM LL-37-containing KSOM. The sperm suspension was mixed gently and incubated for 1 h at 37°C. In the second experiment, P-CAP PGC mouse and human sperm were untreated or treated with 3.6 µM LL-37. In both experiments, control and LL-37 treated sperm were washed with 0.1 M cacodylate buffer containing 0.2 M sucrose (pH 7.4), fixed in 2.5% glutaraldehyde, and then processed for thin sectioning for TEM. The ultrastructure of sperm was viewed under a Hitachi electron microscope 7000 at 75 kV.

In vivo contraceptive effects of LL-37 in female mice

F-CAP mouse sperm (475 µl of 10^7 /ml in KSOM–BSA) were added with 25 µl of LL-37 to the final concentration of 3.6 µM. Immediately, the sperm suspension was centrifuged (300g) and the sperm pellet was resuspended in 50 µl of the supernatant (still containing LL-37). This concentrated sperm preparation (10^8 /ml) was suitable for artificial insemination. A control sperm sample was prepared likewise except that 25 µl of KSOM–BSA was added instead of LL-37 to the original sperm suspension. The 50 µl concentrated sperm suspension was injected transcervically into a female mouse naturally cycling to the oestrous day, as revealed by their vaginal smear patterns (McLean et al., 2012). Pregnancy was then assessed either by scoring the implantation sites in the uterus of sacrificed females 16 days post-insemination, or by counting the number of pups delivered. The uterus and vagina tissues were also collected from the sacrificed females, and fixed in Bouin's solution for paraffin embedding. Tissue sections were stained with haematoxylin/eosin for histological analysis. In an alternate experiment, non-pregnant females preinseminated with LL-37 were individually caged with fertile males for natural mating, as revealed by the presence of a vaginal plug. Pregnancy resulting in pup delivery was then recorded 21 days after the mating.

Flow cytometry

All flow cytometry data were acquired on an EPICS XL MCL (Beckman Coulter, Mississauga, ON, Canada) and were further analysed with FCS Express 4.0 (De Novo Software, Thornhill, ON, Canada).

Statistical analyses

Statistical analyses and graphing were performed using GraphPad Prism 5.0 (San Diego, CA, USA). Significant differences between data from control and LL-37 treated samples were analysed by one-way analysis of variance. Statistical significance was set at $P < 0.05$.

Results

LL-37 bound to mouse and human sperm and inhibited sperm fertilizing ability

Sperm capacitation is a process whereby ejaculated sperm that have swum out from the seminal plasma into the female reproductive tract gain fertilizing ability through biochemical and physiological changes on the cell surface. In order to develop LL-37 as an effective contraceptive, it was important to examine its effects on N-CAP, P-CAP and F-CAP sperm. *In vitro*, N-CAP, F-CAP and P-CAP sperm were prepared for experiments described herein.

LL-37 had a high affinity for SGG ($K_d = 456$ nM; Supplementary data, Fig. S2). LL-37B at 0.36 µM bound to the convex ridge of the mouse sperm head (Fig. 1A). Similar results were observed with sperm co-incubated with 0.09 µM LL-37. To determine whether SGG on sperm functioned as an LL-37 receptor, mouse sperm were treated with anti-SGG O4 IgM or non-immune IgM, or untreated prior to LL-37B (0.09 µM) incubation. Flow cytometry revealed two sperm populations with differential LL-37B binding in the untreated or non-immune IgM treated samples. For the untreated sperm samples, the populations with the higher and lower level of LL-37B binding were 70 and 30%, respectively. For non-immune IgM treated sperm, the corresponding values were 61 and 39%. Significantly, anti-SGG O4 treatment resulted in the loss of the sperm population with high LL-37B binding and minimal levels of LL-37B binding were observed in these SGG-blocked sperm (Fig. 1B). Similar results were obtained when adamantyl SGG, an SGG mimetic, was present in the sperm–LL-37B co-incubate (Supplementary data, Fig. S3), suggesting that SGG is an LL-37 receptor. However, since both anti-SGG and adamantyl SGG treatments did not abolish LL-37B binding to sperm, there are other LL-37 receptors on the sperm surface. Supporting this postulate was the observation that LL-37B at 3.6 µM bound to not only the mouse sperm head but also the tail, where SGG is absent (Fig. 1A).

Similar to the results in mouse sperm, LL-37B at 0.36 µM preferentially bound to human sperm at the head region, where SGG is located (Veerachatanukul et al., 2001), although a strong staining was also present at the sperm neck. At 3.6 µM LL-37B, the staining appeared over the anterior head and tail regions in all sperm, although the post-acrosomal region of the human sperm head seemed to be less bound by the peptide (Fig. 1A). The ability of LL-37 to bind to sperm was confirmed by immunoblotting (Fig. 1C). Notably, LL-37 extracted from sperm preincubated with the peptide was mainly in the monomer form, although dimers and oligomers of extracted LL-37 were also observed especially at the higher LL-37 concentration.

