Development of 17BIPHE2 into a vaginal multipurpose prevention technology (MPT) agent with spermicidal effects and microbicidal activity against *Neisseria gonorrhoeae*

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Abstract

We have shown that LL-37, its truncated (GI-20, GF-17) and engineered (17BIPHE2) forms possess spermicidal effects and microbicidal activity against *Neisseria gonorrhoeae*. However, 17BIPHE2 has the highest spermicidal activity on human sperm resuspended in cervicovaginal fluid-containing medium due to its resistance to proteases. 17BIPHE2 should therefore be developed into a vaginal MPT agent. However,<5% of 17BIPHE2/LL-37/GI-20/GF-17 remained in the mouse reproductive tract after its intravaginal administration. A proper delivery formulation is thus needed. We have chosen the universal placebo gel, hydroxyethylcellulose (HEC), as an excipient in 17BIPHE2 formulation. HEC has been used safely as a vaginal lubricant and the uterus of mice injected with 2% HEC was still normal. 17BIPHE2 (32.4 or 86.4 μM) was solubilized in 2% HEC in a bicarbonate-5% CO_2 buffered isotonic salt solution (HEC-BS). 17BIPHE2-HEC-BS possessed in vitro spermicidal activity on mouse and human sperm. The contraceptive effects of 17BIPHE2-HEC-BS were then evaluated in mice. Since semen ejaculated in the mouse vagina is immediately swept into the uterine lumen, 17BIPHE2-HEC-BS was transcervically administered. Immunoblotting of uterine fluid collected from mice sacrificed 3 hours after transcervical injection indicated that 17BIPHE2 was present in a substantial amount, but the amount was decreased ~50% 24 hours after the administration. Through reaction with orcinol, HEC was shown to be present in the uterine fluid with amounts temporally declined like 17BIPHE2. Pregnancy of mice administered with 17BIPHE2 (86.4 μM)-HEC-BS was then determined. Only three of seven mice (43%) cycling in estrus, which were transcervically injected with 17BIPHE2-HEC-BS and then co-caged with fertile males for 3 days, became pregnant In contrast, 100% pregnancy was observed in mice transcervically injected with HEC alone (n=5). While the results were promising, the experiments need to be done with more mice with modifications (e.g., with one-day co-caging with males and/or different percentages of HEC in formulation).

Background

The ongoing high rates of unplanned pregnancies and sexually transmitted infections (STIs) have launched the World Health Organization toward the development of multipurpose prevention technology (MPT), which involves administration (mainly intravaginal) of a single application/device or a single agent for both contraception and anti-STI purpose (Hemmerling et al., 20[illegible]; Dohadwala et al., 2023). Till now several agents with confirmed spermicidal property, including a surfactant spermicide, nonoxyn 9 (N-9), have been examined/trialed for their anti-STI or anti-HIV effects without successful outcomes (Dohadwala et al., Op Access J Contracept 2023, 14:83-94; Tanphaichitr et al., Pharmaceutics 2016, 9:13; Van Damme et al., Lancet 2002, 360:971-977).

We have therefore embraced a different approach by starting with known microbicides, in particular, those that exert an infective effects on vaginal microbes that cause STIs. This is followed by screening for their spermicidal activity. We have chosen t work with antimicrobial peptides (AMPs) as they are part of innate immunity and are produced by all domains of life. As natural peptides, AMPs will be less prone to induce reaction/toxicity to the body. After the trial runs, it appears that the human cathelicidin, LL-37, is a promising AMP with spermicidal activity.

We have shown that LL-37, and its truncated peptides, GI-20 and GF-17 (see sequences below), **at a physiological concentration exert spermicidal activity on both human and mouse sperm in vitro. The spermicidal concentration of LL-37/GI-20/GF-17 on human sperm is 10.8 μM and on mouse sperm is 3.6 μM.**

The alpha helicity of these cathelicidin peptides is important for the spermicidal activity.

LL-37: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES M.W. 4493

GI-20: GIKEFKRIVQRIKDFLRNLV 2473

GF-17: GFKRIVQRIKDFLRNLV 2102

I, I, L in GF-17 were changed into corresponding D-amino acids

GF-17d3: GFKRIVQRIKDFLRNLV 2102

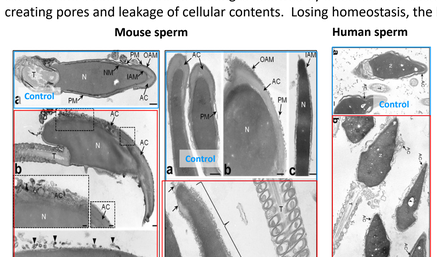
The two F's were replaced with biphenylalamines (B's) and all D-amino acids were Leu..

