**CONTRACEPTION**

Topical reinforcement of the cervical mucus barrier to sperm

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Close to half of the world’s pregnancies are still unplanned, reflecting a clear unmet need in contraception. Ideally, a contraceptive would provide the high efficacy of hormonal treatments, without systemic side effects. Here, we studied topical reinforcement of the cervical mucus by chitosan mucoadhesive polymers as a form of female contraceptive. Chitosans larger than 7 kDa effectively cross-linked human ovulatory cervical mucus to prevent sperm penetration in vitro. We then demonstrated in vivo using the ewe as a model that vaginal gels containing chitosan could stop ram sperm at the entrance of the cervical canal and prevent them from reaching the uterus, whereas the same gels without chitosan did not substantially limit sperm migration. Chitosan did not affect sperm motility in vitro or in vivo, suggesting reinforcement of the mucus physical barrier as the primary mechanism of action. The chitosan formulations did not damage or irritate the ewe vaginal epithelium, in contrast to nonoxynol-9 spermicide. The demonstration that cervical mucus can be reinforced topically to create an effective barrier to sperm may therefore form the technological basis for mucocervical barrier contraceptives with the potential to become an alternative to hormonal contraceptives.

INTRODUCTION

Family planning promotes education, reduces gender inequalities, improves child and maternal health, and, by doing so, decreases poverty and hunger (1). In some regions such as the United States and European Union, access to effective female contraceptives beginning in the 1960s with the advent of hormonal birth control has facilitated family planning and contributed to increased equality between men and women (2, 3). However, 40% of pregnancies globally are still unintended (4), mainly due to the lack of contraceptive use. In the United States, 47% of women have discontinued a contraceptive due to side effects (5), highlighting the need for new reliable and more desirable contraceptive options (6). Ongoing efforts to create long-acting contraceptives and male contraceptives will address part of the need for contraception. Another part can be addressed by creating contraceptives with user preference in mind to ensure maximum compliance. In particular, several studies have shown that there is a large interest for “on-demand” contraceptives that prevent pregnancy at or around the time of intercourse. For instance, Foster et al. showed that 70% of women in abortion clinics and more than 50% of women in family planning clinics were interested in a hypothetical pericoital contraception pill (7, 8).

Although hormonal contraception offers high efficacy rates, a substantial proportion of users express a nonhormonal preference for their contraceptive (9) or discontinue their use, driven in large part by their perceived risks of side effects (10). Existing nonhormonal alternatives are hampered by lack of efficacy or side effects and result in high discontinuation rates. In clinical trials, the 1-year discontinuation rate was at 49% for diaphragm with spermicides (11) and above 50% for nonoxynol-9 (N9) spermicides (12, 13). Even long-acting copper intrauterine devices (IUDs) suffer a 20% discontinuation rate, in part due to dislocation and pain (14, 15). Condoms and spermicides are not only less invasive than copper IUDs but also much less effective in typical use than hormonal methods (13, 16). The withdrawal of the U.S. pharmaceutical industry from contraceptive research and development since the 1980s has led to only minor improvements of existing methods and has maintained our dependence on 19th and 20th century approaches to birth control (17). There is thus a clear need for female contraceptives with alternative mechanisms of action.

Cervical mucus (CM) is a gel secreted in the cervical canal isolating the bacteria-rich vagina from the uterus and the upper reproductive tract. It is mainly composed of high–molecular weight mucin glycoproteins, which adopt an extended linear conformation and interact to form a hydrated network. The mucins interact with other globular proteins, lipids, and salts that define the physical, mechanical, and biological properties of CM. In the time leading up to ovulation, high serum estradiol concentrations drive changes in rheological properties, microstructure, and chemical composition of the CM that allow the CM to transition to a selective barrier, allowing the passage of a few thousand sperm from tens or hundreds of millions of a typical ejaculate (18, 19). The cervix and

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its mucus thus act as regulators of sperm transport in the female reproductive tract, and reinforcement of the CM to block sperm passage has been proposed as a possible contraceptive strategy (20). For instance, progesteronly pills and levonorgestrel intrauterine systems act, at least in part, by thickening the CM (20). However, the successful reinforcement of CM through a nonhormonal method, topical or systemic, has remained elusive (21–24).

Mucus engineering approaches to alter the mucus gel properties via topical treatments are emerging as promising approaches to address mucus-related conditions such as mucosal dryness (25), infections, inflammation (26), and microbiome dysbiosis (27). However, they lack thorough in vivo validation and have not been successfully applied to contraception. In this work, we developed a nonhormonal approach to reinforce the CM barrier to sperm by physically cross-linking the mucin network of the mucus gel. We used chitosan, a biopolymer of β-linked N-glucosamine and N-acetyl-d-glucosamine, obtained by the deacetylation of chitin extracted from fungal cell wall or crustacean shell. Chitosan has been extensively studied in biomedical applications, in particular because of its mucoadhesive properties (28). We have previously shown that chitosan polysaccharides could cross-link porcine gastric mucin gels, thereby reinforcing its barrier properties (29). We here demonstrated that chitosan could cross-link ovulatory CM and, by doing so, could impair sperm penetration both in vitro using human ovulatory CM and in vivo in the ewe without evidence of cytotoxicity toward vaginal epithelial tissues and sperm. This work suggests the feasibility of a novel mucus-centric mechanism of action for a female contraceptive.

RESULTS
Chitosan diffuses into human CM
We first investigated the ability of chitosans dissolved in low concentration of lactic acid (32.5 mM, pH 5.5) to penetrate human ovulatory CM. The low pH of the lactic acid solution used to dissolve chitosan is necessary to ensure that the chitosan is soluble when exposed to the vagina and then to the cervical canal (fig. S1); chitosan typically precipitates above pH 6 (30). In addition to lactic acid, we also tested the addition of a thickening agent to the chitosan formulation. Hydroxyethyl cellulose (HEC) is commonly used in placebo vaginal formulations applied in clinical trials of vaginal products (31). Thickening agents such as HEC are used in vaginal formulations to increase the residency time of the chitosans in the vagina to optimize the gel’s spreading and coverage of the cervix, while preventing leakage of the formulation. Transmittance measurements of the HEC formulation were not affected by the addition of chitosan, suggesting that HEC and chitosan do not strongly interact and that HEC is an appropriate excipient (fig. S1).

