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ORIGINAL ARTICLE ***Andrology***

Antimicrobial peptide LL-37 and its truncated forms, GI-20 and GF-17, exert spermicidal effects and microbicidal activity against *Neisseria gonorrhoeae*

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STUDY QUESTION: Do the truncated LL-37 peptides, GI-20 and GF-17, have spermicidal activity and microbicidal effects on the sexually transmitted infection (STI) pathogen *Neisseria gonorrhoeae* with equivalent potency to LL-37?

SUMMARY ANSWER: GI-20 and GF-17 exhibited spermicidal effects on both mouse and human sperm as well as microbicidal action on *N. gonorrhoeae* with the same efficacy as LL-37.

WHAT IS KNOWN ALREADY: The antimicrobial peptide LL-37 exerts microbicidal activity against various STI pathogens as well as spermicidal effects on both mouse and human sperm.

STUDY DESIGN, SIZE, DURATION: Spermicidal activities of GI-20 and GF-17 were evaluated *in vitro* in mouse and human sperm and *in vivo* in mice. Finally, *in vitro* antimicrobial effects of LL-37, GI-20 and GF-17 on an STI pathogen, *N. gonorrhoeae* were determined. All experiments were repeated three times or more. In particular, sperm samples from different males were used on each experimental day.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The plasma membrane integrity of peptide-treated sperm was assessed by cellular exclusion of Sytox Green, a membrane impermeable fluorescent DNA dye. Successful mouse *in vitro* fertilization was revealed by the presence of two pronuclei in oocytes following co-incubation with capacitated untreated/peptide-pretreated sperm. Sperm plus each peptide were transcervically injected into female mice and the success of *in vivo* fertilization was scored by the formation of 2–4 cell embryos 42 h afterward. Reproductive tract tissues of peptide pre-exposed females were then assessed histologically for any damage. Minimal inhibitory/bactericidal concentrations of LL-37, GI-20 and GF-17 on *N. gonorrhoeae* were determined by a standard method.

MAIN RESULTS AND THE ROLE OF CHANCE: Like LL-37, treatment of sperm with GI-20 and GF-17 resulted in dose-dependent increases in sperm plasma membrane permeabilization, reaching the maximum at 18 and 3.6 μM for human and mouse sperm, respectively ($P < 0.0001$, as compared with untreated sperm). Mouse sperm treated with 3.6 μM GI-20 or GF-17 did not fertilize oocytes either *in vitro* or

in vivo. Moreover, reproductive tract tissues of female mice pre-exposed to 3.6 μM GI-20 or GF-17 remained intact with no lesions, erosions or ulcerations. At 1.8–7.2 μM, LL-37, GI-20 and GF-17 exerted bactericidal effects on *N. gonorrhoeae*.

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: Direct demonstration of the inhibitory effects of GI-20 and GF-17 on human *in vitro* and *in vivo* fertilization cannot be performed due to ethical issues.

WIDER IMPLICATIONS OF THE FINDINGS: Like LL-37, GI-20 and GF-17 acted as spermicides and microbicides against *N. gonorrhoeae*, without adverse effects on female reproductive tissues. With lower synthesis costs, GI-20 and GF-17 are attractive peptides for further development into vaginal spermicides/microbicides.

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Key words: spermicide / antimicrobial peptide / LL-37 / GI-20 / GF-17 / microbicide / *Neisseria gonorrhoeae* / *Lactobacillus crispatus* / sperm-fertilizing ability / vaginal contraceptive

Introduction

The continuing and concurrent increases in global population and sexually transmitted infections (STIs) (Bloom, 2011; https://www.cdc.gov/std/stats/default.htm) demand the search for compounds that can act as both contraceptives and microbicides. Recently, we have shown that the naturally occurring cathelicidin antimicrobial peptide (AMP), LL-37, within a physiological concentration range, can exert spermicidal action on both human and mouse sperm (Srakaew *et al.*, 2014). LL-37 has microbicidal activity on numerous pathogens and in particular on STI bacteria and viruses (Tanphaichitr *et al.*, 2016). Its antimicrobial activity is achieved through damaging effects on bacterial cytoplasmic membranes as well as its immunomodulatory action (Mansour *et al.*, 2014; Wang, 2014; Tripathi *et al.*, 2015; Xhindoli *et al.*, 2016). LL-37 is therefore a potential candidate to be developed into a vaginal spermicide/microbicide (Tanphaichitr *et al.*, 2016). Its wound-healing property (Mangoni *et al.*, 2016) would also be beneficial in repairing minor vaginal tissue damage after intercourse. However, the cost of synthesizing the 37-mer long LL-37 peptide for clinical use is a major challenge.

Attempts to create truncated peptides of LL-37 to be used as microbicides have been based on the amino acid composition and 3D structure of LL-37 (Wang *et al.*, 2014). Like other AMPs, LL-37 (MW, 4493) is cationic and amphipathic, and upon interaction with anionic micelles such as sodium dodecyl sulfate (SDS) or phosphatidylglycerol model membranes (both modeling the anionic bacterial cytoplasmic membrane), LL-37 adopts a long alpha helical structure extending over almost the whole peptide (from Leu2 to Leu31), as revealed by NMR analyses. The remaining C-terminal region is disordered and not involved in membrane binding. Significantly, the cationic hydrophilic residues are localized on one face of the peptide alpha helix, whereas the hydrophobic amino acids are situated on the other face (Wang, 2008). This segregation facilitates LL-37 in electrostatic interaction with anionic lipopolysaccharides in the outer membrane of Gram-negative bacteria and lipoteichoic acid in the cell wall of Gram-positive bacteria and subsequently with the head groups of bacterial anionic phospholipids (phosphatidylglycerol and cardiolipin) in the cytoplasmic membrane. This is followed by the insertion of the LL-37 helices into the membrane bilayer, resulting in pore formation, eventual loss of cellular homeostasis and finally cell death (Wang *et al.*, 2014; Xhindoli *et al.*, 2016).

