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A trifunctional contraceptive gel enhances the safety and quality of sexual intercourse

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ABSTRACT

Current contraceptive methods come with a number of drawbacks, including low efficacy, in the case of commercial contraceptive gels, and a reduction in the quality of sexual intercourse, in the case of condoms. Adding pharmacologically-active agents to contraceptive gels holds the potential to improve sexual experience, and hardbor safety and hygiene. In this study, we fabricated a Carbomer-based contraceptive gel consisting of three agents: tenofovir, gossypol acetate, and nitroglycerin (TGN), with pH adjusted to 4.5 (to be compatible with the vagina). *In vitro*, the gossypol component of the contraceptive gel proved to be a significantly effective spermicide. When the concentration of gossypol acetate was 10 mg/ml, the spermicidal ability reached 100% after 30s. In addition, tenofovir in the gel significantly inhibited lentiviral transfection efficiency in cell-containing media. In 6 pairs of rats, the gel successfully prevented all females from conceiving after successful mating. Moreover, increased sexual frequency and enhanced erection, which were promoted by the nitroglycerin in the components, were observed in male rats that had the gel applied to their penises. This novel TGN contraceptive gel yielded a higher contraceptive success rate than that of the commercial contraceptive gel (Contragel®). In addition, it has the added benefits to prevent sexually transmitted diseases and improve male libido and erection function during sexual intercourse. Combining three FDA-approved and marketed agents together, our trifunctional TGN gel has a great potential for further translation and commercialization.

1. Introduction

The population explosion has become a major worldwide problem [1]. Globally, more than 200 million pregnancies occur each year, and 50% of them are unintended [2,3]. Therefore, the research and development of contraceptives has received much attention in the past decades. Currently, condoms are the most commonly used method of contraception, which is featured by the function of preventing sexually transmitted diseases [4,5]. Whereas, it is universally agreed that using condoms reduces the quality of sexual intercourse. Intrauterine device (IUD) placement is generally considered to be the most effective method of contraception [6]. However, it is an invasive operation which needs surgery for the placement. And postpartum IUD insertion may increase the risk of adverse events such as perforation or bleeding, as well as effectiveness [6]. Short-acting oral contraceptives usually have good effects, but requires the user to strictly follow the medication time. And secondly, because of the androgenic activity it contains, oral contraceptives may cause side effects, such as abnormal glucose or lipid metabolism, weight gain and acne [7]. In addition, oral contraceptives do not have the function of preventing sexually transmitted diseases. Calendar-based contraceptive methods are a group of methods of calculating the fertile period and the safe period of females, based on the

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record of female menstrual cycle [8]. So that couples can choose to have sexual intercourse during the safe period. However, the individual menstrual cycle varies greatly, and each menstruation suffers too much interference from the external environment and internal hormone levels, which could be difficult to estimate accurately [8]. Therefore, calendar-based contraception is one of the contraceptive methods with the lowest success rate.

Spermicides are a group of contraceptive drugs that can be applied in the vagina before intercourse to avoid pregnancy [9]. They have the potential to be combined with other carriers and to become stable on the genitals [9], achieving contraception, and at the same time, improving the sexual experience when compared to condoms. As a consequence, the improvement of spermicidal drug formulations in contraceptive gels, in terms of both efficiency and efficacy, has become the focus of research [10,11]. One of the most common spermicidal agents is nonoxynol-9 (N-9), but recent research showed that a N-9-containing marketed formulation was only 67% effective at killing sperm *in vivo* [12,13]. Also, the long-term use of N-9 can destroy the normal vaginal flora, causing vaginal irritation, hyperemia, edema, or even cervical epithelial damage [14,15]. Damage to endometrial epithelial cells may increase the likelihood of STD transmission, including AIDS [15]. Thus, the use of alternative spermicidal agents in contraceptive gels, along with other additives that improving user experience meanwhile lowering the risks associated with N-9, will contribute to safer and more enjoyable intercourse [10,14,16,17]. Gossypol is one of the alternatives of N-9, and tons of data have proved the potentials of gossypol as a male contraceptive in both of animal studies and clinical trials [18–20]. Either *in vitro* or *in vivo*, gossypol can significantly inhibit sperm motility, making it a promising spermicidal candidate [21–23]. In addition, tenofovir is a first-line medication for the treatment and transmission reduction of HIV [24–27]. It can inhibit the transcription and replication of viruses by inhibiting reverse transcription. tenofovir has been reported to inhibit viral infection *in vitro*, which was determined by observing the efficiency of viral transfection in cells [25]. Moreover, nitroglycerin, an FDA-approved drug, can produce nitric oxide (NO) transdermally to promote the mechanism of cGMP and the expansion of vascular smooth muscle, locally increasing blood flow. It is considered to be an effective male erectile enhancer [28–31].