We let chitosan passively diffuse into CM rather than actively mixing the two components to mimic a topical treatment of the mucus. This was conducted by exposing one side of the CM-filled capillary to the chitosan solution and letting the chitosan diffuse into the CM (Fig. 1A). All chitosans not only accumulated at the interface of the CM gel but also diffused within the gel, forming a concentration gradient (Fig. 1, B to E). After 30 min of diffusion, we analyzed the concentration gradient that formed through the capillary (fig. S2). The maximum penetration distance of the chitosans was dependent on the molar mass of the chitosan (Fig. 1, F to I), with detection at 3.5 mm into the mucus for 1.4-kDa chitosan versus only 1.5 mm for the 150-kDa chitosan tested. The diffusion coefficients calculated from the penetration distances showed an 11-fold difference between the more diffusive 1.4-kDa chitosan oligomer and the less diffusive 150-kDa chitosan (Fig. 1J). Faster diffusion of the smaller chitosans also led to more chitosan accumulation into the ovulatory CM, with the calculated area under the curve being 15 times larger for the 1.4-kDa chitosan than for the 150-kDa chitosan (Fig. 1K). We also found that the presence of HEC did not affect maximum diffusion distance, chitosan diffusion coefficient, or accumulation (Fig. 1, I to K).

Chitosan reinforces the barrier properties of human ovulatory CM
We then asked whether exposing chitosans to human ovulatory CM could affect the ability of sperm to penetrate and swim through the mucus. We used a sperm penetration assay, in which we first exposed a chitosan solution to the opening of a square capillary filled with ovulatory CM (Fig. 2A). We then replaced the chitosan with a solution of fresh undiluted semen and assessed sperm penetration. We selected the fertile phase of CM with a “good” or “excellent” Insler score (32) and selected high-quality semen used within minutes of collections (tables S1 and S2).

Pretreatment of the ovulatory CM with 1.4-kDa chitosan allowed sperm to penetrate the whole length of the capillary (Fig. 2B). However, treatments with chitosans larger than 7.1 kDa limited the maximum penetration distance, defined as the distance at which there is less than 10 cumulative sperm present in the three fields of view analyzed (Fig. 2B). Substantial decreases in sperm numbers compared to treatment with lactic acid solution only were observed with chitosans larger than 18.9 kDa. Exposure of CM to lactic acid also lowered sperm numbers, possibly due to residual lactic acid in the mucus or to structural changes caused by transient acidification (Fig. 2, C to K). The addition of HEC to the formulation did not alter the effectiveness of the 35-kDa chitosan (Fig. 2, J and K). Close to the CM-semen interface (0.1 mm into the gel phase), treating the CM with lactic acid solution without chitosan allowed more sperm into the mucus gel, likely owing to a slight dilution of the mucus (Fig. 2L). All chitosan treatments except 1.4-kDa chitosan markedly reduced the sperm count compared to the control CM (Fig. 2L).

Mixing short oligo-chitosans with larger chitosans inhibited the barrier reinforcing effect (fig. S3), likely because short chitosans diffused in the CM faster than did larger chitosans, blocking binding sites for the larger chitosans. The 36.2- and 35-kDa chitosans struck a balance between reproducible barrier reinforcement and diffusion into CM. We thus selected a 36.2-kDa fungal-derived chitosan for further studies because the animal-free formulation could facilitate further technological development. This chitosan was also able to rapidly reinforce the human CM barrier to sperm, with a reduction in sperm penetration after 1 min of exposure and full sperm blockage after 5 min (fig. S4), enabling the possibility of rapid onset of action.

Chitosan treatment reinforces the CM barrier in the ewe model
Although the sperm penetration assays validated the feasibility of forming a barrier resistant to sperm penetration on human ovulatory CM, they did not take into account the challenge of delivering the chitosan, the contractions, the movements, the resulting shear...
Fig. 1. Diffusion of oligo- and polymeric chitosans into human ovulatory CM. (A) Square capillaries filled with human CM are exposed to solutions of fluorescently labeled chitosan (CS) with or without hydroxyethyl cellulose (HEC) to evaluate the ability of chitosan to interact and diffuse into the mucus. (B to D) Overview of the chitosan distribution over the length of the capillary, highlighting chitosan accumulation at the CM gel interface. (E) Representative images of ovulatory CM-filled capillaries after exposure to chitosan solutions for 30 min at 20 ms. (F to H) Chitosan distribution profiles obtained from overexposed images (800 ms) revealing the diffusion fronts of the chitosan into the mucus. Each curve is the mean of three replicates, and the colored area represents the SD. The measurements were conducted with CM collected from one or more donors. (I) The maximum diffusion distance and (J) the diffusion coefficient for chitosan in CM were calculated as described in Supplementary Materials and Methods. (K) Relative total amounts of chitosan accumulated in the CM as measured by the area under the chitosan distribution profile curve. Graphs (I) to (K) show individual values from independent experiments, their mean values, and the SD. Normality was tested using the Shapiro-Wilk test. Statistical differences between groups were tested via Tukey’s test or Dunn’s test for the area under the curve and maximum diffusion distance or the diffusion coefficient. Asterisks denote statistically significant increases or decreases (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001).
on the formulation and mucus during intercourse, and the dynamic mucus turnover that this approach would face in humans. Thus, we tested whether a chitosan formulation could reinforce the barrier to sperm in vivo, using the ewe as a model (Fig. 3, A and B).

We first tested whether chitosan could reinforce the barrier properties of ovulatory CM collected from ovulating ewes in a sperm penetration assay using ram sperm. Two chitosans that we found to be effective on human ovulatory CM were also effective on the ewe ovulatory CM, albeit with higher sperm counts at the entrance of the capillary (Fig. S5 and Table S3). We attributed this difference to the low viscosity of the ewe ovulatory CM because it was mixed with vagina secretions during collection. We tracked the distribution of the formulation in the ewe’s reproductive tract using chitosan labeled with the Atto-665 fluorescent dye. We detected

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**Fig. 2. Penetration of sperm through human ovulatory CM.** (A) The sperm penetration assay consists of counting sperm through the length of a 5-cm capillary filled with treated or untreated CM after 30 min of exposure to semen. (B) The maximum penetration distance is shown, defined as the distance at which less than 10 cumulative sperm were detected in the three fields of view analyzed. The graph represents individual values, their mean values, and the SD. (C to K) Sperm counts through capillaries treated with chitosan solutions of varying molar masses with and without hydroxyethylcellulose (HEC), showing mean values and SDs. Each point is the mean of three replicates, and the colored area represents the SD. (L) Sperm counts normalized to CM without treatment (w/o), for lactic acid–treated CM (LAC) and for treatment with various chitosans of different molar masses, 0.1 cm inside the CM, close to the semen/CM interface. Each measurement was conducted in triplicate, using one or more CM samples and semen from at least two donors. Replicates are the mean of the sperm count at three different fields at each distance. The graph represents the nine resulting values, their mean values, and the SD. Statistical differences between groups were tested using the D’Agostino and Pearson test followed by Tukey’s test. Asterisks denote significant increases or decreases (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001).
high fluorescence emission in the vagina at the moment of artificial insemination (AI) using probe confocal laser endoscopy (pCLE), 1 hour after gel administration (Fig. 3C). Then, 4 hours after AI (5 hours after gel administration), fluorescence measurement of explanted tract showed that the chitosan spread throughout the vagina and penetrated up to 2 cm into the cervix, corresponding to where the first cervical ring is (33) (Fig. 3, D and E, and fig. S6).