17BIPHE2: GBKRLVQRLKDBLRNLV 2254

(Wang et al., ACS Chem Biol 2014, 9:1997-2002)

The mechanism of the spermicidal action of LL-37/GI-20/GF-17 is likely to be analogous to that of their bactericidal action. The surface of the bacterial cell membrane contains negatively charged lipids, phosphatidylglycerols and cardiolipins, to which the cationic AMPs bind. This initial binding is followed by insertion of a cluster of AMPs into the bacterial membrane bilayers, thus creating pores and leakage of cellular contents. Losing homeostasis, the bacteria die.

Mouse sperm **Human sperm**



The sperm head surface is enriched in a negatively charged sulfogalactosylglycerolipid (SGG, aka seminolipid). We have shown that LL-37 binds to SGG and presumably this binding, analogous to the interaction between LL-37 and phosphatidylglycerol and cardiolipins, leads to the disruption of the sperm head surface membranes (sperm plasma membrane, outer acrosomal membrane and inner acrosomal membrane). **Sperm treated with LL-37/GI-20/GF-17 lose motility and viability** (Srakaew et al., Human Reprod 2014, 29: 683-696; Kiattiburut et al., Human Reprod 2018, 33:2175-2183).

LL-37/GI-20/GF-17 all exert in vivo contraceptive effects in mice. Female mice in estrus were transcervically injected with each peptide (3.6 μM) + sperm or with sperm alone (control). In vivo fertilization was assessed by the presence of two-cell embryos in the oviduct 42 h post-injection. Pregnancy rates were evaluated by pup delivery 21-25 day post-injection.

Inhibitory effects of 3.6 μM LL-37, GI-20, GF-17 on in vivo fertilization

Transcerv. injection with	% 2-4 cell-embryos
Sperm alone (4 females) (n= 48)	89 ± 11
Sperm + LL-37 (3 females) (n = 46)	0
Sperm alone (4 females) (n = 48)	83 ± 13
Sperm + GI-20 (6 females) (n = 61)	0
Sperm alone (6 females) (n = 75)	78 ± 17
Sperm + GF-17 (9 females)(n = 104)	0

n = oocytes + 2-4 cell embryos

Kiattiburut et al., Human Reprod 2018

Inhibitory effects of 3.6 μM LL-37 on pregnancy

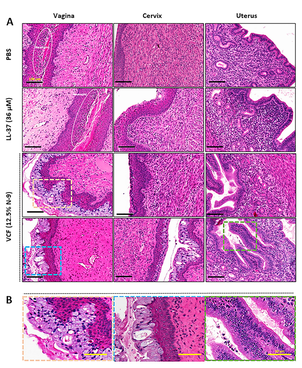
Treatment	No. of pregnant females (%)	Litter size
Control	24/26 (92)	9±5
3.6 μM LL-37	0/26 (0)	0

Srakaew et al., Human Reprod 2014

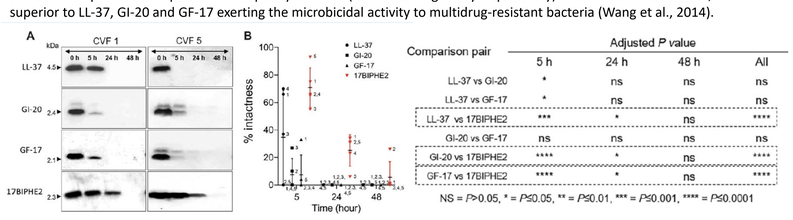
Multiple uterine exposure to LL-37 does not cause any histological damage to the vagina, cervix and uterus. Female mice was transcervically injected with LL-37 (36 μM) in three consecutive estrus phases. Negative control females were injected with PBS, whereas positive control females were injected with VCF (vaginal contraceptive foam containing 12.5% N-9) in parallel. The histology of the vagina, cervix and uterus of multiply-treated with LL-37 was the same as that of PBSs-treated mice. In contrast, VCF-treated mice showed obvious histological abnormality in the three tissues. Low magnification images are in A, whereas the higher magnification of selected areas in A is shown in B.