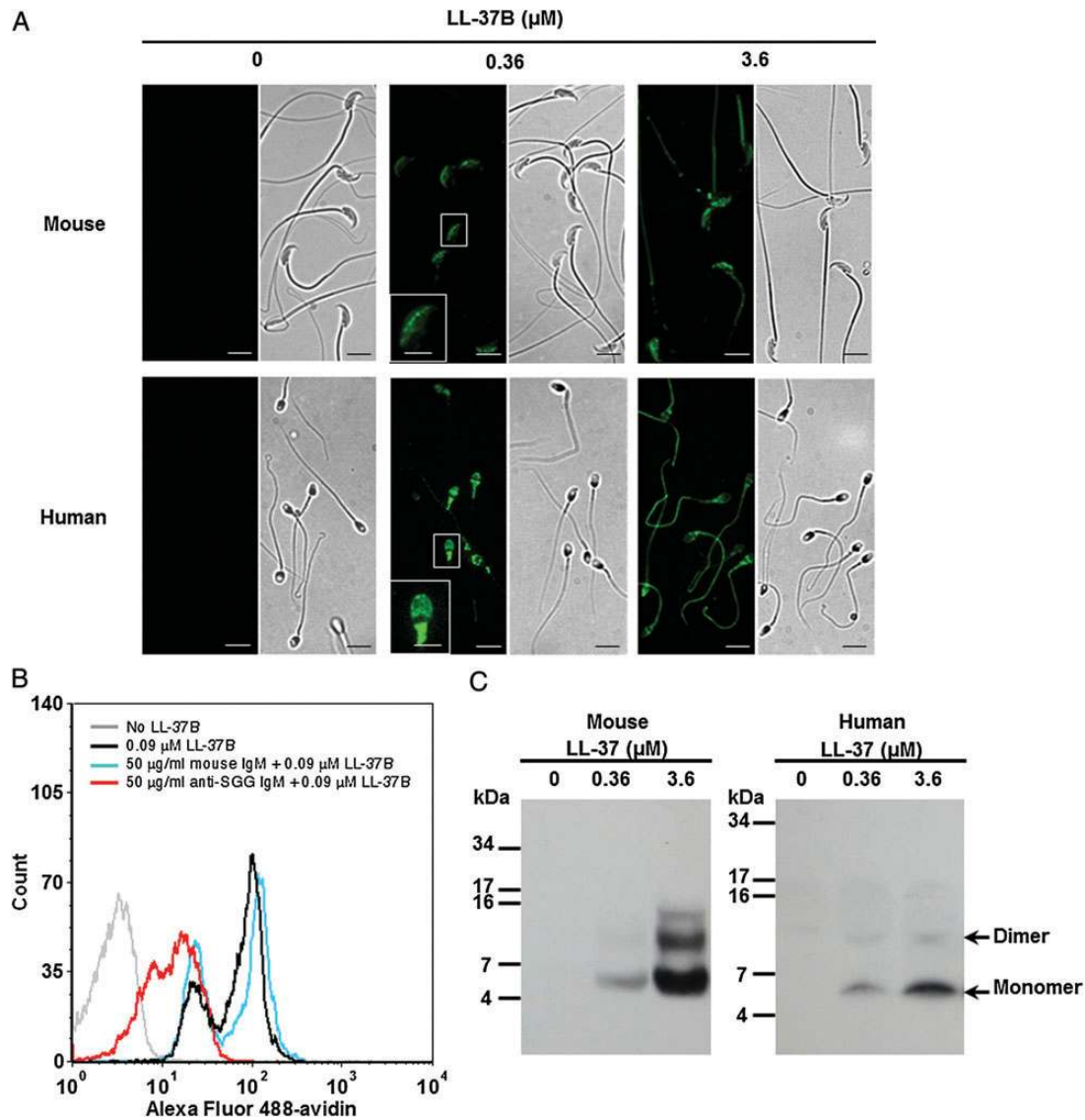


Figure 1 Exogenous LL-37 has affinity for mouse and human sperm. Sperm were incubated (30 min) with LL-37B (**A** and **B**) or LL-37 (**C**) and washed to remove unbound peptide. (B) Sperm were treated (30 min) with anti-SGG O4 (50 $\mu\text{g/ml}$) prior to LL-37B incubation. Sperm bound LL-37B was detected via reactivity with Alexa Fluor 488-avidin by fluorescence imaging (A) or flow cytometry (B). Phase contrast and fluorescent images are shown in (A); bar = 10 μm , and 5 μm (inset). Note that pretreatment with anti-SGG O4 resulted in marked inhibition of LL-37B binding to sperm. (C) Sperm bound LL-37 was revealed by immunoblotting. Results shown were representative of three replicate experiments using P-CAP sperm; experiments performed with F-CAP sperm revealed the same results. P-CAP, partially capacitated.

Figure 2 shows that LL-37 pretreated F-CAP mouse sperm had a reduced ability to fertilize eggs in a dose-dependent manner and at 3.6 μM LL-37, the treated sperm completely lost fertilizing ability. This functional loss was partly attributed to sperm immotility, dose-dependently induced by LL-37. Approximately 50% of F-CAP mouse sperm became immotile upon treatment with 0.36 μM of LL-37 and only $\sim 10\%$ of sperm remained motile at 3.6 μM (Fig. 3). Figure 3 shows that LL-37 induced acrosomal exocytosis dose-dependently on F-CAP sperm, and at 3.6 μM LL-37 $\sim 70\%$ of sperm showed acrosomal exocytosis. LL-37 was also able to induce immotility and premature acrosome reaction on P-CAP mouse sperm, to similar extents as in F-CAP sperm

(Fig. 3). Similar trends of LL-37 effects on motility and acrosomal status were observed in both P-CAP and F-CAP human sperm, although the rise of these effects occurred more gradually in human sperm, with the maximum inductions at 10.8 μM (Fig. 3). In mice, similar effects of LL-37 on premature acrosome reaction were also observed in N-CAP sperm. This indicated that the induction on premature acrosome reaction by LL-37 was not correlated with the capacitation status *per se*. Since N-CAP mouse sperm showed very low levels of forward motility, it was not practical to measure immotility induced by LL-37. Studies on various effects of LL-37 on N-CAP human sperm were not performed as the gametes became completely immotile in the non-capacitation medium.

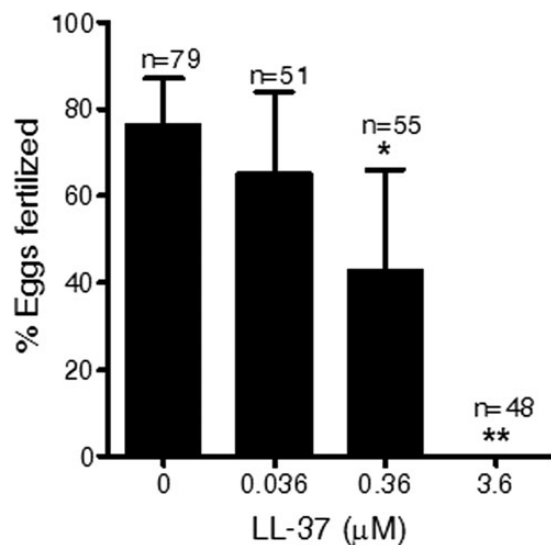


Figure 2 Inhibitory effects of LL-37 on mouse sperm fertilizing ability. F-CAP mouse sperm were treated with LL-37 (30 min, 37°C, 5% CO₂) prior to co-incubation with mouse eggs. Fertilized eggs were scored 6 h post-insemination by the presence of two pronuclei. Data were expressed as mean \pm SD of three replicate experiments. *n* = total eggs scored. * ** denote significant differences (analysis of variance) with *P* < 0.05 and 0.001, respectively. F-CAP, fully capacitated.

LL-37 induced damage to sperm surface membranes

LL-37 might cause membrane damage to the sperm surface in a manner analogous to that occurring on microbial membranes. Damage to the F-CAP mouse sperm head convex ridge where zona pellucida-binding molecules are localized would result in a decrease in the ability of sperm to bind to egg zona pellucida following gamete co-incubation. To test this hypothesis, we used sperm treated with LL-37 at concentrations that did not cause complete sperm immotility. At 0.036 and 0.36 μ M LL-37, 90% (similar to the control untreated sperm) and 50% of the total sperm population, respectively, remained motile, whereas >80% of treated mouse sperm at both LL-37 concentrations remained acrosome intact. Consequently, we used 60 000 sperm treated with 0.036 μ M LL-37, the same number as untreated control sperm, for co-incubation with eggs in a medium droplet. This treatment showed a significant 20% decrease in sperm binding to the zona pellucida, when compared with control sperm (Supplementary data, Fig. S4). Since only 55% of sperm treated with 0.36 μ M LL-37 remained motile, two egg droplets were separately set up for insemination with 60 000 and 103 000 sperm, being 50 and 100% of the number of motile sperm, as in the egg droplet of control sperm. The number of zona pellucida-bound sperm per egg was the same for both sperm motility-adjusted and non-adjusted egg droplets (~60% of the untreated control sperm sample; Supplementary data, Fig. S4). These results thus indicated that the loss of motility was not the primary factor of LL-37 treated sperm that determined the extent of sperm–zona pellucida binding. Thus, we proposed that it was the intactness of the plasma membrane overlying the sperm head convex ridge that was important for this event.