NMR results also indicate that the 3D helical structure of LL-37 bends between Gly14 and Glu16, thus separating the structure into the N-terminal helix from Leu2 to Ile13 and the longer central helix spanning from Gly14 to Leu31 (Wang *et al.*, 2014). Accumulated studies using truncated LL-37 peptides of various lengths and sequence locations reveal that the central helix of LL-37 is much more relevant than the N-terminal helix for microbicidal/virucidal activities (Wang *et al.*, 2014). To date, the two truncated LL-37 peptides, GI-20 (MW, 2473) and GF-17 (MW, 2012) (see sequences in Supplementary Fig. S1) display the best microbicidal/virucidal properties (http://aps.unmc.edu/AP/main.php; He *et al.*, 2018) and are the most promising candidates to be assessed for spermicidal activity. This study is necessary considering that the mechanisms of spermicidal and microbicidal actions of LL-37 are not exactly the same. Although the surface membranes of sperm are the targets of LL-37, analogous to what was observed on the bacterial cytoplasmic membrane, sperm surface membranes are actually ruptured and dissociated from LL-37-treated sperm (Srakaew *et al.*, 2014), in contrast to the more limited pore formation induced by LL-37 in the bacterial membrane (Xhindoli *et al.*, 2016). Since previous studies indicate the significance of the central helical structure of truncated LL-37 peptides on microbicidal and anti-HIV activity (Li *et al.*, 2006; Wang *et al.*, 2008, 2012; Epand *et al.*, 2009; Tripathi *et al.*, 2015), the relevance of this helical structure on spermicidal activity should also be investigated.

The potential use of LL-37 as a vaginal contraceptive will also allow the simultaneous prevention of STIs. The microbicidal activity of LL-37 has been shown in a number of STI microbes (Tanphaichitr *et al.*, 2016), although it would be most relevant to *Neisseria gonorrhoeae*, which has continuously exhibited resistance to various classes of antibiotics at a dramatic rate (Unemo and Shafer, 2014). Although the microbicidal activity of LL-37 has been demonstrated in *N. gonorrhoeae* MS11 (Bergman *et al.*, 2005), it is important that similar microbicidal activity of GI-20, GF-17 and LL-37 is shown in other geographically distinct clinical isolates of *N. gonorrhoeae*. Furthermore, their effects on the commensal bacterium, *L. crispatus*, which is relevant for vaginal health (Reid, 2017), must be determined.

Our research objectives were 2-fold. We asked: (i) whether the two truncated LL-37 peptides, GI-20 and GF-17, possessed spermicidal activity, with dependence on their helical structure and (ii)

whether GI-20, GF-17 and LL-37 could exert microbicidal action on *N. gonorrhoeae* but exhibit minimal deleterious effects on *L. crispatus*.

Materials and Methods

Peptides

All peptides used in our study were chemically synthesized with >95% purity (by CPC Scientific (San Jose, CA, USA) for LL-37; AnaSpec (Fremont, CA, USA) for scrambled LL-37; and Genemed (San Antonia, TX, USA) for GI-20, GF-17 and GF-17D3). GF-17D3 has the same sequence as GF-17 but with Ile20, Ile24 and Leu28 substituted with the corresponding D-amino acids (see Supplementary Fig. S1).

Analyses of the secondary structure of peptides by circular dichroism

Circular dichroism (CD) spectra were obtained for ~15 μM peptide in 10 mM sodium phosphate buffer, pH7.4, containing 10 mM SDS on a Jasco J-815 CD spectrometer (Jasco Inc., Easton, MD, USA). Averaged spectra from four replicates normalized for protein concentration (SPARC BioCentre, Amino Acid Analysis Facility, Toronto, Canada) were analyzed in CDPro (Jasco) employing the CONTIN method with the SMP56 reference set (Sreerama and Woody, 2000).

Ethical approval

The use of mice (protocol 2567) was approved by the University of Ottawa Animal Care committee, which endorses the use of ARRIVE checklists and guidelines. Recruitment for sperm donors and the handling of human sperm for experimental purposes were authorized by the Ottawa Health Network Research Ethics Board (protocol 2005256-01 H).

Mice and their boarding

Specific pathogen-free CD-1 male mice (8–10 weeks old) and CF-1 female mice (6–8 weeks old), obtained from Charles River Laboratories (Senneville, QC, Canada), were kept in a temperature-controlled (22.5°C) room with a 14-h light/10-h dark cycle and fed with food chow and water ad libitum.

Mouse and human sperm preparation

The motile population of mouse sperm, collected from the cauda epididymis and vas deferens, was prepared by 45%/90% Percoll-gradient centrifugation and was either partially capacitated (P-CAP) or fully capacitated (F-CAP) as previously described by Srakeaw *et al.* (2014).

Fertile and healthy human sperm donors with normal semen parameters (WHO, 2010) were recruited. Sperm samples were collected by masturbation after 2–3 days of abstinence and selected by Percoll-gradient centrifugation for the motile population, from which P-CAP and F-CAP sperm were prepared. In additon, swim-up sperm were prepared from ejaculated semen. All procedures were previously described by Srakaew *et al.* (2014).

Treatment of sperm with peptide

Mouse and human sperm (1×10^7/ml) with different capacitation states were treated (30 min, 37°C) with various concentrations of LL-37, GI-20, GF-17 or GF-17D3. Excess peptides were removed from sperm by centrifugation (350 × *g*, room temperature (RT), 5 min). Washed sperm were resuspended in either PBS or medium for further analyses.

Binding of GI-20 and GF-17 to sperm

Following treatment of mouse and human sperm with GI-20 or GF-17 (0.36 and 3.6 μM for mouse sperm, and 3.6 and 10.8 μM for human sperm), binding of the peptide to sperm was detected by indirect immunofluorescence as described by Srakaew *et al.* (2014).