Hydrogel, as a type of synthetic biomaterial, has been popularly applied in the area of cell engineering [32–37], tissue engineering [32,38] and drug delivery [39]. As an unique derivative of polyacrylic acid, carbomer has the potential to become a hydrogel carrier when it is dissolved in water at a concentration of 1% [40]. The pH of 1% carbomer aqueous solution is about 2.5. With alkaline substance its pH could be adjusted slowly, and the viscosity of the gel would increase as pH value increases [41–43]. It has the capability to form a good adhesion and high stability as a drug carrier. When the pH reaches about 4.5, which is a stable value of the pH in intravaginal environment, the gel strength will get strong enough, making it popular as a carrier for intravaginal administration [41–43].

Stepping from the strategies of biomedical engineering, therefore we conceived the contraceptive gel by employing carbomer as the carrier of above functional agents tenofovir (T), gossypol (G), and nitroglycerin (N), and aiming at the fulfill of the goals of contraception, prevention of STD as well as improvement of the male erection function. Taking full advantages of these bioactive agents, our contraceptive gel has been successfully tested for safety and functionality *in vitro* and *in vivo* as a novel biomaterial.

2. Materials and methods

2.1. Synthesis of the TGN contraceptive gel

Commercial available National Formulary (NF)-grade carbomer (Fisher Scientific, Waltham, MA, USA) was used to prepare the gel. Briefly, 1 g carbomer was added into 100 mL deionized (DI) water at the ratio of 1:100 (w/w) and fully stirred into a solution. Then, tenofovir (Sigma-Aldrich, Missouri, USA) was added to the solution at a concentration of 2 mg/ml. Subsequently, gossypol acetate (Sigma-Aldrich, Missouri, USA) was dissolved in 70% ethanol and added to the get at a concentration of 10 mg/ml. The pH of the solution was adjusted to 4.5 (same as the intravaginal environment) with triethanolamine (Sigma-Aldrich, Missouri, USA) titration, which made the carbomer gelatinous. Finally, nitroglycerin (Sigma-Aldrich, Missouri, USA) was added at a concentration of 1% (by weight) and stirred thoroughly to yield the final contraceptive gel.

2.2. Characterization of the gel

In order to observe the subtle morphological structures and properties of the gel, we used a JEOL JCM-7000 scanning electron microscope (SEM) (JEOL, Tokyo, Japan). Regarding to isothermal shear rate sweep analysis of the gel, all measurements were performed at ambient temperature using a TA Instruments Discovery Series Hybrid Rheometer HR-3 (TA Instruments, New Castle, DE, USA) outfitted with a 40 mm parallel plate (sand blasted). Sample were analyzed by shear rate ($\dot{\gamma}$) in the range of 0.01–1000 s^{-1}. The viscoelastic properties of the gel at different pH levels were characterized by a controlled stress rheometer (Anton Paar Physcia MCR 302, Graz, Austria) in which a cone-and-plate measuring system (50 mm diameter cone, 1° angle) was mounted. Two oscillation based analytical methods were used for characterizing the samples: 1. strain amplitude sweep; 2. frequency sweep. Storage modulus (G') and loss modulus (G'') are calculated values based on the measured torque during the oscillatory analyses. The measuring principle is explained in a previous work [44]. All measurements were performed at a temperature of 37 °C. The strain amplitude sweep tests were performed to identify the linear viscoelastic region (LVE) for each sample. Shear strain (γ) range and frequency were set as 0.1–100%, 1 Hz respectively for this method. Frequency sweep tests were performed only within the LVE, the frequency range was set as 0.01–80 Hz. Two replicates were performed for each measurement.