The formation of a barrier to sperm was then assessed by localizing with pCLE the sperm throughout the reproductive tract. As expected, we found large numbers of highly motile sperm in the vagina immediately after AI (Fig. 3F). In untreated ewes \( (n=13) \) or ewes treated with HEC gel only \( (n=7) \), sperm effectively migrated up the reproductive tract. We found high numbers in the vagina and at the entrance of the cervix and lower numbers in the distal part of the cervix and in the uterus. In sharp contrast, we measured...
a marked reduction in the number of sperms in the distal cervix and in the uterus in chitosan-treated ewes \((n = 8, \text{Fig. } 3G)\). Of the eight animals in the chitosan-treated group, only one ewe had two sperm detected in the uterus (see raw data in table S4), corresponding to a 98% decrease in average uterine sperm numbers compared to the untreated control animals. This effect was partially attributed to the HEC and lactic acid excipients, which can account for 44% decrease in sperm number.

These results suggested that an effective barrier to sperm was formed in the cervical canal between the cervical os and the uterus, preventing sperm migration within this 4-hour time frame. Four hours after AI, the sperm in the vagina of untreated, HEC-only, or HEC with chitosan-treated ewes still showed a high rate of motility compared to immediately after AI (Fig. 4, A and B). This is in part because the ewe vagina has a pH that is close to neutral \((34)\) (fig. S7), which is in contrast to the human vagina, in which the acidic pH \((35)\) and immune response immobilize sperm within hours \((36)\). The HEC gel alone was not sufficient to form an effective barrier in the ewe, although a small reduction in sperm numbers was seen, probably due to the physical barrier that the HEC gel creates. The lactic acid content of the HEC gels did not have the effect seen in the in vitro sperm penetration assays (Fig. 2, C to K), likely because the ratio of lactic acid to mucus in vivo was much smaller than in vitro in which 100 μl was exposed to 4 μl of mucus.

**Chitosan barrier reinforcement is not mediated by spermicidal action**

In addition to the direct effects of chitosan on CM, the chitosan formulation could interfere with sperm migration by affecting viability and motility. We have previously shown that oligo-chitosans are relatively inert toward human sperm \((37)\); however, these differ in

![Image](https://via.placeholder.com/150)

**Fig. 4. Effect of chitosan on ram and human sperm motility and velocity.** (A) Proportion of motile sperm in the vaginal cavity of the ewe with no treatment \((w/o, n = 10)\), with HEC only \((n = 7)\), and with chitosan in a HEC gel \((CS 36.2 \text{kDa} + \text{HEC}, n = 8)\) immediately after AI \((1 \text{ hour after administration of formulation})\) and (B) throughout the ewe’s reproductive tract 4 hours after AI \((5 \text{ hours after administration of formulation})\). The graphs represent individual values of independent biological samples, their mean values, and the SD. Statistical differences between groups were tested using the Kolmogorov-Smirnov test followed by Dunn’s test. (C) Progressive motility and (D) velocity of human sperm exposed to chitosan-containing gels \((CS 36.2 \text{kDa} + \text{HEC}, n = 5)\) measured in vitro by CASA and normalized to motility and velocity of human sperm exposed to the gel without the chitosan \((\text{HEC}, n = 5)\). A semen sample containing sperm that exhibits high progressive motility \((\text{mean } 88\%)\) was used. The graphs represent individual values of technical replicates, their mean values, and the SD. Statistical differences between groups are tested via the D’Agostino and Pearson test followed by the Sidak’s test. Asterisks denote significant increases or decreases \(*P < 0.05, **P < 0.01, ***P < 0.001)\).
molar mass to the chitosan used here. In the ewe, the proportion of motile sperm in the vagina and ecto-cervix was near 100% immediately after AI, even in the presence of HEC and HEC/chitosan formulations (Fig. 4A). In the vagina and at the cervical entrance, the proportion of motile sperm remained unaffected compared to control, even after thorough spreading and mixing of the formulation had occurred 4 hours after AI due to the ewe’s movements (Fig. 4B). Deeper in the reproductive tract, at the internal cervix and uterus, the number of sperms was too low to assess motility. However, in the external os, sperm were present but with decreased motility likely due to the presence of crossed-linked mucus, which immobilized a fraction of the sperm. The same phenomenon was observed in the in vitro sperm penetration assay in which spermatozoa were immobilized by the chitosan–complexed mucus but still actively beating their flagella (movies S1 and S2).

For a more quantitative assessment of the direct effect of chitosan on sperm motility, and on their ability to penetrate CM, we exposed human sperm to HEC (pH 5.5) with or without chitosans and measured the impact on sperm kinematic parameters by computer-aided sperm analysis (CASA). The chitosan formulation did not affect the kinematic parameters motility and velocity of human sperm compared to HEC gels only (Fig. 4, C and D). Although the kinematic values of sperm decreased over time due to the presence of seminal fluid (38) and the acidity of the HEC gels (39), the chitosan did not have any additional effect (fig. S8). A direct effect of chitosan on sperm motility was thus considered unlikely to be the main contributor to the barrier. Because HEC formulations alone were poorly effective at blocking sperm in vivo (Fig. 3G), we concluded that the blockage of sperm transport to the distal cervix and uterus by the chitosan treatment in the ewe was mediated by chitosan and its physical alteration of the CM barrier.

Chitosan formulations are noncytotoxic, induce low concentrations of inflammatory cytokines, and do not alter vaginal epithelial thickness

Spermicides have a history of vaginal epithelial irritation leading to discomfort, and possible increased risks of infections (40). Decades of chitosan research have shown that chitosan-based materials are generally biocompatible with a range of implantation sites (41). This is further validated by the successful approval in the United States and in the European Union of several medical devices including chitosans such as hemostatic materials (for example, HemCon, Syvek, Clo-Sur, and ChitoSeal) (42–44), nerve guides (NeuroShield) (45), and in carboxymethylated versions as injections to treat osteoarthritis of the knee (KioMedine™one) (46). However, the biocompatibility profile of chitosan is dependent on its molecular features, including molar mass, deacetylation degree (DDA), and impurities, and it could be tissue dependent.