Effects of LL-37, GI-20, GF-17 and GF-17D3 on sperm-fertilizing parameters

Sperm surface membrane intactness

Sytox Green (3 μM) (Life Technologies, Burlington, ON, Canada) was used for surface membrane intactness assessment as previously described (Srakaew *et al.*, 2014). Only sperm with compromised plasma membranes showed positively stained nuclei.

In vitro sperm-fertilizing ability

Untreated and peptide-pretreated (37°C, 5% CO_2, 30 min) F-CAP mouse sperm were co-incubated (37°C, 5% CO_2, 6 h) with mature oocytes in 500 μl of 25BmKSOM +0.3% BSA medium. Oocytes were then microscopically scored for two pronuclei as evidence of fertilization (Tantibhedhyangkul *et al.*, 2002).

In vivo sperm fertilization ability

F-CAP mouse sperm (1×10^7/ml) were untreated or treated (37°C, 5% CO_2, 30 min) with 3.6 μM of each peptide in 500 μl of KSOM+0.3% BSA. The sperm concentration was then increased to 1×10^8/ml by centrifugation (350 × *g*, 5 min, RT) followed by the removal of 450 μl of the supernatant. The pelleted sperm were then resuspended in the remaining supernatant, which still contained 3.6 μM of the peptide. The concentrated sperm suspension (50 μL) was transcervically injected using a 1-ml syringe attached with a blunt-ended 23.5 G needle (1.8 cm long) into each female mouse naturally cycling to the estrous phase 1 to 1.5 h before the earliest expected ovulation time, following the previously described method (Srakaew *et al.*, 2014). Various phases in the estrous cycle was evaluated by cytology of the vaginal lavage (Byers *et al.*, 2012). Embryos (2–4 cell) and unfertilized oocytes were collected from the oviducts of the female mice sacrificed 42 h post-insemination (Behringer *et al.*, 2014). The *in vivo* fertilization rate was calculated as a percentage of 2–4 cell embryos over the sum of 2–4-cell embryos and unfertilized oocytes. The 2–4 cell embryos were further assessed for their normal development by their progress into blastocysts upon culturing (37°C, 5% CO_2) for another 3–4 days in KSOM (Lawitts and Biggers, 1993) supplemented with essential and non-essential amino acids (Sigma, Mississauga, ON, Canada) (Biggers *et al.*, 2000).

Histological assessment of the female reproductive tract

Female reproductive tract organs (the vagina, cervix and uterus) were removed from mice 24 h post-transcervical insemination with or without GI-20 or GF-17 peptide, fixed in Bouin's solution and processed for paraffin embedding following the standard protocol. Tissue sections were stained with hematoxylin and eosin. Images on each slide recorded by microscopic scanning on an Aperio CS2 Digital Pathology Scanner (Leica Biosystem, Buffalo Grove, IL, USA) were analyzed *in silico* using ImageScope software (Leica Biosystem) for the integrity of the vagina, cervix and uterus tissues. As well, various phases of the estrous cycle was determined from the histology of the vagina sections based on previously described criteria (Westwood, 2008).

Resumption of fecundity in females previously treated with LL-37, GI-20 and GF-17

Females previously transcervically injected with sperm + peptide were individually mated with fertile males 4 days post-injection. Once the vaginal plug was observed, males were removed from the female cages and the females were monitored for pregnancy and pup delivery in the next 25 days. The same experiment was also performed with females transcervically injected with PBS (positive controls).

Bactericidal effects of LL-37, GI-20 and GF-17

A clinical isolate strain NRCC 6879 of *N. gonorrhoeae* was obtained from NRCC, Ottawa, Ontario, Canada, whereas *Lactobacillus crispatus SJ-3C* was purchased from ATCC (Manassas, VA, USA). A mixture of Todd Hewitt (Sigma) and Columbia (Becton, Dickinson and Company (BD), Sparks, MD, USA) (TH + CB, 1:1, *v/v*) broth was used for culturing (37°C, 95% relative humidity (RH), 5% CO_2) *N. gonorrhoeae*, whereas MRS (de Man, Rogosa and Sharpe) (Sigma) broth was employed for culturing *L. crispatus* (37°C, 95% RH). Initially, the dilution factor of the stock bacterial suspension was determined on the basis of the appearance of a bacterial button in the bottom of a 96-well plate after 24 h in culture. The highest dilution of bacteria that produced a visible button was subsequently used in the minimal inhibitory concentration (MIC) assay for each peptide. The MIC assay was set up, following the standard broth microdilution method (Wiegand *et al.*, 2008). Briefly, 180 µl of appropriately diluted *N. gonorrhoeae* or *L. crispatus* were pipetted into each microtiter well, which already contained 20 µl of 10× concentrated peptide solution. The final concentrations of each peptide tested were 0, 0.36, 1.8, 3.6, 7.2, 10.8 and 21.6 µM. After 24-h incubation, the presence of a bacterial button in the bottom of each well was examined. The MIC was defined as the most dilute peptide concentration that inhibited formation of the bacterial button. Bacteria suspensions in the wells with the peptide of the MIC value and higher concentrations were then used for the minimal bactericidal concentration (MBC) determination. For this, the well contents were transferred into another 96-well plate and diluted 2-fold serially with fresh broth over eight wells. After 24-h incubation, the presence of a bacterial button was determined. The original peptide concentration (before serial dilution) that did not allow bacterial button formation in the second plate was defined as its MBC.

Sample size and statistical analyses

All experiments were repeated three times or more using sperm samples collected from different male individuals. Statistical analyses were performed using GraphPad Prism Software 6.0 (San Diego, CA, USA). One-way ANOVA and two-way ANOVA, both with Tukey's multiple comparison, were used for determining significance differences ($P < 0.05$) between samples.