These females, which were previously transcervically injected with LL-37 in three consecutive estrus phases, could resume fecundity. After resting for one week, they were artificially inseminated with sperm from fertile males. All of these females (n = 7) became pregnant.

Lee et al., Mol Human Reprod 2023, 29(7), gaad023



While LL-37/GI-20/GF-17 are promising AMP spermicides, they are highly prone to degradation by body fluid proteases. **Therefore, 17BIPHE2 is engineered based on the GF-17 sequence to circumvent this problem. 17BIPHE2 is much resistant to proteases,** as it contains three D-amino acids (substituting the corresponding L-amino acids in GF-17). These D-amino acids, however, decrease the helicity of 17BIPHE2, thus causing hydrophobic defects. To correct these defects, the two phenylalanines in the GF-17 sequence are replaced with biphenylalanines (with much higher hydrophobicity) in 17BIPHE2. As a result, 17BIPHE2 is superior to LL-37, GI-20 and GF-17 exerting the microbicidal activity to multidrug-resistant bacteria (Wang et al., 2014).



At the same mass concentration, 17BIPHE2 is a more effective spermicide than LL-37 and GF-17 on human sperm resuspended in cervical fluid (CVF)-containing medium , with the spermicidal concentration of 32.4 μM.

All LL-37, GI-20, GF-17 and 17BIPHE2 exert microbicidal effects on *Neisseria gonorrhoeae* with a minimum inhibitory concentration (MIC) of 1.7, 7.2, 1.7 and 1.8 μM, respectively (Kiattiburut et al., 2018; Lee et al., 2022). *N gonorrhoeae* is the cause of one of the most common sexually transmitted diseases globally and it is becoming resistant to conventional antibiotic treatments. **Therefore, the microbicidal activity against *N gonorrhoeae* of these cathelicidin peptides together with their spermicidal activity make them attractive to be developed into multipurpose prevention technology (MPT) agents. However, due to its low susceptibility to protease degradation, 17BIPHE2 most deserves to be chosen for this MPT development.**

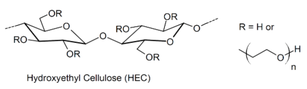
17BIPHE2 formulation for its delivery in the female reproductive tract

Despite its resistance to body fluid proteases, <4% of 17BIPHE2 (20 μl of 72 μM) intravaginally injected into females in estrus remains in the vaginal lumen 1 h afterwards. This indicates fast migration of the peptide into the blood stream or interstitial tissues/spaces. Dosage formulation of 17BIPHE2 for the delivery into the female reproductive tract, is therefore needed.

A number of dosage forms for vaginal delivery have been described including gel/cream/ointment, universal and thermosensitive hydrogel, film strips, vaginal rings, and micro/nanoparticles (dos Santos et al., 2020; Sanchez-Lopez et al., 2021). Among these systems, nanoparticles have an advantage on specific cell targeting. The surface of nanoparticles (such as the polymer/lipid hybrid type) can be functionalized with an antibody that will recognize target cells. In our case, we want 17BIPHE2 nanoparticles to target sperm. We can therefore functionalize the nanoparticle surface with an antibody such as an anti-sulfogalactosylglycerolipid (SGG –seminolipid, present selectively on sperm) antibody, which will enhance the targeting of the nanoparticles to sperm. Therefore, we started our 17BIPHE2 delivery system study by attempting to make polymer/lipid hybrid nanoparticles with biodegradable poly(lactic-co-glycolic acid (PLGA)) as the solid core and a lipid (lecithin/cholesterol) layer as a shell. 17BIPHE2 is supposed to be enveloped inside these nanoparticles during their preparation through chemical steps. However, after 8 months of experimentation by trial and error, less than 50% of the peptide could be incorporated into the nanoparticles. We, therefore, decided to try another delivery approach. Since we are using mice as an animal experimental model in our study, it will be difficult to try the film strip or vaginal ring as the delivery system due to the very small size of the mouse vagina.

We therefore have chosen a hydrogel (water swelling networks of polymers, proteins, small molecules or colloids), which is the simplest dosage form, for our trial study on the 17BIPHE2 vaginal delivery system.