The damage to the membranes of LL-37 treated sperm was shown by their reduced response to a hypo-osmotic buffer. In control gametes, both P-CAP and F-CAP, ~85% of mouse sperm and 60% of human sperm were HOS responsive, as scored by their tail curling (Fig. 4A and B). In contrast, LL-37 treated mouse and human sperm showed reduced HOS responses in a dose-dependent manner. At the LL-37 concentration at which sperm fertilizing ability was lost (3.6 μ M for mouse sperm and 10.8 μ M for human sperm), the HOS response of LL-37 treated mouse and human sperm was 0 and 10%, respectively. When compared with the iso-osmotic condition, the number of sperm with higher forward scatter values (larger heads) increased by 4.5- and 2-fold, respectively, when control P-CAP and F-CAP sperm were incubated in a hypo-osmotic buffer (Fig. 4C). This sperm head size increase was not observed in P-CAP and F-CAP mouse sperm treated with 3.6 and 10.8 μ M LL-37, and the increase was only ~2-fold in P-CAP sperm treated with 0.36 μ M LL-37. These results were thus consistent with surface membrane damage in LL-37 treated sperm.

The hypothesis that LL-37 caused impairment to mouse and human sperm surface membranes was further evaluated by exposing LL-37 pre-treated sperm to Sytox Green, a membrane impermeable fluorescent DNA dye. Cells incorporate Sytox Green only when their surface membranes are permeabilized. Figure 5 indicates that P-CAP and F-CAP mouse and human sperm, as well as N-CAP mouse sperm, which were treated with LL-37, became Sytox Green-positive dose-dependently. At 3.6 μ M LL-37, 100% of the treated mouse sperm under all capacitation conditions became Sytox Green positive, indicating that LL-37 induced damage to the sperm surface membranes, independently of the capacitation state (Fig. 5A and B). For humans, the nuclei of 90% of P-CAP and F-CAP sperm were stained with Sytox Green, following treatment with LL-37 at 10.8 μ M (Fig. 5C and D). The higher concentration of LL-37 required for Sytox Green-positive staining in human sperm, when compared with mouse sperm, appeared to correlate with the results of the decrease in motility and the induction of premature acrosome reaction. The efficiency of LL-37 in permeabilizing the sperm membranes required the overall structure of LL-37 peptide. KR-12, an LL-37 truncated peptide, and a polycationic poly-L-lysine were used to treat human and mouse sperm. The treatment with poly-L-lysine was to determine whether the inhibitory effect of LL-37 on sperm fertilizing ability was simply related to the positive charges of the peptide. Human sperm treated with poly-L-lysine or with KR-12, an LL-37 truncated peptide (Wang, 2008), at 0.36, 3.6 and 10.8 μ M, did not become Sytox Green positive or prematurely acrosome reacted above the background values of untreated sperm. Neither did poly-L-lysine nor KR-12 induce acrosome reaction in mouse sperm (Supplementary data, Fig. S5). Similarly, Sytox Green staining above background was not observed in mouse sperm treated with 0.36 and 3.6 μ M of poly-L-lysine and KR-12. Only at 10.8 μ M, did poly-L-lysine and KR-12 increase Sytox Green staining of the treated mouse sperm by 20 and 10%, respectively, above the control values of untreated sperm (Supplementary data, Fig. S5). Notably, the adverse effects of LL-37 on the three sperm functional parameters (Sytox Green incorporation, premature acrosome reaction and immotility) were observed within 2–5 min of sperm treatment (data not shown). When the extents of these sperm dysfunctions were compared at low doses of LL-37 (\leq 0.36 μ M), in almost all sperm samples the Sytox Green-positive staining parameter appeared to supersede the other two effects (Supplementary data, Fig. S6), suggesting that the initial action of LL-37 on sperm might be at the surface membranes.

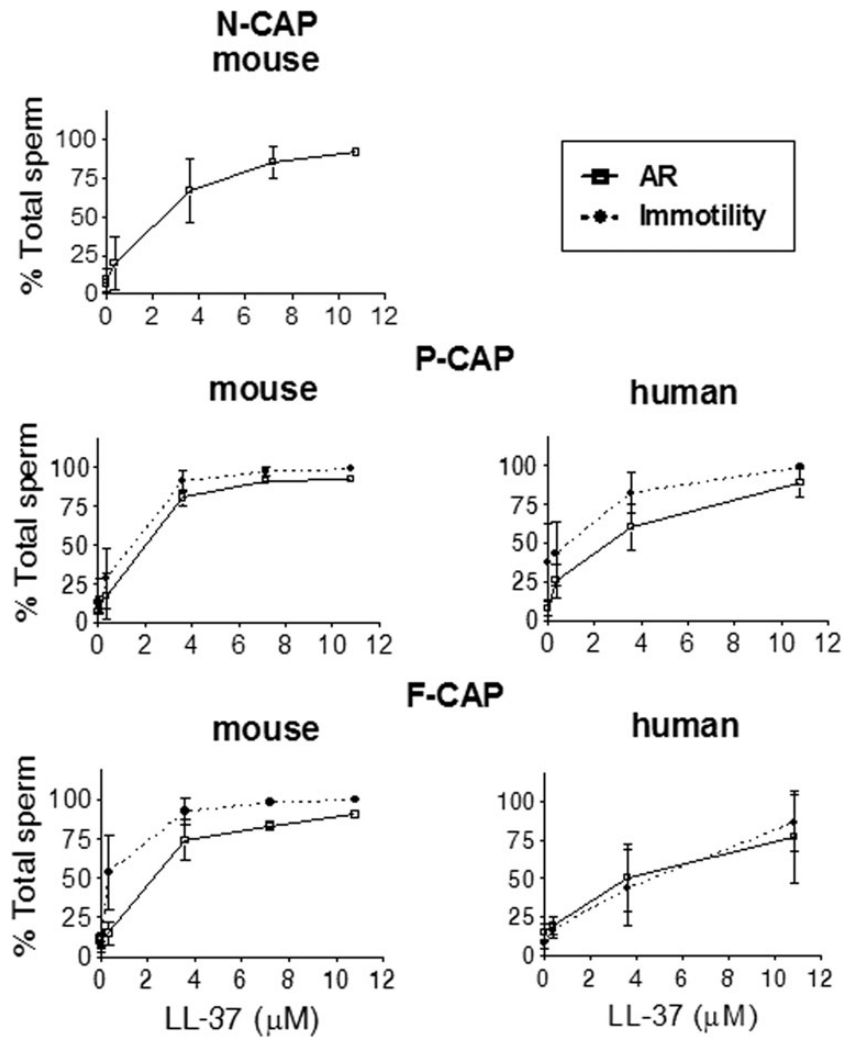


Figure 3 LL-37 induces immotility and premature acrosome reaction of mouse and human sperm. Sperm of various capacitation states were treated with LL-37 for 30 min (37°C, 5% CO₂) and then recorded for the % population showing immotility or having undergone the acrosome reaction (AR). Data are shown as mean ± SD from three replicate experiments. N-CAP, non-capacitated; P-CAP, partially capacitated; F-CAP, fully capacitated.