Results

Spermicidal activity of truncated LL-37 peptides: GI-20, GF-17 and GF-17D3

The sequences of three truncated peptides lie within the central helical structure of LL-37 (Supplementary Fig. S1). Our CD analyses validated their helical structure (Supplementary Fig. S2). Both GI-20 and GF-17, associated with anionic SDS micelles, exhibited 100% helicity. In contrast, the helicity of corresponding SDS-bound GF-17D3 was only 70%. Regardless, GI-20, GF-17 and GF-17D3 could all bind to sperm in a similar manner to LL-37 (Srakaew *et al.*, 2014) (data not shown).

Treatment of P-CAP and F-CAP mouse sperm with GI-20, GF-17 and LL-37 induced membrane permeabilization (Sytox Green positive staining) in a concentration-dependent manner (Fig. 1A) with significant differences over the untreated control sperm except at 0.36 µM LL-37 on P-CAP sperm (Supplementary Table S1A). Close to 100% of P-CAP and F-CAP sperm lost membrane intactness at 3.6 and 1.8 µM, respectively, of these three peptides. Concurrently, the majority of these mouse sperm became immotile (Supplementary Fig. S3A). P-CAP and F-CAP human sperm treated with GI-20, GF-17 and LL-37 likewise lost membrane integrity and motility in a dose-dependent manner with significant differences from the untreated control sperm (Fig. 1B, Supplementary Fig. S3B, Supplementary Table S1B). However, it required 18 µM of each peptide for the maximum spermicidal effects on >80% of P-CAP and F-CAP human sperm. The results indicated equimolar spermicidal potency among GI-20, GF-17 and LL-37 at these maximal concentrations. At lower concentrations, however, the three peptides appeared to have differential effects on inducing sperm membrane permeabilization on P-CAP and F-CAP mouse sperm and P-CAP human sperm. On P-CAP mouse sperm, the effects of GF-17 were higher than those of GI-20, which in turn were higher than those of LL-37. But for F-CAP mouse sperm, the potency of GF-17 and LL-37 was equivalent but higher than that of GI-20 (Fig. 1A). On the other hand, for P-CAP human sperm, GI-20 exerted the highest membrane permeabilization effects at 3.6, 7.2 and 10.8 µM, whereas GF-17 and LL-37 exhibited similar adverse effects (Fig. 1B).

Unlike GF-17, the mutant GF-17D3 peptide did not induce membrane permeabilization and immotility on F-CAP mouse at 3.6 µM (Fig. 1C, Supplementary Fig. S3C). Although there was a slight but significant increase in the F-CAP human sperm population with membrane permeabilization upon treatment with 18 µM GF-17D3, as compared with untreated sperm, this increase was markedly lower than that observed with F-CAP human sperm treated with 18 µM GF-17 (21 ± 1% vs 94 ± 1%) (Fig. 1C).

The direct ability of truncated LL-37 peptides to inhibit fertilization *in vitro* was evaluated using the mouse system. Similar to LL-37 (Srakaew *et al.*, 2014), GI-20 and GF-17 inhibited mouse *in vitro* fertilization in a dose-dependent manner and when F-CAP sperm were treated with 3.6 µM of any of the three peptides, they completely lost their fertilizing ability (Fig. 2A). Notably, GF-17 at 1.8 µM inhibited sperm-fertilizing ability better than GI-20 (Fig. 2A, middle and right panels). In contrast, F-CAP sperm treated with 3.6 µM of scrambled LL-37 did not show any significant inhibition, as compared with control-untreated sperm (Fig. 2A, left panel). Unlike GF-17, GF-17D3 at 3.6 µM did not inhibit sperm-fertilizing ability to the zero level (Fig. 2B). The slight but significant decrease in the percentage of oocyte fertilized by GF-17D3-treated sperm (63 ± 5%, compared with 85 ± 11% by peptide-untreated sperm), however, was somewhat surprising.

Spermicidal effects of GI-20 and GF-17 on human swim-up sperm

Human swim-up sperm still contain a residual amount of seminal plasma, thus resembling sperm that have just swum out from liquefied semen in the vagina into the uterine lumen. Over 80% of capacitated swim-up human sperm treated with 18 µM of GI-20 or GF-17 became