Among several hydrogels already used in the vaginal drug delivery, we have selected **hydroxylethylcellulose (HEC)**, also known as universal gel (https://polymerdatabase.com/Polymer%20Brands/HEC.html) for the following reasons:



1. HEC has been used in several formulations (Li et al., 2018; Richardson et al. 2013, Dimatteo et al., 2018) as well as the integral component of vaginal lubricants (KY Jelly and Astroglide- available over the counter).
2. A protocol has been described for making HEC at a select concentration and pH for administration into the vagina of various animals (mice, rabbits, macaques) (Tien et al., 2005).
3. HEC (2.7%) containing a protease inhibitor (AEBSF) has been transcervically injected into mice with demonstrated contraceptive effects (Barton et al., 2020). However, the pH of this AEBSF containing HEC gel is 4, which is improper as the pH of the mouse uterine lumen is neutral.

Our formulation of 17BIPHE2-HEC

We have chosen 2% HEC as the excipient instead of 2.7% previously described (Tien et al., 2005; Barton et al., 2020). This is because the viscosity of 2.7% HEC still makes it difficult for its delivery into the uterine lumen by our transcervical injection.

We make 2% HEC in KRB or KRB-HEPES but without perishable components such as lactate, pyruvate and glucose (nutrients) and CaCl2. KRB and KRB-HEPES have been used as medium for gamete culture under 5% CO2 and air, respectively. We omit the nutrient components because of their shelf life. 17BIPHE2-HEC is used mainly in in vivo experiments (e.g., transcervical injection into the uterine lumen). There should already exist enough nutrients in the uterine fluid to support survival of sperm, which enter the uterine cavity after mating. $CaCl_2$ is omitted because it tends to speed up the gelatinization of HEC. Components of KRB and KRB-HEPES for solubilizing 2% HEC are in the table.

We include 86.4 μM 17BIPHE2 in 2% HEC and this 17BIPHE2-HEC is used for transcervical injection into female mice naturally cycling in estrus. The injected females are then co-caged with fertile males to allow mating. The 17BIPHE2 concentration of 86.4 μM was from our empirical experiments. There was not much 17BIPHE2 remaining in the uterine lumen 3 h after transcervical injection if the peptide concentrations were lower.

Preparation of 2% HEC gel (pH: 7.4 and osmolality: 285 mOsmc physiological range)

Conditions	Under air		Under 5% CO_2	
Compounds	KRB-HEPES (mM)	Salt-HEPES buffer for HEC solution (mM)	KRB (mM)	Salt-bicarbonate buffer for HEC solution (mM)
Glucose	5.6	—	5.6	—
Na pyruvate	1.0	—	1.0	—
NaHCO3	4.0	4.0	25.0	25.0
HEPES	21.0	21.0	—	—
Na lactate	25.0	—	25.0	—
Penicillin-Streptomycin*	0.1% (strep)/ 100 IU (in 100 ml)	0.1% (strep)/ 100 IU (in 100 ml)	0.1% (strep)/ 100 IU (in 100 ml)	0.1% (strep)/ 100 IU (in 100 ml)
NaCl	99.6	132.8*	99.6	132.8*
KCl	4.8	4.8	4.8	4.8
KH_2PO_4	1.2	1.2	1.2	1.2
MgSO4·7H2O	1.2	1.2	1.2	1.2
$CaCl_2·2H_2O$	1.7	—	1.7	—
Phenol Red***	0.0011%	optional	0.0011%	optional

Protocols for making 17BIPHE2 (86.4 μM)-2% HEC: 2% HEC in KRB components (pre-equilibrated under 5% CO2 to attain pH 7.4) is first made. Immediately, 1/10 volume of 864 μM of 17BIPHE2 is added into 2% HEC solution and the solution is mixed thoroughly. The 2% HEC solution rapidly becomes gelatinous. Therefore, the immediate mixing of the peptide into the 2% HEC solution is essential. 17BIPHE2-HEC is instantaneously loaded into the syringe for transcervical injection.

OBJECTIVE

To determine whether 17BIPHE2 formulated into hydroxylethylcellulose can exert contraceptive in vivo in mice.

RESULTS

Figure1. 17BIPHE2 in HEC COULD EXERT SPERMICIDAL ACTIVITY

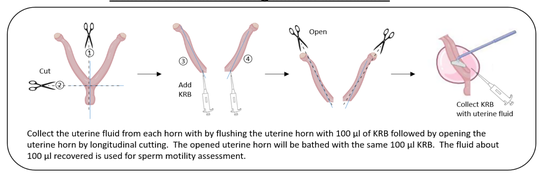
In vitro approach: Motile mouse sperm selected by Percoll gradient centrifugation (PGC) (100 μl in KRB-BSA) with the density of 10 million/ml were layered on top of 100 μl of HEC or 17BIPHE2-HEC solution (the concentration of 17BIPHE2 used to make 17BIPHE2-HEC = 10.8, 32.4 and 86.4 μM). After 15 min of incubation, sperm motility was assessed by videomicroscopy.