To assess the risk of the chitosan formulation to vaginal tissues, we first tested for epithelial irritation by exposing three-dimensional vaginal epithelial tissue to the HEC/chitosan formulation. The chitosan was restricted to the upper layer of the cells of the tissue, which suggests the treatment was localized and that chitosan was unlikely to be absorbed systemically (Fig. 5A). The cells of the tissue model maintained cell viability after 24-hour exposure to chitosan formulation. In contrast, N9 spermicide, which is known to irritate vaginal tissues, led to a 91% reduction in cell viability compared to the untreated tissues after 24-hour exposure (Fig. 5B). In}

| Table 1. Histopathological analysis of vaginal biopsies. Mucosal epithelium was screened for irritation and inflammation using untreated tissues (Untreated), tissues treated with chitosan of 36.2 kDa (CS 36.2 kDa) and hydroxethyl cellulose (HEC), and the spermicide nonoxynol-9 (N9). Biopsies were analyzed for n = 6 (untreated), 4 (CS 36.2 kDa), and 5 (N9) ewes (E). |
|---------------------------------------------------------------|
|                  Untreated                                   |            | CS 36.2 kDa + HEC |            | N9                      |
|                  | E1   | E2   | E3   | E4   | E5   | E1   | E2   | E3   | E4   | E5   | E1   | E2   | E3   | E4   | E5   |
| Submucosa        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Mononuclear cells* | 2   | 2   | 2   | 2   | 2   | 2   | 2   | 2   | 1   | 2   | 1   | 0   | 1   | 0   | 0   |
| Acute hemorrhage  | 0   | 0   | 1   | 1   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| Neutrophilic cells* | 2   | 1   | 1   | 2   | 1   | 2   | 1   | 0   | 1   | 0   | 0   | 0   | 0   | 0   | 0   |
| Erosion           | 1   | 0   | 0   | 1   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 4*  | 0   | 2   |
| Neutrophilic microabscess | 1   | 0   | 1   | 0   | 1   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| Hypertrophy       | 1   | 0   | 1   | 0   | 2   | 1   | 0   | 1   | 0   | 1   | 0   | 0   | 2   | 0   | 0   | 0   |
| Squamous metaplasia | 0   | 1   | 0   | 0   | 1   | 1   | 1   | 2   | 0   | 1   | 0   | 0   | 1   | 1   | 1   | 1   |
| Mucoid cellular shedding | 0   | 1   | 0   | 0   | 0   | 1   | 1   | 1   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   |
| Apoptosis         | 0   | 0   | 1   | 1   | 2   | 0   | 0   | 1   | 0   | 0   | 0   | 0   | 0   | 2   | 0   | 0   |
| Inflammation      | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 3*  | 3   | 0   | 1   |
| Atrophy           | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 2   | 4   | 0   | 0   | 0   | 0   |

*Infiltration. †With evidence of ulceration. ‡Acute, large crust formation. §Acute/subacute, extending into the dermis with neovascularization.
addition to cell viability, proinflammatory cytokine secretion, in particular interleukin-1α (IL-1α), is a risk factor for vaginal irritation by topical formulations (47). We measured a dose-dependent but limited increase in IL-1α cytokine release from tissues exposed to the formulation containing chitosan compared to those exposed to HEC gels, whereas N9 formulation led to release 12 times higher than no treatment control baseline (Fig. 5C).

To test the effect of the treatment in vivo, vaginal epithelial tissue from the efficacy studies (Fig. 3) was collected after the 5-hour exposure. In contrast to the N9 treatment that led to the shedding of the epithelium, the HEC gel with chitosan showed no effect compared to HEC alone (Fig. 5, D and E). In addition, a histopathological scoring of the histology slides showed no effect of the chitosan treatment compared to untreated animals, and increased inflammation and atrophy scores in the N9-treated animals (Table 1).

DISCUSSION
In this work, we hypothesized that the interaction of the mucoadhesive chitosan biopolymer with CM could create an impenetrable barrier to sperm. We first sought to study the interaction of the chitosans varying in molar mass, with human ovulatory CM. We observed an accumulation of all chitosans at the interface of the gel, likely resulting from the strong interactions between chitosan and the CM (Fig. 1, B to E). Several other mucoadhesive polymers and peptides have also been shown to accumulate at the interface...
of mucin gels (48–50). The superior diffusion profile and accumulation of smaller chitosans was likely due to limited steric hindrances between chitosans and mucins of the CM. In contrast, entanglements and multiple physical cross-links between larger chitosan and mucins likely limit their diffusivity. The decrease of chitosan penetration in CM with increasing chitosan molar mass was well corroborated with our previous works on mucin hydrogels (29). Before in vivo testing, it was important to validate the use of the jellifying excipient HEC. The addition of such a large hydrated polysaccharide to the formulation, necessary for the vaginal delivery of chitosan, increased the crowding of the formulation, which could possibly have affected the ability of chitosan to diffuse into the mucus.

Using in vitro sperm penetration assays, we then found that the CM barrier to sperm could be successfully reinforced by exposure to chitosan solutions. The dependence of the CM barrier reinforcement on chitosan molar mass suggested that there is a minimum chitosan size required to effectively cross-link the mucin strands in the CM. Ovulatory CM is very hydrated with a water content of 97 to 98% at ovulation (51), and the volume of CM increases 10- to 20-fold compared to nonovulatory CM (52). Pore sizes have been estimated to be between 20 and 15,000 nm (53, 54). We hypothesize that oligo-chitosans diffuse and interact with mucins but are unable to bridge over these pores, whereas larger chitosans of 35 kDa, which we expect to be in the order of 100 nm in length (55), could.

We next selected the ewe model to further investigate the efficacy of such an approach. Anatomically, the ewe has been well studied (56–58). The ewe’s cervix and vagina share similar length and epithelial layer thickness compared to that of humans (59), supporting the use of ewes to test the safety of contraceptive products such as microbicidal vaginal rings (60), and the efficacy of spermicides (61). In addition, the ram deposits semen vaginally (62), not directly in the uterus like in the rat models also used in reproductive and contraceptive research (63). The ewe model also allowed us to track sperm throughout the reproductive tract, which is not easily performed clinically due to invasiveness (64). The chitosan-containing HEC gel reached the cervix after 1 hour but seemed to be halted by the presence of ring structures, which are found along the ewe’s cervix (65). These ring structures are not found in the human cervix, which could mean that the formulation could penetrate deeper in humans. Previous studies have shown that viscous gels can penetrate the cervical canal in humans but do not typically reach the uterus in notable amounts (66–68). We showed that the chitosan formulation was effective at reducing the sperm numbers in the distal cervix and uterus of ewes 4 hours after AI and investigated the mechanism by which these effects were obtained. We show that the HEC gel, containing lactic acid and adjusted to pH 5.5, also reduced the average number of sperm detected in the upper reproductive tract, although it was not statistically significantly different from untreated animals. The addition of chitosan was necessary to reach the low sperm counts close to or at 0, suggesting that the mechanism of action was different from pH deactivation of sperm or the physical blockage of sperm by the HEC gel. This was further confirmed by the absence of effect of chitosan-containing HEC gels on human sperm motility compared to HEC gel alone. Further studies including other viability markers such as membrane integrity, DNA integrity, and reactive oxygen species production would be required to provide a full toxicology profile of the chitosan toward sperm.