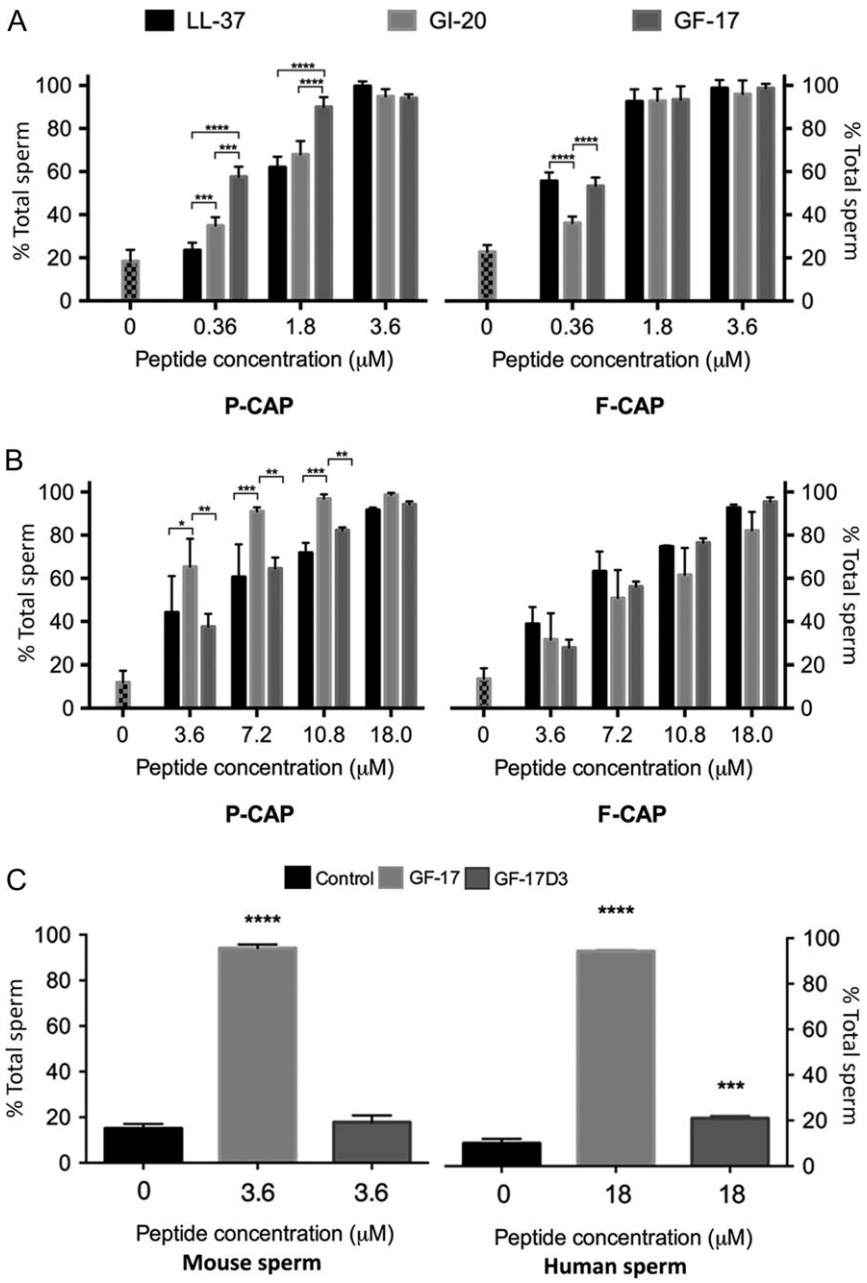

Figure 1 Effects of LL-37, GI-20 and GF-17 on surface membrane permeabilization on mouse (**A**) and human (**B**) sperm. P-CAP and F-CAP sperm untreated (control) or treated with the peptide (0.36–3.6 μM for mouse sperm and 3.6–18 μM for human sperm) were evaluated for surface membrane permeabilization by Sytox Green staining. Percentages of sperm with permeabilized membranes in total sperm population were expressed as means ± SDs of three experiments using different sperm samples. The symbols *, **, *** and **** denote $P < 0.05$, 0.01, 0.001 and 0.0001, respectively, for the significant differences among the three peptides. (**C**) Zero and minimal adverse effects of GF-17D3 on mouse and human sperm surface membranes, respectively. F-CAP sperm were treated with GF-17 or GF-17D3 at 3.6 μM for mouse sperm and at 18 μM for human sperm. Expression of data from three different sperm samples and statistical analyses for significant differences of data from GF-17- and GF-17D3-treated samples vs those of control-untreated samples followed the same approaches as in A and B.

Sytox Green stained and lost motility, similar to results observed in both F-CAP and P-CAP Percoll-gradient centrifuged human sperm (Fig. 3 vs Fig. 1B, and Supplementary Fig. S4 vs Supplementary Fig. S3B). At lower peptide concentrations, the membrane permeabilization effects of GI-20 and GF-17 on capacitated swim-up sperm were also dose-dependent, with significant differences from control-untreated sperm (Fig. 3, Supplementary Table SII), although GI-20 at 10.8 μM exerted a higher effect than GF-17 of equivalent molarity (Fig. 3). Similar dose-dependent adverse effects of GI-20 and GF-17 on swim-up sperm motility were observed, but the higher immotility induction of GF-17 was demonstrated throughout the three sub-maximal concentrations (3.6, 7.2 and 10.8 μM) (Supplementary Fig. S4).

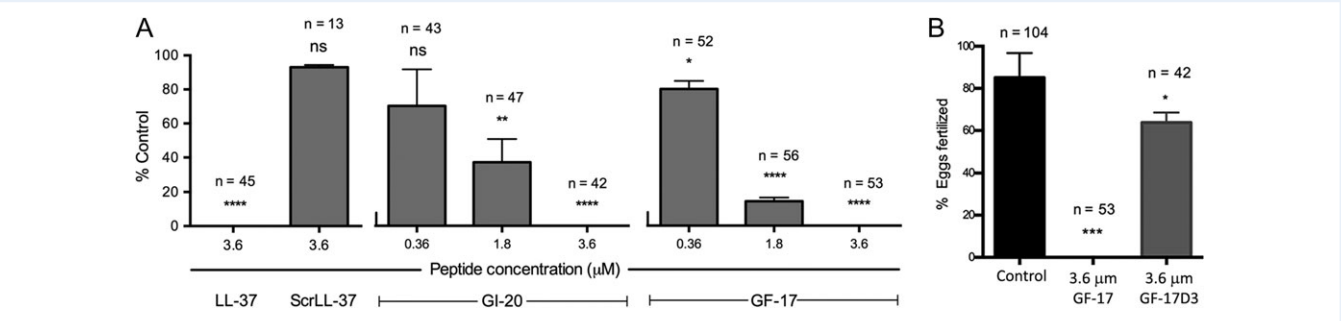

Figure 2 (**A**) Decreased ability of GI-20- and GF-17-treated mouse sperm to fertilize mature oocytes *in vitro*. F-CAP mouse sperm, untreated or pretreated with various concentrations of GI-20 or GF-17, were co-incubated with mature oocytes. F-CAP sperm pretreated with 3.6 μM LL-37 and 3.6 μM scrambled LL-37 were also used for oocyte co-incubation. More than 80% of oocytes co-incubated with control sperm were fertilized and designated as 100% control. Fertilization data of peptide-treated sperm were expressed as means ± SDs of percent controls from three or more replicate experiments performed on different days. (**B**) Inefficiency of the mutant GF-17D3 peptide in inhibiting mouse *in vitro* fertilization. F-CAP mouse sperm untreated (control) or pretreated with 3.6 μM GF-17 or GF-17D3 were co-incubated with mature oocytes. Data from three replicate experiments performed on different days were expressed as means ± SDs of percent fertilized oocytes of the total oocytes in the gamete co-incubates. In both A and B, *n* = total number of inseminated oocytes from all experimental days. The symbols *, **, *** and **** indicate significant differences with $P < 0.05$, 0.01, 0.001 and 0.0001, respectively, as analyzed by one-way ANOVA.