Results: HEC alone slowed down sperm movement but most of them were still motile. 17BIHE2 at 10.8 μM in 17BIPHE2-HEC did not inhibit sperm motility, but at 32.4 μM motility was about 50%, and at 86.4 μM, about 10% of sperm remained motile and tended to aggregate.

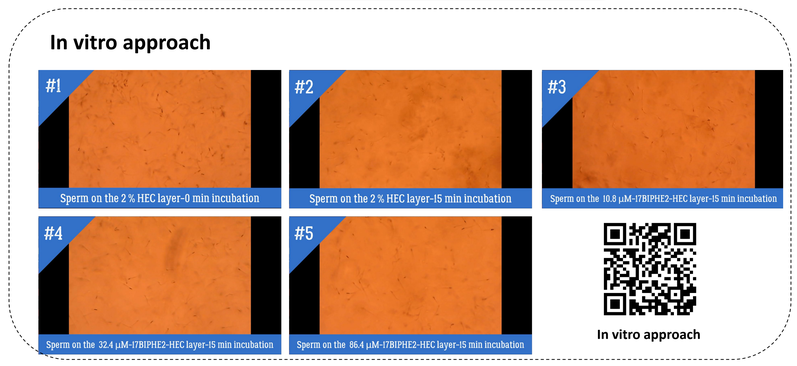
In vivo approach: Female mice were superovulated by PMSG/hCG IP injections. After hCG injection (12 pm), females (in the estrus) were transcervically injected with HEC or 17BIPHE2 (10.8, 32.4 or 86.4 μM)-HEC (pm) and were then individually mated with fertile males (1 pm). Mating occurred during the estrus night. On the following morning, females with a vaginal plug were sacrificed and uterine fluid containing semen was collected (see below). It was diluted 4X with KRB and assessed for sperm motility.

Results: About 50% of sperm remained motile in females injected with HEC alone (note that semen containing uterine fluid was collected a number of hours after mating). Motility of sperm collected from females injected with 10.8 μM 17BIPHE2-HEC was the same as that of sperm from HEC alone-injected females. Motility of sperm from females injected with 32.4 μM 17BIPHE2-HEC and 86.4 17BIPHE2-HEC was ~20% and 0%, respectively.

Uterine fluid containing semen collection



Collect the uterine fluid from each horn with by flushing the uterine horn with 100 μl of KRB followed by opening the uterine horn by longitudinal cutting. The opened uterine horn will be bathed with the same 100 μl KRB. The fluid about 100 μl recovered is used for sperm motility assessment.



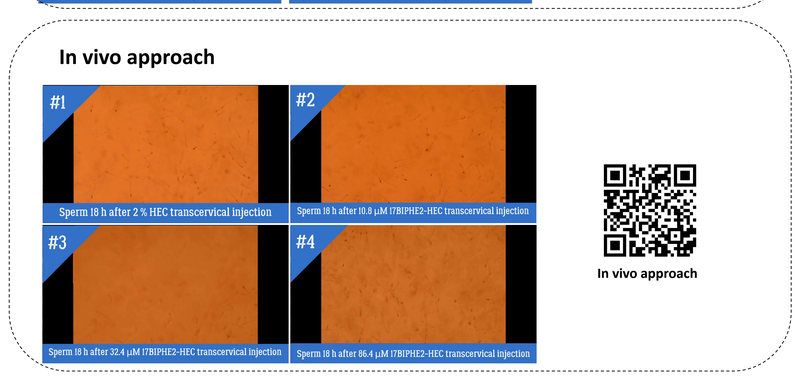


Figure 2A. The vagina/cervix and uterus of females transcervically injected with HEC (2%) remained histologically normal.