Direct evidence of the impairment of the sperm surface membranes induced by LL-37 was revealed by TEM. In the first experiment in mice, caudal epididymal and vas deferens sperm were collected into medium containing 3.6 μM LL-37, and these sperm were centrifuged only once at 300g prior to immediate fixation with aldehyde for TEM processing. All membrane layers (plasma membrane, outer and inner acrosomal membranes, nuclear membrane) of the control sperm head (Fig. 6A, panel a) and tail (data not shown) were intact, as expected. In contrast, sperm treated with LL-37 showed distinctive defects on their surface membranes, whether or not they contained the acrosome (Fig. 6A, panels b and c). Small vesicles were selectively present on the sperm head surface, reflecting the damage to the head plasma membrane, and outer and inner acrosomal membranes. These vesicles were clearly discernible on LL-37-treated sperm that had lost the acrosome (Fig. 6A, panel c). The vesicles were also present on the surface of LL-37 treated sperm that still contained the acrosome, although they were still adjacent to each other (Fig. 6A, panel b-insets). Frequently, damage on the acrosome itself was observed (Fig. 6A, panel b, large

inset). Since mouse sperm used in this experiment were not centrifuged prior to LL-37 treatment, all of the structural damage reflected direct actions of LL-37 on sperm. However, the tail plasma membrane of LL-37 treated sperm remained consistently intact. While it is desirable to establish the same quality control for the TEM procedure (whether centrifugation affects the plasma membrane ultrastructure) using human sperm, this is not possible because of a high heterogeneity of sperm morphology (including the inherently abnormal ultrastructure of the plasma membrane) and quality within a single human ejaculate.

In a second experiment, P-CAP mouse and human sperm were prepared by Percoll gradient centrifugation (600g) and then treated (30 min) with 3.6 μM LL-37 as in the other experiments described above. Sperm were then processed for TEM as in the first experiment. Similar effects of LL-37 on the head surface membranes were observed for PGC mouse sperm. Control PGC sperm contained intact acrosomes and plasma membranes (Fig. 6B, panel a). In LL-37-treated PGC sperm, although the sperm tail plasma membrane remained intact (Fig. 6B, panel d), disruption of the surface membranes in the sperm head was

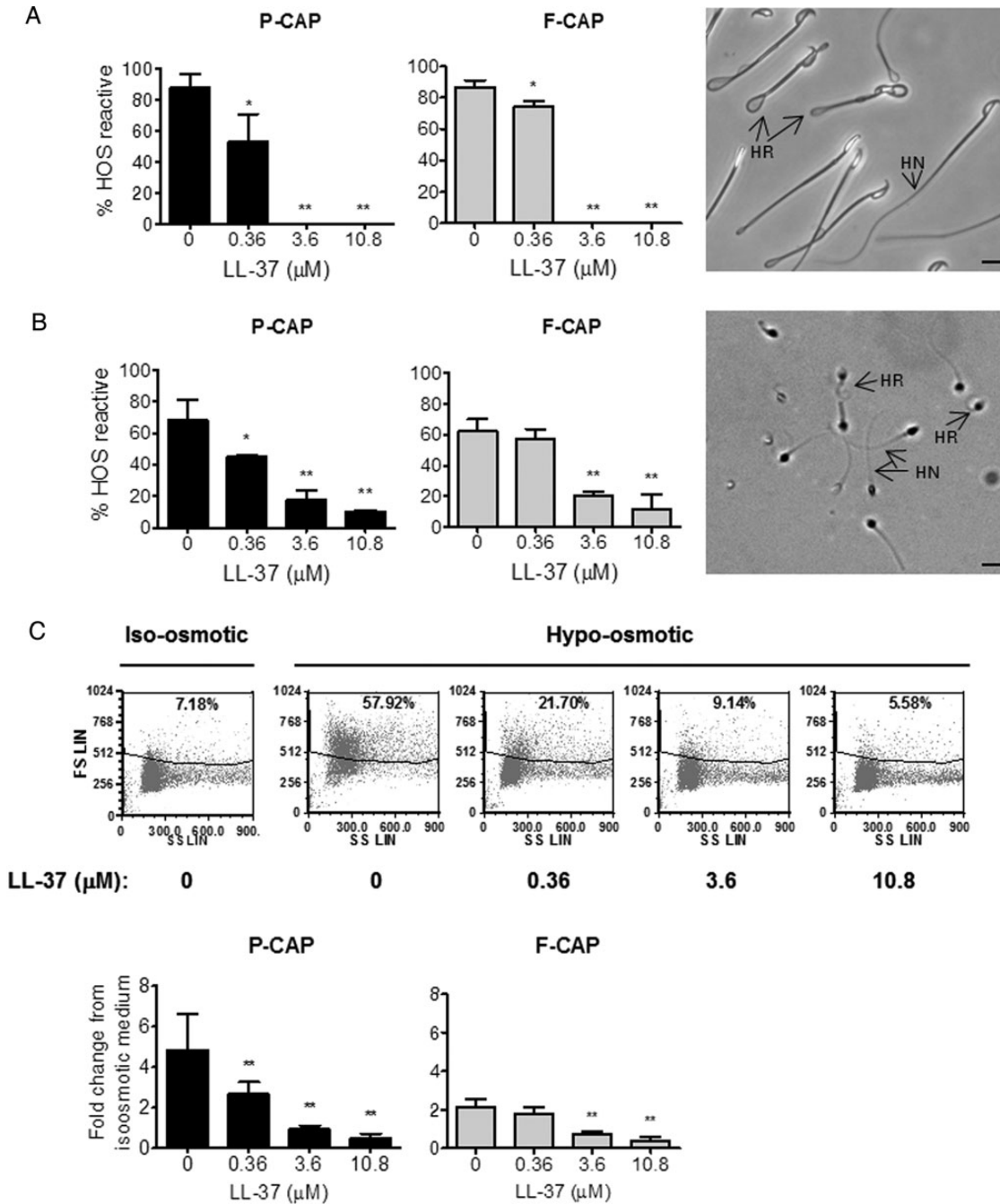


Figure 4 LL-37 treated sperm have reduced responses to exposure to hypo-osmotic buffer. Mouse (**A**) and human (**B**) sperm, P-CAP and F-CAP, were treated with LL-37 (30 min, 37°C, 5% CO₂). Following treatment, sperm were resuspended in a hypo-osmotic solution (25 mM sodium citrate dihydrate and 75 mM d-fructose) and scored for the curling of the sperm tail towards the sperm head as an indication of their plasma membrane response to hypo-osmotic conditions. Examples of responsive (HR) and non-responsive (HN) sperm are shown in the right panels (bar = 10 μm). (**C**) The swelling of the mouse sperm head in response to hypo-osmotic conditions was detected by flow cytometry as shown by an increase in the forward scatter parameter. The percentage in each histogram indicates the distribution of sperm above the gated forward/side scatter values when sperm were incubated in an iso-osmotic buffer (KSOM-HEPES). Summary graphs represent the fold change in the number of untreated and LL-37 treated sperm that increased their forward scatter parameters following hypo-osmotic swelling. Data are expressed as mean ± SD from three replicate experiments. * ** denote significant differences with $P < 0.05$ and < 0.001 , respectively. P-CAP, partially capacitated; F-CAP, fully capacitate.