By demonstrating that the barrier can be attributed in large part to the physical reinforcement of the CM barrier, we establish a new mechanism that can be added to the toolbox available for contraceptive design. The contraceptive efficacy of such an approach can only be determined in contraceptive trials, but it is likely that it will in part rely on the natural or induced deactivation of sperm. This is because the chitosan-modified CM will likely be replaced by nonmodified CM through the natural mucus turnover, progressively weakening the barrier to sperm. Existing on-demand nonhormonal mechanisms of action seem to show intrinsic limitations that hamper their contraceptive efficacies. Contraceptives such as spermicides, gels, and rings have shown 6-month failure rates of 10 to 20% (12, 69) in typical use, whereas 6-month failure rates are limited to 3 to 5% for hormonal contraceptives (70). Even when used consistently and correctly, spermicides stay less effective than hormonal treatments (16), with a 6-month failure rate of 4% for spermicides (71) versus 0 to 1% for oral hormonal contraceptives (72, 73). New mechanisms of action, such as the one highlighted in this work, are opportunities to go beyond such a glass ceiling in efficacy.

We also investigated the safety profile of the chitosan-containing gels. Although chitosans are generally biocompatible (41), studies of inflammatory response of cells exposed to chitosan have produced mixed results, likely due to differences in chitosan characteristics. While some studies indicate no effect on fibroblast cells (74), others indicate increase of IL-1α expression from oral epithelial cells (75) and mild activation of macrophage cells by pure chitosan (76). In this study, the IL-1α expression induced by chitosan was less than the expression induced by the U.S. Food and Drug Administration–approved N9 spermicide. N9 is known to trigger an inflammatory response, especially in repeated use, characterized by a marked cytotoxicity and the increased secretion of proinflammatory cytokines such as IL-1α and IL-1β from epithelial cell cultures (40, 77). These toxicity markers have been validated in several animal models, showing epithelial disruption, epithelial cell death, and inflammatory infiltrations when exposed to N9 (78–80), including using the ewe model (81). In humans, these side effects lead to discomfort (82) and are suggested to be linked with increased risk of HIV transmission (83). Thus, these in vitro and in vivo studies suggest that chitosan is unlikely to cause acute toxicity or short-term inflammation of vaginal tissue. The three-dimensional cell model used here, although a good mimic of the cell composition and structure of vaginal epithelium (47), does not include the layer of cervical-vaginal secretions, which is likely to bind chitosan and shield the cells from the treatment. Even without mucus secretion, the chitosan, likely due to its negative charge and large size, was restricted to the upper layer of the cells of the in vitro vaginal epithelial tissue.

We have identified several limitations of this study. The ewe model did not mimic the mechanical shear of intercourse, which can be beneficial by spreading the formulation quickly and helping it reach the CM and detrimental by possibly breaking the barrier that was formed. Coitus could therefore be simulated in the ewe model to address this concern (84). Still, even without coitus, because the animals were free to move between interventions, the barrier needed to resist some contractions and resulting mechanical shear generated by the animals. This study does not inform on the
contraceptive efficacy of this approach, which will need to be verified, possibly using the ewe model. The safety assessment tests constitute a preliminary assessment of the safety profile of such a treatment. Possible irritation to not only vaginal tissues but also cervical and penile tissues will need to be tested in other dedicated in vivo models and in clinical trials. Considering that chitosan formulation could be administered before each intercourse, it is also clear that chronic or subchronic toxicity and inflammation will need to be tested. The compatibility with other vaginal gels and devices, and with menstrual bleeding would also need to be addressed during the further development of a chitosan-containing contraceptive vaginal gel. A prolonged presence of the chitosan in the vagina could also alter the vaginal microbiome. The effect of vaginal products and contraceptives in general has been understudied but is currently being investigated for hormonal (85) and nonhormonal methods (86). Certain chitosans have known antimicrobial activities (87) and could affect commensal microbiomes and participate in balance or limit pathogen growth, prompting further vaginal microbial health. The antimicrobial activity of chitosan would preferably need to be tested in the in vivo context of the vaginal microbiome, in which secreted mucins and other proteins, lipids, and ions can limit its interaction with microbes.

One other key challenge ahead is the importance of developing the appropriate delivery of the chitosan, which can have a strong impact on efficacy, usability, and overall user compliance. Developers of such a product will need to leverage the conclusions of previous studies on vaginal gels, which have identified ideal volume and rheological properties for optimal coverage of the vaginal wall and cervix (66, 68, 88) and modeling work to predict the spreading of vaginal gels (89). The gel can be applied with syringe-type applicators optimized for gel distribution and selected by user preference (90–92).

In conclusion, we demonstrated that certain chitosans could effectively reinforce the CM barrier to sperm without acute toxicity toward sperm or epithelial cells. Although this study did not demonstrate contraceptive efficacy, we presented a proof of concept for a possible contraceptive mechanism of action, without the potential toxicities of other pericoital methods such as N9 spermicides. Physical reinforcement of the CM barrier could therefore constitute a class of muco-cervical barrier contraceptives that could theoretically be varied in dose, delivery modality, and release kinetics to address a range of user needs in contraception.

**Materials and Methods**

**Study design**

The main goal of this study was to identify chitosan formulation that could successfully block sperm penetration of ovulatory CM. The study first relied on in vitro assays using human ovulatory CM and human sperm, which helped identify chitosan candidates to be further tested in vivo. All experiments were repeated three times with different sperm samples. Although 53 human ovulatory CM samples were collected and analyzed, only a fraction could be used for these assays because of limited sample size or quality. For this reason, some of the assay triplicates were performed only on one or two different mucus samples. The in vitro assays also analyzed the diffusion of fluorescently labeled chitosan into the CM.

Ile de France breed sheep were used to demonstrate the blocking ability of chitosan in vivo. The sheep model allowed the imaging of fluorescently labeled sperm in the vagina, and of sperm up the reproductive tracts on explanted tissues. Animals were allocated to treatment based on age and body condition scores before the day of the experiment to ensure no bias in age or body condition score between the treatment allocations. The body condition scoring technique, scaled from 0 (very thin) to 5 (obese), allowed us to assess the overall fitness of the ewe, taking into consideration skeletal size, breed, and physiological state. All animals were judged healthy and fit for inclusion before us, including lameness. Animals were free of clinical disease, had a condition score of 3 or more, had no obvious signs of damage to the vulva, and had no defects to the udders. All experiments on the ewes were done on the day of ovulation. The number of animals for sperm counting was set to at least $n = 7$ based on previous experience of variability of such experiments. The sheep model was also used to assess the acute toxicity of the formulations. In vitro assays complemented the toxicological data, measuring the effect of chitosan formulation on sperm kinematic parameters and viability and cytokine secretion of a commercial three-dimensional vaginal epithelial tissue model.