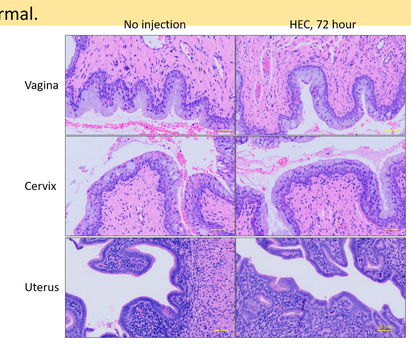
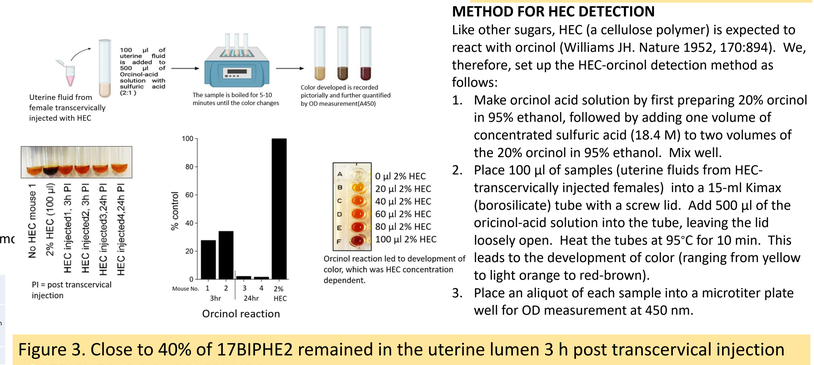


Figure 2B. Close to 40% of HEC remained in the uterine lumen 3hr after transcervical injection of 2% HEC



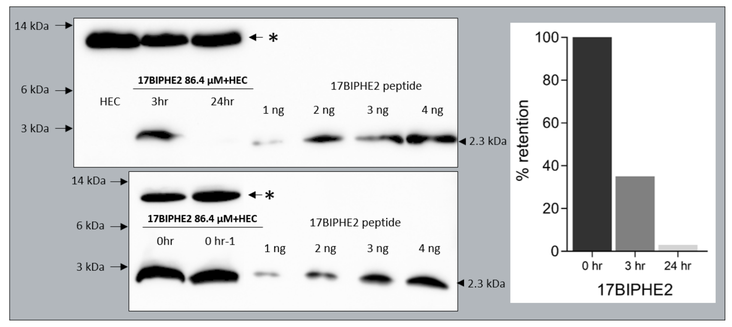
METHOD FOR HEC DETECTION

Like other sugars, HEC (a cellulose polymer) is expected to react with orcinol (Williams JH. Nature 1952, 170:894). We, therefore, set up the HEC-orcinol detection method as follows:

1. Make orcinol acid solution by first preparing 20% orcinol in 95% ethanol, followed by adding one volume of concentrated sulfuric acid (18.4 M) to two volumes of the 20% orcinol in 95% ethanol. Mix well.
2. Place 100 μl of samples (uterine fluids from HEC-transcervically injected females) into a 15-ml Kimax (borosilicate) tube with a screw lid. Add 500 μl of the oricinol-acid solution into the tube, leaving the lid loosely open. Heat the tubes at 95°C for 10 min. This leads to the development of color (ranging from yellow to light orange to red-brown).
3. Place an aliquot of each sample into a microtiter plate well for OD measurement at 450 nm.

Figure 3. Close to 40% of 17BIPHE2 remained in the uterine lumen 3 h post transcervical injection of 17BIPHE2 (86.4 μM)-2% HEC

The experimental scheme for 17BIPHE2-HEC transcervical injection was similar to that of HEC transcervical injection (see above) except that the time point 0 h was also included for this 17BIPHE2-HEC injection. The relative amounts of 17BIPHE2 present in the uterine lumen were determined by immunoblotting of the collected uterine fluid samples. The amount of 17BIPHE2 at time 0 of the transcervical injection was designated as 100% and used for calculation of percentages of 17BIPHE2 remaining in the uterine lumen 3 h and 24 h after the transcervical injection.



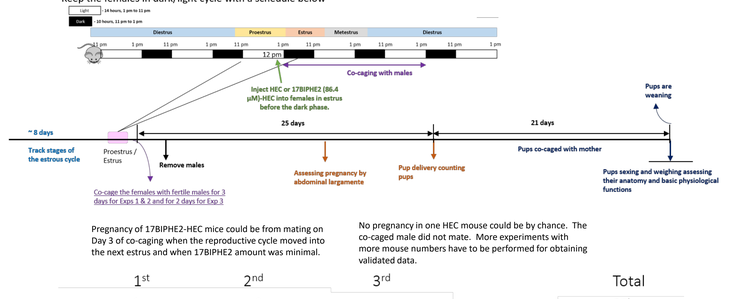
The relative amount of 17BIPHE2 remaining in the uterine lumen 3 h after transcervical injection of 17BIPHE2-HEC was similar to that of HEC (see above). Also, 24 h after the transcervical injection of 17BIPHE2-HEC, only a minimal amount of 17BIPHE2 remained in the lumen.