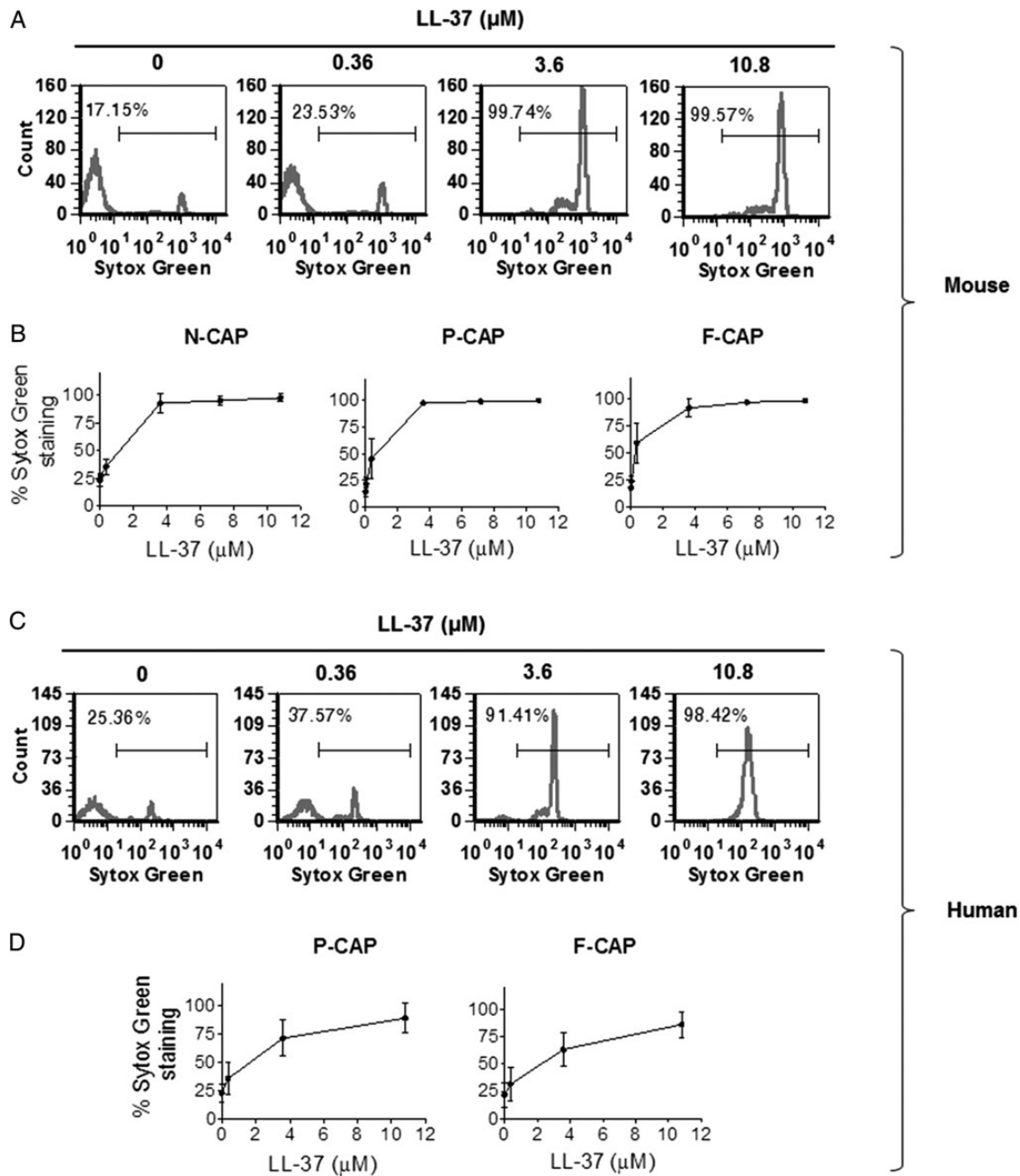


Figure 5 LL-37 induces sperm plasma membrane permeabilization. Membrane permeability was detected by positive staining of sperm with Sytox Green. Untreated and LL-37 treated sperm were incubated with 3 μM Sytox Green (5 min) and analyzed by flow cytometry. **A** and **C**: representative histograms of P-CAP mouse (A) and human (C) sperm showing increased populations of Sytox Green-positive cells with increasing doses of LL-37. **B** and **D**: Dose–response curves of LL-37 treated mouse (B) and human (D) sperm (N-CAP, P-CAP, F-CAP) that were positively stained with Sytox Green. Data are expressed as mean \pm SD from three replicate experiments. N-CAP, non-capacitated; P-CAP, partially capacitated; F-CAP, fully capacitate.

obvious. Small vesicles and ruffling structures were present on the outer acrosomal membrane and the remnant plasma membrane of gametes that still possessed the acrosome (Fig. 6B, panel b), and on the inner acrosomal membrane of those that had lost the acrosome (Fig. 6B, panels c and d). Likewise, PGC human sperm revealed membrane structural damage upon treatment with 3.6 μM LL-37. However, the damage occurred at both the sperm head and tail regions (Fig. 6C).

LL-37 also induced immotility, premature acrosome reaction and surface membrane permeabilization on swim-up human sperm

It was important to determine whether sperm with residual amounts of seminal plasma, such as swim-up human sperm, could be affected by LL-37 treatment. Results shown in Fig. 7 indicated that upon treatment