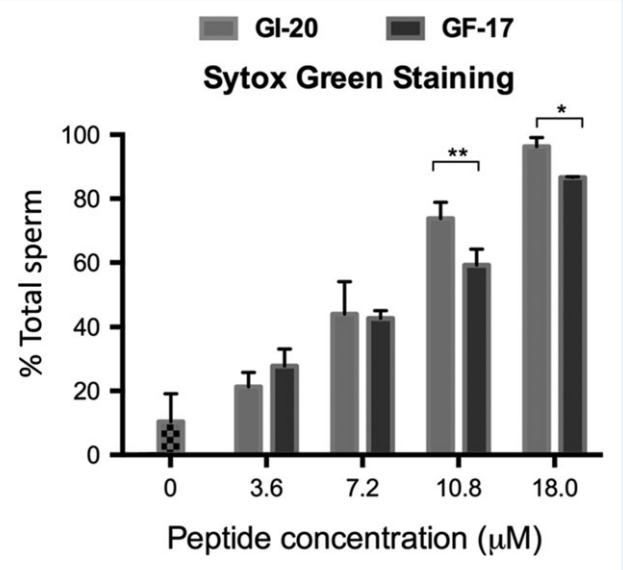

Figure 3 Effects of GI-20 and GF-17 on surface membrane permeabilization on human swim-up sperm. Swim-up sperm treated with the peptide (3.6–18 μM) were assessed for percent of total sperm with Sytox Green staining. Untreated swim-up sperm served as negative controls. Data were expressed as means ± SDs of results from the three donors. Significant differences of the effects between the two peptides at each tested concentration were analyzed by two-way ANOVA followed by Tukey's multiple comparison. The symbols * and ** denote $P < 0.05$ and 0.01, respectively.

Effects of LL-37, GI-20 and GF-17 on mouse sperm-fertilizing ability *in vivo*

Capacitated mouse sperm treated with 3.6 μM of LL-37, GI-20 and GF-17 completely lost the ability to fertilize oocytes in vivo, as shown by the absence of 2–4 cell embryos in the oviduct of the female mice, transcervically injected 42 h prior with these peptide-treated sperm. In contrast, 78 ± 17% to 89 ± 11% of ovulated oocytes were fertilized and developed into 2–4 cell embryos 42 h following transcervical injection with untreated sperm in the three sets of experiments (Table I).

Lack of adverse effects of GI-20 and GF-17 on the histology of the reproductive tract of female mice transcervically injected with sperm + peptide

The epithelial cell layers and the lamina propria of the vagina, cervix and uterus of female mice transcervically injected with sperm + 3.6 μM GI-20 (Fig. 4, panels b, e and h) or GF-17 (Fig. 4, panels c, f and i) remained intact one day after the transcervical injection of sperm + peptide, with the appearance resembling that of the control females injected with sperm alone (Fig. 4, panels a, d and g). Cytological assessment of cellular materials in the vaginal lumen also indicated that the reproductive cycle of all mice (control, GI-20 and GF-17 exposed) progressed to the next phase of the estrous stage, when the transcervical sperm injection was performed. In all of the three control females, their cycles were in diestrus (revealed by a few layers of the vaginal epithelium and existence of leukocytes in the lumen) (Fig. 4, panel a, and Supplementary Fig. S5, panels a and b). Two GI-20 exposed mice (Fig. 4, panel b, and Supplementary Fig. S5, panel d) and one GF-17 exposed mouse (Fig. 4, panel c) were in metestrus (revealed by the presence of nucleated epithelial cells, cornified cells and leukocytes in the vaginal lumen). The cycle of the other GI-20 exposed mouse (Supplementary Fig. S5, panel c) and the other two GF-17 exposed mice (Supplementary Fig. S5, panels e and f) were between the diestrus and proestrus phases (revealed by the existence of leukocytes in the vaginal lumen and a few layers of epithelial cells with the superficial mucoid layer stained as a lavender color).

Unsurprisingly, females that were previously exposed to GI-20, GF-17 and LL-37 regained fecundity upon individually co-caging with

Table I Inhibitory effects of LL-37 and its truncated peptides on mouse *in vivo* fertilization.

Transcervical injection with[a]	Oocytes + 2–4 cell embryos	2–4 Cell embryos	Blastocysts
Sperm alone	48[c]	43[c]	38[c]
n[b] = 4	(100%)	(89 ± 11%)[d]	(90 ± 10%)[e]
Sperm + 3.6 μM LL-37	46	0	0
n = 3	(100%)	(0%)	(0%)
Sperm alone	48	40	39
n = 4	(100%)	(83 ± 13%)	(81 ± 15%)
Sperm + 3.6 μM GI-20	61	0	0
n = 6	(100%)	(0%)	(0%)
Sperm alone	75	59	57
n = 6	(100%)	(78 ± 17%)	(76 ± 18%)
Sperm + 3.6 μM GF-17	104	0	0
n = 9	(100%)	(0%)	(0%)

[a]Each peptide was separately evaluated for its effects on sperm-fertilizing ability *in vivo*. Transcervical injection experiments were performed with peptide-treated sperm in comparison with untreated sperm on three experimental days.
[b]*n* denotes the total number of female mice used for transcervical sperm injection on the three experimental days.
[c]The numbers of oocytes, two cell embryos and blastocysts shown here are the sums from the total female mice transcervically injected with either sperm alone or peptide-treated sperm on the three experimental days.
[d]Percentages of *in vivo* fertilization were calculated from the ratio of 2–4 cell embryos over total unfertilized oocytes + 2–4 cell embryos. Data were expressed as means ± SDs from the three replicate experiments.
[e]Percentages of blastocysts formed were from 2–4 cell embryos (designated as 100%). Data were expressed as means ± SDs from the three replicate experiments. Note that blastocyst formation from 2–4 cell embryos, retrieved from females transcervically injected with sperm alone, was >75% in the three sets of experiments, indicating that *in vivo* fertilization occurred normally following transcervical sperm injection.

fertile males 4 days after the transcervical peptide injection. All of these females became pregnant with litter sizes and gestation periods similar to those of females that were transcervically injected with PBS (Supplementary Table SIII).