**Chitosan labeling, desalting, and characterization**

Chitosans and the chitosan oligomers were labeled with fluorescein isothiocyanate (FITC; Sigma-Aldrich) for in vitro diffusion testing, using a modified method previously reported by Kootala et al. (29). Briefly, chitosan of was dissolved in 2 ml of ultrapure water at 40 mg/ml to which 2 ml of methanol was added. The solution was adjusted to pH to 5.5 with 37% HCl or 2 M NaOH. Then, a solution of FITC (10 mg/ml) in dimethyl sulfoxide (DMSO) was added to reach a ratio of 1:50 (1 fluorescein for every 50 repeating units) followed by 2 hours of shaking at room temperature in the dark. Chitosans were precipitated by the successive addition of 2 ml of 2 M NaOH then 4 × 6 ml of EtOH, increasing the pH to 9. The supernatant was removed after centrifugation at 20,000g for 25 min at 4°C, and the pellets were rinsed three times with ethanol to extract un conjugated FITC. After removing ethanol by rotator vaporization for 2 hours, the pellet was frozen with liquid nitrogen, lyophilized, and stored at 4°C.

For in vivo tracking, the chitosan was labeled with Atto-665 dye. Chitosan (29 m. 3.75 g, c = 35 mg/ml; Happe Medical Chitosan) was dissolved in an aqueous solution of 100 mM lactic acid overnight. The pH of the solution was adjusted to pH 6 with 0.5 M NaOH. Atto-665 N-hydroxysuccinimide (NHS) ester (15 mg) was dissolved in 200 μl of DMSO and added to the chitosan solution. The solution was stirred for 4 hours at room temperature. Sodium hydroxide (2 ml, 0.5 M) was added to the mixture to raise the pH to a range of 7 to 8 and precipitate chitosan polymer in large lumps. The suspension was transferred into falcon tubes to be centrifuged for 10 min at 4500 rpm. The supernatant was discarded, and precipitates of chitosan were resuspended in Milli-Q water. Suspensions were centrifuged for 10 min at 4500 rpm. Rinsing steps were repeated using 96% ethanol to remove excess of the Atto-dye. Chitosan precipitate was finally rinsed and centrifuged (10 min, 4500 rpm) with Milli-Q water four times to remove traces of ethanol. The blue solid was filtered on a Buchner vacuum. Labeled chitosan was freeze-dried to give a colored blue powder as a final product (3.2 g, η = 82%). 1H nuclear magnetic spectroscopy (NMR) (400 MHz, D2O) δ 4.96 to 4.77 (m, 0.6H), 4.3 to 3.32 (m, 4.2H), 3.11 (s, 1H), 1.97 (s, 0.04H).

To desalt the chitosan Z10 (36.2 kDa), a 50 mg/ml solution of chitosan was prepared in 32.5 mM lactic acid (LAC). The solution

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was stirred for 4 hours and adjusted in pH to 5.5 by the addition of 1 M HCl. The chitosan solution was purified by dialysis for 5 days (molecular weight cut-off of 12 to 14 kDa, 25 mm; SPECTRA/Por 4, Spectrum, USA), using ultrapure water adjusted to pH 4.0. The sample was subsequently lyophilized.

The solubility of the chitosans was assessed by transmittance measurements of chitosan solutions (5 mg/ml) at 600 nm using a Varian Cary 50 Bio UV-Visible spectrometer and Cary WinUV Simple Reads Software (Agilent Technologies). Samples containing 2.7% HEC were transferred to a cuvette and centrifuged at 600g for 5 min to remove air pockets before turbidity testing. Osmolarities [mOsm/kg H2O] were determined in 100 µl of samples with a Roebking automatic micro-osmometer using freezing point depression. The pH was measured and adjusted with the SevenCompact pH S210 meter and pH electrode InLab Ultra-Micro (Mettler Toledo AB). Average molar mass [weight-average molecular weight (Mw) and number-average molecular weight (Mn)] and dispersity (D) of chitosans were determined by size exclusion chromatography (SEC). DDA of chitosans was determined by 1H NMR. SEC and 1H NMR spectra were acquired as described elsewhere (37). Results on chitosan oligomer and chitosan characterization are compiled in Table 2.

**Table 2. Characteristics of chitosans and chitosan solutions.** The degree in deacetylation (DDA), the weight-average molecular weight (Mw) and number-average molecular weight (Mn), ratio of Mn/Mw, and osmolality in millimole per kilogram water (mOsm/kg H2O) and measured impurities are shown. The variability of the values is given as SD. NA, not analyzed.

| Chitosan | DDA % | Mw kDa | Mn kDa | D | Osmolality at pH 5.5 mOsm/kg H2O | Impurities                     |
|---------|-------|--------|--------|---|---------------------------------|--------------------------------|
| 0–10 kDa |       |        |        |   |                                 |                                |
| CO      | 78.0  | 1.4 (±0.7%) | 0.9 (±1.3%) | 1.6 (±1.5%) | 12.3 ± 0.6 (±106.3 ± 3.3) / 173.7 ± 2.1 | 2-Propanol, undefined impurities |
| Z49     | 98.9  | 7.1 (±0.4%) | 4.8 (±0.7%) | 1.5 (±0.9%) | 16.4 ± 6.4 |                                |
| Z56     | 93.7  | 18.9 (±0.3%) | 13.1 (±0.4%) | 1.4 (±0.5%) | 71.7 ± 2.1 | Isopropanol, ethanol, methanol |
| Z13     | 91.6  | 27.9 (±0.3%) | 21.1 (±0.4%) | 1.3 (±0.5%) | 70.7 ± 2.1 | Isopropanol                     |
| 95/5    | 98.5  | 35.0 (±0.1%) | 27.2 (±0.2%) | 2.0 (±0.3%) | 26.4 ± 2.9 | 130.0 ± 1.0 | NA                              |
| Z10     | 97.4  | 36.2 (±0.2%) | 27.9 (±0.3%) | 1.3 (±0.4%) | 105.7 ± 2.9 | Isopropanol                     |
| Z10/desalted | 95.0  | 51.2 (±0.4%) | 36.9 (±0.5%) | 1.4 (±0.7%) | 69.3 ± 0.6 | NA                              |
| >50 kDa |       |        |        |   |                                 |                                |
| 95/1000 | 94.7  | 209.9 (±0.4%) | 163.4 (±0.4%) | 1.8 (±0.6%) | 111.0 ± 0.6 | NA                              |