2.3. In vitro spermicidal capability test

All pig sperm samples were taken twice a week from boars at the Swine Unit of North Carolina State University's College of Veterinary Medicine. The Androstar Premium extender (Minitube, Germany) was used to maintain the sperm's viability at or above 90%. The gel was diluted into 3 concentration gradients of gossypol acetate in carbomer: 0 mg/ml, 5 mg/ml, 10 mg/ml. Fresh pig sperm was pipetted into the 1 ml pipette tip. Then, gels with each of the three different concentrations were applied to different pipettes to cover the tips. The sperm in the pipette tip was shot onto a glass slide to simulate the ejaculation process. The sperm activity was immediately observed under a microscope and recorded, at three time points (30s, 1min, and 3min). As a control, pig sperm was shot onto the slide with a pipette without applying the gel. The experiment was repeated three times.

2.4. Biosafety test of TGN gel in vitro

VK2/E6E7 vaginal epithelial cells (ATCC®, Manassas, VA) were seeded in two 96-well plates at a density of 5000 cells per well, and then incubated at 37 °C in a 5% CO_2 incubator until cells attached. The gel was diluted with culture medium adjusted to a pH of 4.5 (same pH as vaginal secretions) to concentrations of 10 mg/ml, 5 mg/ml, 1 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0 mg/ml. The culture medium in the 96-well culture plates was replaced with the gel-infused medias at different concentrations. Each group had six duplicates. After 2 h and 6 h of incubation, respectively, 10 μL of Cell Counting Kit-8 (Sigma-Aldrich, Missouri, USA) solution were added to each well and incubated for another 2 h. The 450 nm absorbance for each group was measured with a microplate reader and the viability/proliferation data was analyzed to