High efficacy of antimicrobial effects of LL-37, GI-20 and GF-17 on *N. gonorrhoeae* but not so on *L. crispatus*

All three peptides exhibited bactericidal effects on *N. gonorrhoeae* with MICs and MBCs of 1.8 μM for LL-37 and GF-17, and 7.2 μM for GI-20 (Table II). However, their effects on the commensal *L. crispatus* bacterium in the vagina were either none (for LL-37 up to 22 μM) or lower than those observed with *N. gonorrhoeae*. GI-20 exerted only bacteriostatic effects on *L. crispatus* with an MIC of 7.2 μM. For GF-17, its MIC and MBC on *L. crispatus* was 3.6 and 10.8 μM, respectively (Table II).

Discussion

We demonstrated herein that the two truncated LL-37 peptides, GI-20 and GF-17, exerted spermicidal effects on human and mouse sperm with the same efficacy as LL-37. Close to 100% of Percoll-gradient selected human and mouse sperm with high fertilizing ability became immotile and lost their membrane intactness upon treatment with GI-20, GF-17 or LL-37 at 10.8–18 μM (for human sperm) and 3.6 μM (for mouse sperm). Similar effects were observed in human swim-up sperm, which resemble sperm that have swum out from liquefied semen in the vagina (Prins and Lindgren, 2015). The direct ability of mouse sperm treated with 3.6 μM GI-20, GF-17 or LL-37 to fertilize oocytes was further shown to be completely lost both *in vitro* and *in vivo*. Notably, the *in vivo* fertilization results with LL-37-treated sperm shown herein provide the key mechanism explaining our previous findings on the no pregnancy outcome in female mice transcervically injected with sperm + 3.6 μM LL-37 (Srakaew *et al.*, 2014). All of these results indicate that GI-20 and GF-17 have equal spermicidal efficacy to LL-37. Notably, LL-37 is a natural peptide and its propeptide, hCAP-18, which is convertible to ALL-38 (LL-37 with an additional Ala at the N-terminus) in the vagina (Sorensen *et al.*, 2003), is present at a high concentration (2–10 μM) in seminal plasma. Consequently, exposure of the female reproductive tract to the spermicidal concentration of LL-37 or its truncated forms, GI-20 and GF-17, should bear minimal adverse effects. This expectation was validated by the absence of histological damages of the epithelia and lamina propria of the reproductive tract of female mice, pre-exposed to 3.6 μM GI-20, GF-17 or LL-37 (Srakaew *et al.*, 2014). The same results were also obtained in females transcervically injected with 18 μM LL-37 (our unpublished observation). Moreover, female mice previously exposed to LL-37/GI-20/GF-17 resumed fecundity. All of these results indicate that GI-20, GF-17 and LL-37 warrant development into vaginal spermicides. However, given the much lower synthesis cost for the truncated peptides vs LL-37, GI-20 and GF-17 should be more appropriate for this development.

Our current studies also indicate the significance in spermicidal action of the central alpha helix of LL-37 (which cover the sequences of GI-20 and GF-17). As shown by CD analyses, GF-17D3, a mutant GF-17 peptide with three amino acids substituted with corresponding D-amino acids contained 74% helicity upon interaction with SDS micelles. Yet, GF-17D3 at 3.6 μM exerted minimal adverse effects on sperm surface membrane permeabilization and motility in contrast to full effects of equimolar GF-17. These results indicated that the uninterrupted helicity of GF-17 and likely also GI-20 was needed for the spermicidal action, and future creation of bioactive mimetics of GI-20 and GF-17 must consider this requirement. Nonetheless, F-CAP mouse sperm treated with 3.6 μM GF-17D3 fertilized 62% mature oocyte *in vitro*, a value significantly lower than 84% of mature eggs fertilized by control-untreated sperm. The slight but significant inhibition of the sperm-fertilizing ability by GF-17D3 could be from the possibility that GF-17D3, having the same cationic charge (+5) as GF-17, still bound electrostatically to the anionic sperm head surface (Ainsworth *et al.*, 2005). However, unlike the case with GF-17, binding of GF-17D3 to sperm did not lead to further sperm surface membrane permeabilization or damage and immotility and subsequently incomplete loss of sperm ability to fertilize oocytes *in vitro* (Fig. 2). However, the sperm-bound GF-17D3 might mask certain oocyte-binding proteins/molecules on the sperm surface, leading to a slight decrease in sperm ability to fertilize the oocytes. As alluded to in the Introduction section, the mechanisms through which LL-37 and its truncated peptides exert spermicidal activity are likely not identical to their microbicidal mechanisms of action despite the fact that the surface membranes of both sperm and microbes are initial targets of the peptides. Using GF-17D3, we showed herein that interruption of peptide helicity led to