* Dissolved in endotoxin-free H2O.  † Dissolved in 0.05 M phosphate-buffered saline.  ‡ Dissolved in 100 mM lactic acid.  § Dissolved in 32.5 mM lactic acid.
inside diameter (ID), 0.3 × 0.3 mm; outside diameter (OD), 0.45 × 0.45 mm, borosilicate glass] with Luer-connector (Hilgenberg GmbH) and 1-ml Soft-Ject syringe (Henke-Sass Wolf GmbH). Next, the tubes were broken off at the Luer connection, and the broken end was sealed with wax (Paul Marienfeld GmbH & Co. KG). The capillary was gently inserted through the septa of vial caps (55° shore, Teknolab Sorbent AB), puncturing the septum from the inside to the outside with the broken and sealed end of the capillary. The cap with capillary was then screwed onto the glass vial (ND9, 1.5 ml; Avantor), and the open end of the capillary was immersed into 300 μl of preheated buffer solution or chitosan-FITC in buffer placed in glass vials. Identical square capillaries without Luer-connector (ID, 50 mm; OD, 0.3 × 0.3 mm, borosilicate glass; CM Scientific Ltd.) filled with chitosan-FITC solution at 1 or 5 mg/ml were used for calibration. After 30-min incubation at 37°C and 5% CO₂, the capillaries were observed by fluorescence microscopy and images were acquired using an Eclipse Ti inverted microscope with a 2× objective (Nikon), Zyla sCMOS camera (5.5 MP, Andor, Oxford Instruments), and the light source pE-300L (CoolLED) connected to the NIS-Elements BR 4.60.00 software (Nikon). Images were captured at an exposure time of not only 20 ms to avoid saturation but also 800 ms to measure low fluorescence signals of the diffusion front deeper inside the capillary (fig. S2). Assays were conducted in triplicate. The limited availability of CM and its limited storage time determined the number of CMs used for the three replicates. Images were analyzed by ImageJ software (version 2.0.0-rc-43/1.52b). A region of interest for fluorescence profiles is a rectangle (h = 15, w = 1314), in the middle of the capillary starting at 5 mm before the air/CM interface in the capillary. The chitosan concentration estimated on the basis of the calibration capillaries or the fluorescence intensity normalized to the saturated fluorescence intensity was plotted over the distance in pixels. Each pixel corresponded to 3 μm. At an exposure time of 800 ms, the autofluorescence of buffer-treated CM was subtracted from all measurements.

Sperm penetration assays
Glass vials (ND9, 1.5 ml, VWR) containing microinserts (flat bottom, 200 μl, VWR) were filled with 100 μl of lactic acid solution with or without chitosan or with liquefied semen and heated at 37°C. An aliquot of the transparent section of CM was aspirated into square capillaries (ID, 0.3 × 0.3 mm) and sealed at the broken end. The capillary filled with CM was introduced through the septum of a cap (55° shore, Teknolab Sorbent AB), with the sealed end first, and then the cap was placed on the vial to immerse the capillaries. Nontreated capillaries were directly exposed to semen and left for 30 min at 37°C. The incubation period was no longer than 30 min to limit the spontaneous loss in sperm motility (37). After incubation, the capillary penetrated by sperm was removed from the vial through the septum of the cap, stripping sperm and seminal plasma off the capillary's surface. Treated capillaries were first exposed to lactic acid with or without chitosan for 1, 5, or 30 min at 37°C and then transferred to a vial containing semen for another 30 min at 37°C. To observe the distribution of sperm, a customized microscopic glass slide marked with the distances 0.5, 1, 2, 3, 4, and 5 cm was used. The slides were positioned on a prewarmed (37°C) stage heater DC 95 (Linkam Scientific Instruments), and the capillaries were positioned onto the glass slide. Videos were recorded at the enumerated distances, including the beginning of the capillary (0.1 cm), using the Eclipse Ci phase contrast microscope (Nikon) equipped with UI-3240LE-C-HQ camera (IDS Imaging Development Systems) using a 10× phase contrast objective (total magnification of ×100), and the software Picosara. The recorded microscopic field was 0.21 mm × 0.27 mm, equivalent to 0.0567 mm². The recording started at the upper, outer surface of the capillary, followed by focusing through the capillary until reaching the lower surface, resulting in a three-dimensional scan through the capillary (movies S3 and S4). Sperm was counted in the three-dimensional zone of 0.017 mm³ corresponding to 17 nl of CM. The assay was conducted in triplicate using the semen of different volunteers. Sperm penetration assays using ewe ovariolyte CM and ram semen were conducted in a similar way than for human samples, albeit using a BH2-RFCA microscope (Olympus) at 10× and Phase 3, a pco.edge sCMOS camera (Photon Lines) of 10× (total magnification of ×100), and the software CamWare V3.17. Capillary tubes were primarily assessed by phase contrast microscopy; however, when assessing chitosan formulated in HEC, the sperm were first stained with 4’,6-diamidino-2-phenylindole (DAPI) (10 μg/ml, Hoechst Sigma) and then observed using the fluorescence mode of the microscope.

In vivo efficacy and safety testing of chitosan formulation in the ewe
Estrus synchronization
A fluorogestone acetate sponge was inserted in the vagina for 14 days, and 600 IU of pregnant mare serum gonadotropin (PMSG; Sanofi Animal Health Ltd.) was intramuscularly injected at the withdrawal of sponges. Oestrus occurred 55 hours after sponge removal.

Sperm collection and labeling
Ejaculates were obtained after natural ejaculation using an artificial vagina. Semen volume and mass motility were assessed immediately after collection. The sperm concentration was assessed using a standard precalibrated spectrophotometer. Collected semen was labeled for 8 min with R18 fluorochrome (Invitrogen, O246) and Mitotracker Green FM (Invitrogen, M7514) (300 and 20 μM, respectively) according to (83). Semen fluid and dyes were washed out by centrifugation (800g, 40 min, 37°C) in a discontinuous Percoll gradient [45% (v/v) and 90% (v/v)]. Washer spermatozoa were then diluted in a warm (37°C) skim milk extender (11.1 g/100 ml of water) at a final concentration of 1.0 × 10⁹ sperm/ml. Diluted semen was packaged in 0.25 ml of straws and stored at 15°C until cervical insemination was performed.

Administration of formulation and insemination
The chitosan formulation [5 ml of chitosan–Atto-665 at 5 mg/ml, HEC 2.7% (w/w), pH 5.5 in lactic acid] or a formulation without chitosan [5 ml of HEC 2.7% (w/w), pH 5.5 in lactic acid] was deposited in the vagina, using a syringe and a silicone tube extension. The formulation was gently deposited close to the cervix but not into the cervix. The ewes were then set free for 1 hour and then vaginally inseminated with 1 × 10⁹ labeled spermatozoa.

pCLE and IVIS spectrum assessment of chitosan and sperm penetration in the ewe reproductive tract
In vivo imaging using pCLE
The distribution of labeled chitosan and labeled sperm was assessed in vivo using pCLE (Cellvizio Dualband, Mauna Kea Technologies).
The confocal microscope delivered laser light at an excitation wavelength of 488 and 600 nm and collected emitted fluorescence signal simultaneously from sperm cells in the visible range at 505 to 633 nm and chitosan in the near-infrared range at 673 to 800 nm. In vivo examination of vagina and cervical os with pCLE 2-min video sequences was performed before and after vaginal application of formulation and sperm insemination.