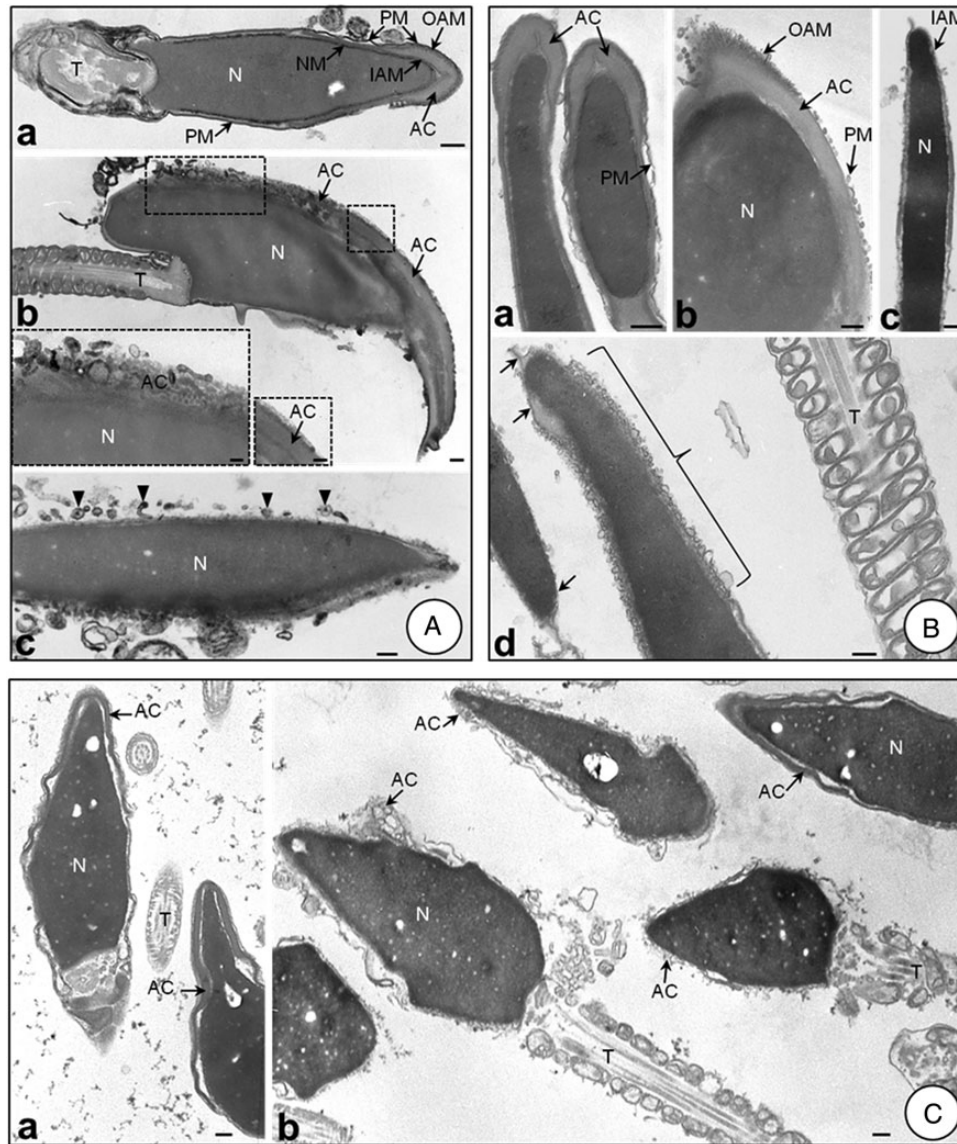


Figure 6 Electron microscopy showing the disrupting effects of LL-37 on sperm membranes. **(A)** Caudal epididymal mouse sperm were incubated in KSOM containing 0 or 3.6 μM LL-37. a: Tangential section of control sperm showing intact head and tail (T) components, including plasma (PM), outer acrosomal (OAM), inner acrosomal (IAM) and nuclear (NM) membranes. N, nucleus. b: LL-37 treatment showing a longitudinal section through the sperm head and tail. Note that the distal segment of the acrosome (AC) was undergoing changes, including ruffling of sperm PM and OAM (small inset) and disruption (large inset). The sperm tail appeared intact. c: LL-37-treated sperm head, obliquely cut, showing absence of acrosomal matrix and vesiculation of sperm membranes (arrow heads). **(B)** Mouse PGC sperm resuspended in KSOM containing 0 or 3.6 μM LL-37. a: In control sperm the AC and PM were intact. b, c, d: In LL-37-treated sperm, vesiculation and ruffling of the PM and OAM (b) eventually led to disruption of the AC (c, d). In d, the IAM was exposed in the dorsal part of the AC (arrows) and membrane vesiculation even affected the more stable ventral part (equatorial segment) of the AC (bracket); however, the sperm tail and its membranes appeared intact. **(C)** Human PGC sperm resuspended in HTF containing 0 (a) or 3.6 μM (b) LL-37. Compare the membrane- and acrosome-intact control sperm (a) to the membrane- and acrosome-disrupted LL-37-treated sperm (b). Bars = 0.2 μm ; inset bars = 0.1 μm . PFC, percoll gradient centrifuged.

with LL-37 (0–10.8 μM), the numbers of swim-up sperm showing immotility, Sytox Green staining and premature acrosome reaction increased in a dose-dependent manner, and the values of these three parameters at 10.8 μM LL-37 had reached or almost reached the maximum. These observations were comparable with those of P-CAP and F-CAP PGC human sperm (Figs 3 and 5). However, the percentages of acrosome reacted sperm as assayed by the exposure of the inner

acrosomal membrane protein, CD46, appeared to decrease in swim-up sperm treated with higher LL-37 concentrations (16.2 and 21.6 μM). These decreases in the numbers of ‘acrosome reacted’ sperm were very likely not real. The lower numbers of sperm expressing CD46 on their head surface may rather reflect acrosome reacted sperm with further LL-37 induced damage to the inner acrosomal membrane that caused CD46 to be shed from the sperm surface.

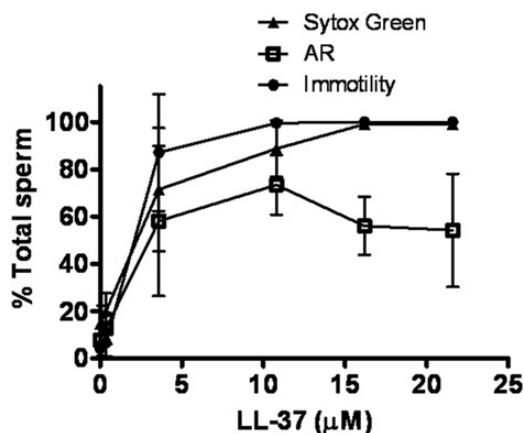


Figure 7 LL-37 also induces immotility, premature acrosome reaction and plasma membrane permeabilization to swim-up human sperm. Swim-up human sperm carrying residual amounts of seminal plasma were treated with LL-37 and assessed for their motility, acrosomal status and plasma membrane intactness in the same manner as PGC human sperm (Figs 3 and 5). LL-37 adversely affected these three functional parameters of swim-up human sperm to similar extents as PGC human sperm. Data are expressed as mean \pm SD from greater than or equal to three replicate experiments. PFC, percoll gradient centrifuged.

LL-37 exerted *in vivo* contraceptive effects in female mice with minimal adverse effects to the genital tract

The contraceptive effects of LL-37 were confirmed *in vivo* in mice. Co-injection of sperm and LL-37 into the uterine cavity of naturally cycling female mice resulted in no pregnancy ($n = 26$), while 24 out of 26 females inseminated with sperm only became pregnant with a normal litter size (mean \pm SD: 9 ± 5 ; Fig. 8A). Significantly, no histological damage was observed in the vagina and uterus tissues (Fig. 8B) of the females that were inseminated with LL-37. When the females previously inseminated with LL-37 ($n = 6$) were naturally mated with fertile males, they became pregnant delivering pups with normal anatomy and the average litter size was similar to that from naturally mated control females ($8, n = 6$ versus $9, n = 6$). These results demonstrated the pre-administered LL-37 did not impact subsequent fertility of these female mice.