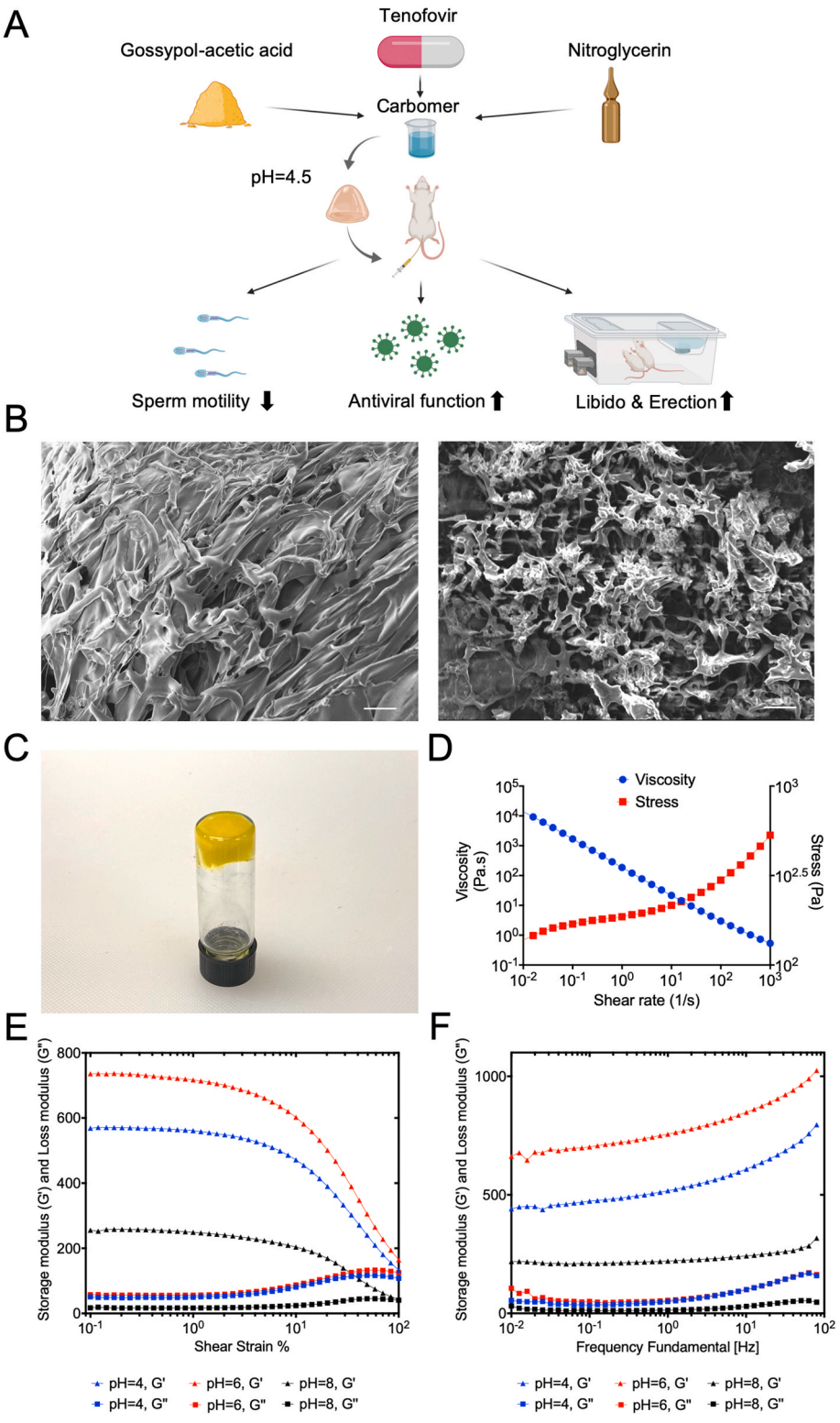

Fig. 1. Fabrication and characterization of the trifunctional TGN contraceptive gel. **A.** Schematic image showing the process of gel preparation and the functional evaluation. **B.** Representative SEM images of the trifunctional TGN contraceptive gel. Left panel, low power field of the gel, scale bar, 100 μm. Right panel, high power field of the gel, scale bar, 10 μm. **C.** Gel in a glass bottle that has been flipped upside down. **D.** Rheological properties of the trifunctional contraceptive gel. Following the increase of the stress, the viscosity of the gel is decreased. **E.** Dependency of storage modulus and loss modulus on shear strain for TGN gel at different pH levels (f = 1Hz). **F.** Dependency of storage modulus and loss modulus on frequency for TGN gel at different pH levels.

calculate the 50% cytotoxicity concentration (CC50). GraphPad (GraphPad Software, California, USA) software was used to graph the data. Cytotoxicity was also examined with a Live/Dead imaging kit (ThermoFisher Scientific, Waltham, MA). In short, 500,000 cells per well were seeded in a 12-well plate and incubated overnight. The next day, the gel was diluted with culture medium to concentrations of 10 mg/ml, 6 mg/ml, 4 mg/ml, 0 mg/ml. Each group had three replicates. After a 2-h incubation, components A and B of the Live/Dead imaging kit were mixed and added to each well, followed by a 15-min incubation. The plate was then put under a fluorescence microscope for observation and cell counting.

2.5. *In vitro virus infection*

VK2/E6E7 cells were seeded in three 4-well plates at a density of 50,000 cells per well and then incubated overnight at 37 °C in a 5% CO_2