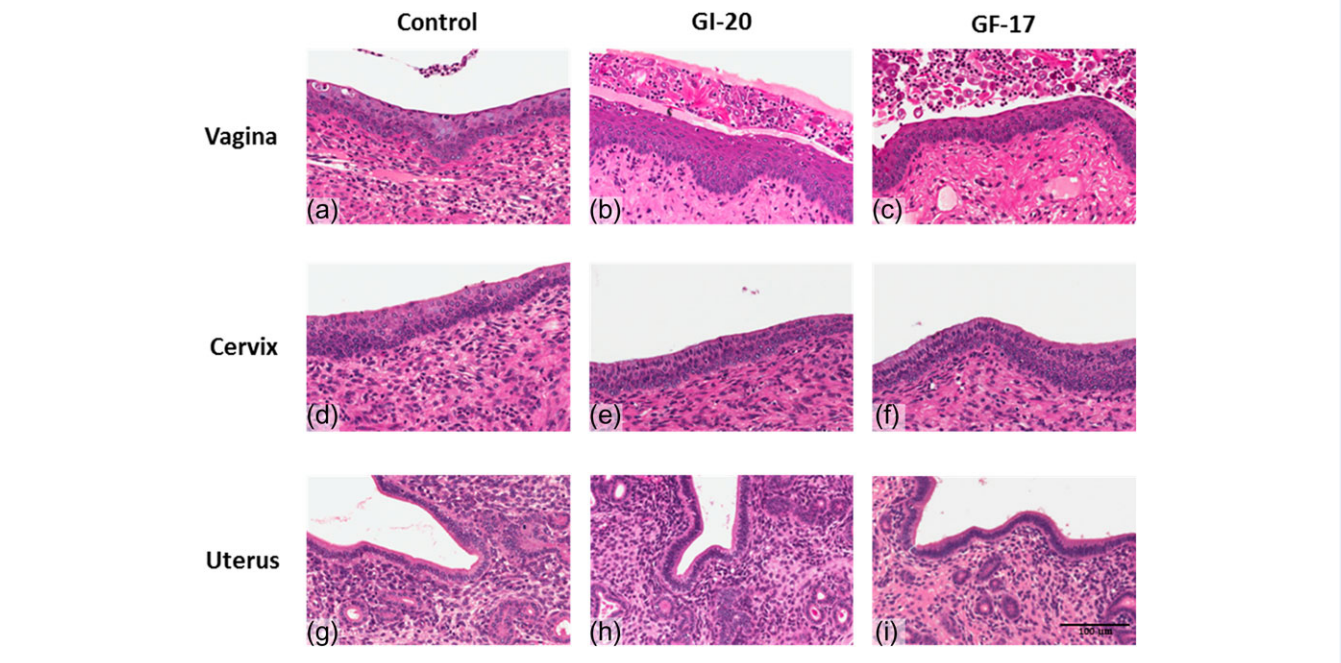

Figure 4 Lack of histological damages to the reproductive tract tissues of the female mice inseminated with GI-20 or GF-17. The vagina (top panels), cervix (middle panels) and uterus (bottom panels) were collected from the female mice 24 h post-insemination for paraffin block preparation. Sections were stained with hematoxylin/eosin. Control tissues were those from female mice transcervically injected with sperm alone. Bar = 100 µm. Shown here are representative results from one of the three females, transcervically injected with sperm alone (panels a, d and g), sperm + GI-20 (panels b, e and h) or sperm + GF-17 (panels c, f and i).

Table II High bactericidal efficacy of LL-37, GI-20 and GF-17 on *N. gonorrhoeae* but not so on *L. crispatus*.

Bacterial species	MIC (µM)			MBC (µM)		
	LL-37	GI-20	GF-17	LL-37	GI-20	GF-17
N. gonorrhoeae	1.8	7.2	1.8	1.8	7.2	1.8
L. crispatus	–	7.2	3.6	–	–	10.8

very minimal spermicidal activity. In contrast, GF-17D3 still possesses moderate microbicidal activity (Li *et al.*, 2006; Wang *et al.*, 2008; Epand *et al.*, 2009). A better understanding of the spermicidal action of LL-37 should be obtained from mechanistic studies, which would be much less costly if performed with truncated LL-37 peptides with known spermicidal activity, i.e. GI-20 and GF-17.

Another important attribute of LL-37, or its truncated forms, that should inspire its further development into a vaginal spermicide/contraceptive is its known properties as a microbicide especially against STI pathogens (including HIV). Herein, we focused our microbicidal work on *N. gonorrhoeae* as it is a common sexually transmitted pathogen that has shown continuing resistance to various types of antibiotics for over 80 years (Unemo and Shafer, 2014). We demonstrated that LL-37, GI-20 and GF-17 each had MBCs against the clinical isolate strain NRCC 6879 of *N. gonorrhoeae* that were lower than the spermicidal concentration of human sperm. We further demonstrated that LL-37 up to 22 µM did not have any adverse effects on the commensal bacterium, *L. crispatus*, a finding that is consistent with previous results describing no effects of LL-37 on other *Lactobacillus* species (Smeianov *et al.*, 2000). However, both GI-20 and GF-17 exhibited antimicrobial effects on *L. crispatus* at concentrations lower than those required for spermicidal action. GI-20 only exerted bacteriostatic effects, whereas GF-17 was bactericidal to *L. crispatus* (Table II). For GI-20, if its vaginal use is not very frequent, *L. crispatus* should be able to repopulate. On the other hand, the vaginal use of GF-17 may need to be followed with *Lactobacillus* probiotic supplementation (Reid *et al.*, 2013).

Regardless, our findings described herein should inspire future development of these two truncated LL-37 peptides into vaginal spermicides/microbicides as well as further investigation of their microbicidal properties against a spectrum of STI microbes. The search for less costly mimetics of GI-20/GF-17 with spermicidal and microbicidal activity but fewer side effects on commensal vaginal microbiota should also be considered.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

NT first described the concept that LL-37 and its truncated forms could act as spermicides. NT designed all experimental approaches, but with significant inputs from GW, WC, JBA, NKG and JWK. Detailed planning of experiments, thelaboratory work and the analyses of results were carried out mainly by WK, as well as RZ, SGL and ACF, also with help from DRH. All authors, including WL, gave comments and interpretations on the results along the way. NT and WK mainly prepared the manuscript, on which all authors gave comments and/or suggestions for improvement. In particular, GW, JBA, JWK, NKG and WC suggested meaningful editorial modifications. All authors approved the final version of the submitted manuscript.

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Conflict of interest

The authors have declared that there is no conflict of interest on this work.

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