Discussion

Our study revealed an efficient contraceptive effect of LL-37 both *in vitro* (human and mice) and *in vivo* (mice), which was mediated through its adverse action on certain sperm parameters. Specifically, LL-37 caused sperm to become immotile and to acrosome react prematurely, thus hindering them from fertilizing the egg. The primary adverse action of LL-37 appeared to be on the sperm surface membranes; LL-37-treated sperm became positively stained with Sytox Green and unable to respond to a hypo-osmotic buffer. In addition, TEM results revealed structural damage on LL-37-treated sperm surface membranes. The damage at the sperm head, especially at the plasma membrane overlying the acrosome, the site where sperm first interact with the egg zona pellucida

(Florman and Ducibella, 2006), would directly make sperm lose the ability to bind to the zona pellucida (Supplementary data, Fig. S4) and to fertilize eggs (Fig. 2). The structural damage caused by LL-37 was not reversible. When P-CAP mouse sperm pretreated with LL-37 were washed in fresh medium and incubated in capacitating medium, they could not regain their functional parameters (e.g. motility; our unpublished results). The adverse effects of LL-37 on the integrity of the sperm surface membrane and the acrosome as well as on motility were observed not only on PGC human and mouse sperm but also on swim-up human sperm (Fig. 7), which contain residual amounts of seminal plasma surrounding their surface. Since the swim-up sperm are analogous to sperm that have just swum out from the liquefied semen in the vagina into the uterine cavity, our results suggest that LL-37 administered into the vagina (for example in a form of vaginal gel) should be able to exert its contraceptive effects on motile sperm that are leaving the vagina.

The induction of premature acrosome reaction by LL-37 may also be a consequence of LL-37 induced membrane structural damage. As part of the normal sperm–egg interaction that culminates in fertilization, F-CAP sperm are induced by a zona pellucida glycoprotein to undergo the acrosome reaction on the zona pellucida through a calcium signalling pathway (Florman and Ducibella, 2006). A massive calcium influx into sperm leads to the dissociation of actin filaments that hold the acrosome structure in place (Breitbart *et al.*, 2005). The fusion between the outer acrosomal membrane and the plasma membrane overlying the acrosome then results in the release of the acrosomal content containing hydrolases, which digest the zona pellucida layer to create tracks for sperm to move towards the egg plasma membrane (Florman and Ducibella, 2006). With LL-37 treatment, a premature acrosome reaction occurred even in N-CAP sperm, which were incubated in a calcium-free medium. The high calcium level required for actin filament dissociation in these LL-37 treated N-CAP sperm could have come from the intra-acrosomal storage pool, which was released due to the structural damage to the outer acrosomal membrane (Fig. 6). In addition, LL-37 can bind to actin, which is an anionic protein (Bucki *et al.*, 2007). The actin-LL-37 binding, together with the rise in the intracellular calcium level, may lead to the destabilization of the acrosome structure, and thus a rapid premature acrosome reaction (within 5 min) with a subsequent loss of sperm fertilizing ability (Florman and Ducibella, 2006).

Interestingly, the membrane structural damage of mouse sperm treated with $3.6 \mu\text{M}$ LL-37 was confined to the sperm head. One possible mechanism for this selective action of LL-37 may arise from its substantial affinity for SGG (Supplementary data, Fig. S2), which is present only in the sperm head (White *et al.*, 2000; Weerachayanukul *et al.*, 2001). SGG, existing at 10 mol% of total sperm lipids, is integral to the formation of sperm lipid rafts (Bou Khalil *et al.*, 2006). The liquid-ordered property of lipid rafts would facilitate LL-37 interaction with SGG accumulated in these microdomains. SGG-bound LL-37 peptides would be in very close proximity to each other, and this might enhance LL-37 oligomerization and subsequently membrane permeabilization (Sood *et al.*, 2008). It is likely that the small membrane vesicles (~ 35 – 100 nm in diameter) observed on the head surface of mouse sperm treated with $3.6 \mu\text{M}$ LL-37 (Fig. 6A and B) reflect this reorganization of the membrane following LL-37 treatment. The membrane perturbing effect of LL-37 on the anionic SGG-containing sperm plasma membrane would be analogous to its action on microbial membranes, which is dependent on negatively charged molecules (Bucki *et al.*, 2010). However, since anti-SGG