Figure 4. 17BIPHE2 (86.4 μM)-2% HEC exerted contraceptive effects when transcervically administered into female mice in estrus.

Overall scheme

1. Check estrous cycle for 6-8 days
2. Inject HEC or 17BIPHE2 (86.4 μM)-HEC into females in estrus before the dark phase.
3. Co-cage the females with fertile males for 3 days for Exps 1 & 2 and for 2 days for Exp 3
4. Assess pregnancy and determine litter size during the 21 ,25 day period after mating.
5. Weigh, examine for anatomical normalcy and identify sex of 18-21 day old pups.

Keep the females in dark/light cycle with a schedule below



Pregnancy of 17BIPHE2-HEC mice could be from mating on Day 3 of co-caging when the reproductive cycle moved into the next estrus and when 17BIPHE2 amount was minimal.

No pregnancy in one HEC mouse could be by chance. The co-caged male did not mate. More experiments with more mouse numbers have to be performed for obtaining validated data.

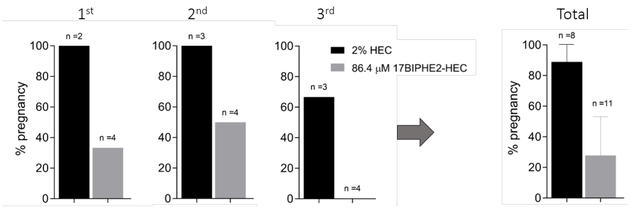


Figure 5. 17BIPHE2 (86.4 μM)-2% HEC exerted contraceptive effects when transcervically administered into female mice in estrus.

Two of the three pregnant 17BIPHE2-HEC mice had a normal litter size, but the litter size of the other one was only 6, suggesting the contraceptive effect of 17BIPHE2-HEC still manifested. Gender distribution of the delivered pups was normal ~50% female/50% male. Seven out of eight 17BIPHE2-HEC mice that did not get pregnant could become pregnant in the second co-caging a few weeks afterwards, indicating that the contraceptive effect of 17BIPHE2-HEC was reversible. The effect of 17BIPHE2 (86.4 μM)-HEC (2%) on the histology of the female reproductive tract is being investigated.

SUMMARY AND DISCUSSION

1. Sperm placed next to HEC for 15 min in vitro had reduced motility rates. However, >90% of sperm placed next to 86.4 μM- 17BIPHE2-HEC for 15 min lost motility, indicating that 17BIPHE2 even in HEC could still inhibit sperm motility. In the in vivo experiment, sperm entered into the uterine cavity of female mice transcervically injected with 86.4 μM-17BIPHE2-HEC completely lost motility indicating that 17BIPHE2 present together with HEC in the uterine fluid exerted the spermicidal action on sperm.
2. Approximately 30-40% of 2% HEC transcervically injected into females in estrus remained in the uterine lumen 3 h after the injection. However, 24 h after the injection, the HEC level in the uterine lumen became minimal. HEC did not have any toxicity to the uterus/cervix/vagina. These tissues remained normal in females 72 h post-transcervical injection of HEC.
3. Similar levels of 17BIPHE2 (~ 40%) remained in the uterine lumen 3 h after transcervical injection of 17BIPHE2 (86.4 μM)-2% HEC into females in estrus. Likewise, 24 h after the injection, the 17BIPHE2 level in the uterine lumen became minimal. This result together with that in #2 indicated that the contraceptive effect of the injected 17BIPHE2-HEC was best within a few hours after its transcervical injection.
4. From the three in vivo experiments performed, it appeared that the transcervically injected 17BIPHE2-HEC could exert contraceptive effects but not at 100%. The timing of co-caging needs to be adjusted with the hope to reach 100% contraceptive efficacy. The numbers of mice to be injected with HEC and 17BIPHE2-HEC also need to be increased. Various concentrations of 17BIPHE2 included in 2% HEC should also be tried to determine the optimum contraceptive effect.
5. Since the concentration of 17BIPHE2 included in 2% HEC is rather high (86.4 μM), it is important to determine whether 17BIPHE2-HEC has any adverse effects to the female reproductive tract. This experiment is ongoing.

Reference

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