**Ex vivo imaging using pCLE**

The animals were euthanized by a bolt gun and destruction of the brain via pithing, followed by confirmation of permanent cessation of the circulation 4 hours after sperm insemination. After a 10-min delay following euthanasia, the reproductive tracts of the ewes were collected and dissected. The vagina, posterior cervix, anterior cervix, and uterus were dissected for pCLE analysis. An S1500 microscope (1.5 mm diameter, 3.3 μm lateral resolution) was used for recording 2-min video sequences for each anatomical region (vagina, posterior cervix, anterior cervix, and uterus) at a frame rate of 12 images per second. All images from the videos were blindly analyzed by particle tracking analysis software. Sperm cells were quantified using an ImageJ macro (CASA) (96) before being manually reviewed to confirm that all particles counted were sperm.

**Ex vivo IVIS spectrum imaging on ewe reproductive tracts**

Fluorescence imaging on ex vivo ewe reproductive tracts was performed using the Imaging System IVIS Spectrum (PerkinElmer). Detection and quantification of Atto-665 from labeled chitosan were obtained after acquisition using the filter pair mode: excitation at 640 nm (bandwidth of 30 nm) and emission at 680 nm (bandwidth of 20 nm). Detection and quantification of octadecyl rhodamine from labeled spermatoides were performed using the spectral unmixing mode acquisition: excitation at 570 nm (bandwidth of 30 nm) and emission at 620, 640, 660, 680, and 700 nm (bandwidths of 20 nm). Fluorescent signals and tissue autofluorescence were unmixed. Quantifications of Atto-665 signal and octadecyl rhodamine signal were applied on vagina, cervix, and uterus regions. Results were expressed in average radiant efficiency [p/s/cm²/sr]/[μW/cm²]. Fluorescent data were uniformly acquired and analyzed with the PerkinElmer Living Image software (version 4.7, PerkinElmer).

**In vitro toxicity profiling**

**Sperm kinematics**

In 50-ml Falcon tubes, fresh human semen (1 ml) was placed on 2 ml of a HEC gel, containing 32.5 mM lactic acid (pH 5.5) with or without Z10 chitosan (36.2 kDa) and incubated at 37°C. After different time intervals, the semen was sampled (6 μl) and assessed by CASA for sperm count, progressive motility, motility, immotility, velocity, sperm size, and cell count as described for semen assessment in the Supplementary Materials.

**Human vaginal epithelium culture**

Three-dimensional models of human vaginal epithelium [SkinEthic Human Vaginal Epithelium (HTS 0.33 cm²) (Episkin)] developed for toxicity testing (47) were cultured in the provided maintenance medium according to the manufacturer’s protocol for 24 hours before treatment. The tissue inserts were cultured in a 24-well flat-bottom plate (Corning) in 600 μl of medium and incubated at 37°C, 5% CO₂. The medium was changed immediately before the treatments.

**PrestoBlue cell viability assay**

Gels composed of chitosan at various concentrations and HEC (2.7%, w/v) or N9 (2%, v/v) and HEC (2.7%, w/v) were prepared, and the pH was adjusted to 5.5 with HCl and NaOH. For each gel tested, 250 μl of the gel was applied directly onto the apical side of the tissues and incubated for 24 hours at 37°C. After the treatments, the cell medium on the basal side of the cell culture was removed and 540 μl of fresh SMM medium was added to each well. In each well, 60 μl of the PrestoBlue Cell Viability Reagent was added directly to the medium [10%, (v/v)], and the plate was incubated for 1 hour at 37°C. Then, the fluorescence was read on the microplate reader (Biotek, Synergy 4) at an excitation and emission of 560 and 590 nm, respectively. Values were corrected for background fluorescence (medium only) and normalized to values of untreated control tissues.

**Interleukin release measurements**

Twenty-four hours after the treatments of the human vaginal epithelium tissues, the cell medium was removed from the 24-well plate. IL-1α release was quantified in the medium by enzyme-linked immunosorbent assay (ELISA) (DuoSet ELISA, R&D Systems) according to the manufacturer’s instructions. The absorbance signal was read at 450 nm using a microplate reader (Biotek, Synergy 4).

**Ewe vaginal tissue histology**

After a 10-min delay following euthanasia, the reproductive tracts of the ewes were collected and dissected as described above. A biopsy of vaginal tissue of 2 × 2 cm was cut out (approximately mid-vagina). The tissue biopsy was fixed in 4% paraformaldehyde for up to 72 hours at 4°C. Following the fixation, the tissues were placed in 1x PBS and stored at 4°C. The fixed tissues were processed by Histocenter AB. The tissues were dehydrated through a series of ethanol organic solutions (4% formaldehyde at 50°C, 70% ethanol, 2× absolute ethanol, absolute ethanol at 65°C, and isopropanol at 68°C; vaporization) and embedded in paraffin in a tissue processor (LOGOS, Milestone MI-61504). Cross-sections of the embedded tissues with a 4 μm thickness were cut and mounted on glass slides. The paraffin-embedded sections were then stained with hematoxylin and eosin in an automated slide stainer (Medite TST 44.200S). The slides were imaged on Panoramic Scanner P250 (3D Histech) and analyzed using the CaseViewer software (3DHistech). Histopathological analysis was performed by a veterinary toxicopathologist (Atlantic Bone Screen), according to the following scoring system: 1, minimal; 2, slight; 3, moderate; 4, marked; 5, severe.

**Statistical analysis**

Statistic calculations were conducted with the GraphPad software Prism 9. Normality was examined by the Shapiro-Wilk, D’Agostino & Pearson, or Kolmogorov-Smirnov test. The Kruskal-Wallis post hoc Dunn’s test was performed when datasets did not pass the test for normal distribution. When the values were normally distributed, statistical significance was calculated using either ordinary one-way or two-way analysis of variance (ANOVA). Multiple comparisons were conducted with Tukey’s or Sidak’s test. Significant differences in results are marked as asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001). The repeatability of results is indicated in figures and tables through calculated standard deviations. The area under curve (AUC) was conveyed as total peak area. To calculate the top, bottom, logIC₅₀, and HillSlope, a curve fitting in the form of the nonlinear regression “sigmoidal, 4PL, X is log(concentration)” was conducted.
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