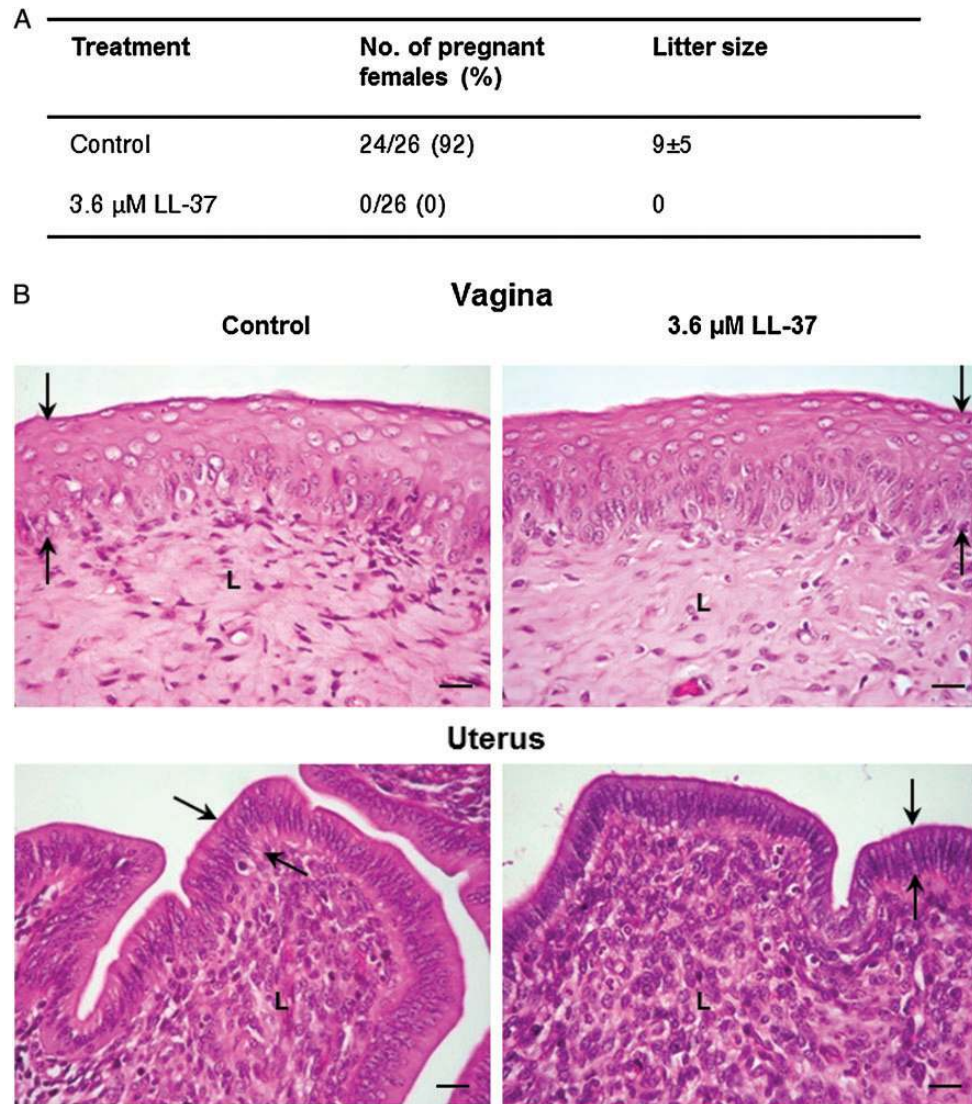


Figure 8 Females inseminated with sperm + LL-37 fail to become pregnant. **(A)** All naturally cycling female mice inseminated with sperm + 3.6 μM LL-37 ($n = 26$) showed no pregnancy, whereas 92% of females inseminated without LL-37 ($n = 26$) became pregnant. **(B)** Stratified epithelial cell layers in the vagina and single epithelial cell layer in the endometrium (denoted by area in between the two opposing arrows) were still preserved in the females inseminated with LL-37, and the lamina propria (L) in both tissues of LL-37 inseminated females were also as intact as in control females (inseminated without LL-37). Bar = 20 μm. Results were representative of five replicate experiments.

O4 and adamantyl SGG treatments could not completely block LL-37 binding to mouse sperm (Fig. 1B and [Supplementary data, Fig. S3](#)), other LL-37 binding partners may exist besides SGG. This implication was supported by the fact that LL-37 at 3.6 μM bound not only to the sperm head but also to the tail, which did not contain SGG ([Tanphaichitr et al., 2003](#)). In the case of human sperm, a large variety of anionic molecules, which exist in quantity, may exist on the tail surface, and they can interact with LL-37 with the downstream membrane disruption effects. This would explain the differential effect of LL-37 in disrupting sperm functions as opposed to other human and mouse cell types that generally require much higher concentrations for cytotoxicity ([Lau et al., 2005](#)). The LL-37 induced disruption of the sperm plasma membrane would facilitate the ability of the peptide to traffic towards the axoneme (motility

apparatus) and mitochondria (energy producing organelles) in the sperm tail, and LL-37 might interact with the axonemal and mitochondrial components leading to spermstasis.

The multiplicity of immediate and irreversible adverse actions of LL-37 on sperm (i.e. cell membrane damage, immotility and premature acrosome reaction) provide the peptide with highly contraceptive effects both *in vitro* and *in vivo*. While attempts have been made to use other antimicrobial peptides (e.g. nisin made by the bacterium *Lactococcus lactis*, and magainin and dermaseptin, both made by frog skin) as contraceptives by interfering with sperm functions, these antimicrobial peptides act mainly as spermstasis agents, and high concentrations are needed for their contraceptive effects ([Reddy et al., 1996](#); [Aranha et al., 2004](#); [Zairi et al., 2005](#)). Our attempt to use a rodent epididymal antimicrobial

peptide SPAG1IE (aka Bin1b), with a known function in initiating sperm forward motility (Zhou *et al.*, 2004), to inhibit sperm to fertilize co-incubated eggs also revealed only a 60% decrease in sperm fertilizing ability (unpublished results). The most significant advantage of LL-37 over other antimicrobial peptides in terms of its development into a vaginal contraceptive with microbicidal properties is that LL-37's close analogue, ALL-38, could be naturally produced up to $\sim 10 \mu\text{M}$ in the human vagina (Sorensen *et al.*, 2003). ALL-38 and LL-37 are structurally very similar. Based on the 3D structure of LL-37, which reveals segregation of hydrophobic amino acids and hydrophilic residues into two opposite lateral faces of the peptide helix (Wang, 2008), the alanine residue (hydrophobic) in the N-terminus of ALL-38, next to leucine (also hydrophobic) of the N-terminal sequence of LL-37, should not change the overall 3D structure of LL-37 (Wang, 2008). Therefore, LL-37 at contraceptive concentrations ($3.6 \mu\text{M}$ for mouse sperm, and $10.8 \mu\text{M}$ for human sperm) should exhibit minimal adverse effects on the female reproductive tract. Our results in the mouse system confirm this postulate. Furthermore, LL-37 does not exert adverse effects on vaginal microflora (e.g. lactobacilli; Smeianov *et al.*, 2000). Therefore, LL-37 is a promising candidate as a dual vaginal contraceptive/microbicide. Producing a device to deliver LL-37 effectively in the female genital tract, such as a vaginal/cervical ring that can release the peptide bidirectionally (downwards towards the vaginal opening and upwards into the uterine cavity), will be the key to inhibiting the fertilizing ability of both N-CAP and capacitated sperm and to protect the wide area of the female reproductive tract from microbial attacks. Alternatively, a LL-37 formulated vaginal gel can be used on demand to prevent pregnancy and protect women from microbial attacks. Our preliminary results indicate that a vaginal hydroxyethylcellulose gel formulated with LL-37 can effectively prevent pregnancy in mice. Following natural mating, only 3 out of 11 female mice administered the LL-37 vaginal gel became pregnant, whereas 100% pregnancy was observed in all eight females treated with the placebo gel. Ongoing and immediate future experiments include modifications of the LL-37 vaginal gel formulation and a higher number of experimental animals (mice and rabbits) for testing the efficacy of the LL-37 vaginal gel in pregnancy prevention. These experiments will provide the basic information to guide long-term studies in non-human primates and eventually clinical trials. Ultimately, the use of such LL-37 vaginal rings/gel will provide women with empowerment over family planning and healthy sexual activities. However, we are aware of LL-37's immunomodulatory properties, which include its activation of the production of cytokines such as interleukin (IL)-8, IL-6 and interferon- α (Mookherjee *et al.*, 2009; Pistolic *et al.*, 2009) and studies are being conducted to assess the effects of long-term exposure of the female genital tract to LL-37.

Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

Authors' roles

The three aspects towards the preparation of the manuscript: (i) conception and design, acquisition of data, analysis and interpretation of data, (ii) drafting the article and revising it critically for important intellectual content and (iii) final approval of the version to be published were contributed the most by N.S., C.D.Y., A.S., and N.T. who also executed all

experiments. H.X., K.L.Q., R.B., L.P., T.W.P., R.E.H., R.J.O., L.S.H. also substantially contributed to these three aspects. For the first aspect, K.K., D.F., G.H., W.W., L.F., F.C. contributed to acquisition of data, analysis and interpretation of data, whereas S.H.H., Y.-L.Z. substantially contributed to conception and design in this aspect. All authors in these last two categories contributed to the second and third aspects like other authors.

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Conflict of interest

The authors have declared that there is no conflict of interest on this work. Although R.E.H. has been working on various host defence peptides and has 43 issued patents or numerous patent protection filings, invented by him and assigned to his employer UBC, none of these concern LL-37 or anything to do with fertility.

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