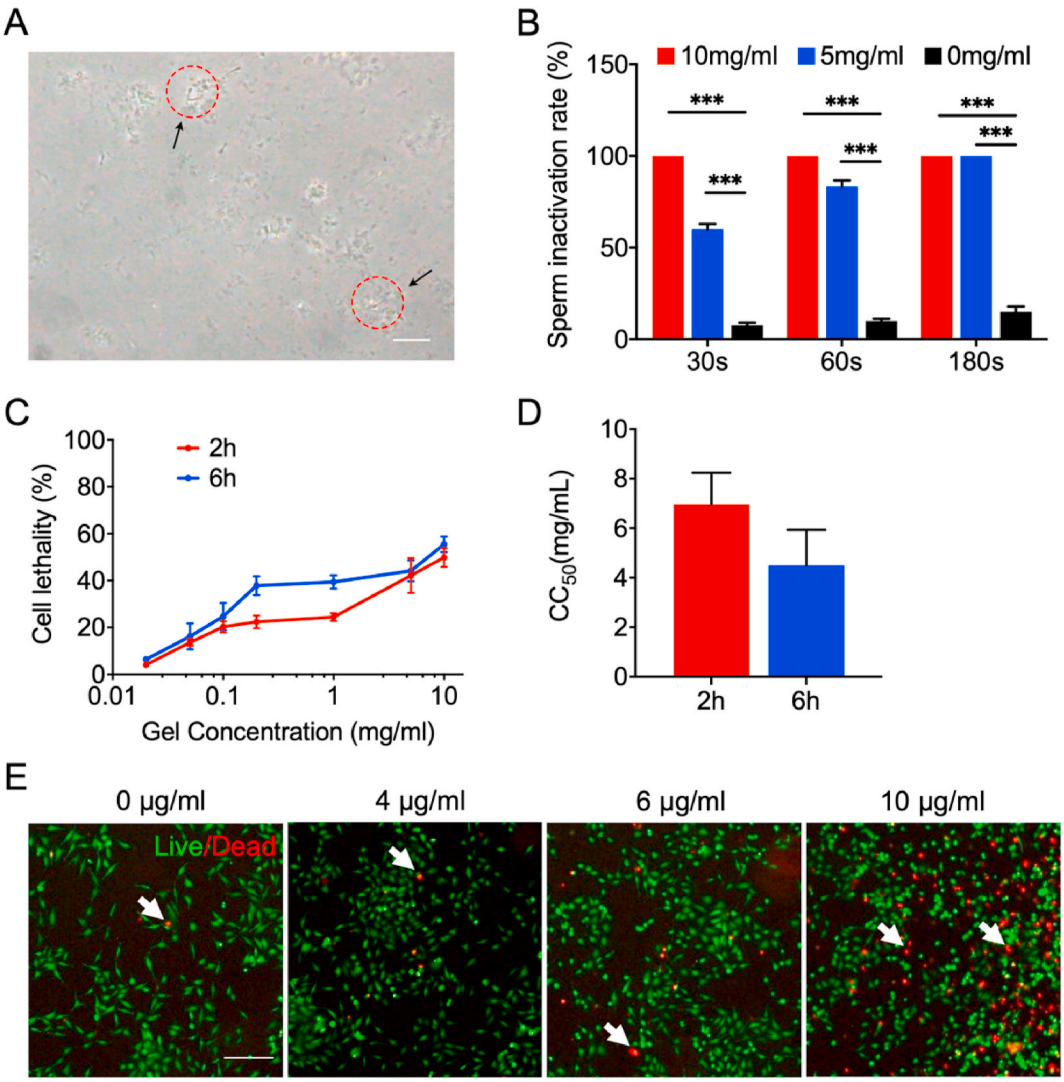

Fig. 2. *In vitro* evaluation of the spermicidal and cytotoxic effects of the TGN gel. **A.** Representative brightfield image of the morphological observation of sperm. Scale bar, 100 μm. **B.** Measurement of spermicidal efficiency of the contraceptive gel containing different concentrations of gossypol acetate. **C.** Measurement of cytotoxicity of the TGN contraceptive gel on VK2/E6E7 cells. **D.** Quantification of CC50 level of the TGN contraceptive gel for cytotoxicity. **E.** Representative live (green)/dead (red) staining images of the cells cultured with gossypol acetate at different concentrations. White arrows indicate dead cells (in red). Scale bar, 100 μm.

incubator. The gel was diluted in cell culture medium to achieve the following concentrations of tenofovir: 20 μg/ml, 40 μg/ml, 75 g/ml. Three of the wells were used to test each of the tenofovir concentrations stated, and one well was used as a blank control. The cell culture medium in the three test wells was replaced with tenofovir-infused gels diluted in medium. The cell culture medium in the last well was simply replaced with fresh cell culture medium. Then, GFP-Lentiviruses (Cellomics Technology LLC, Halethorpe, MD) were used to transduce the cells (MOI = 2). After incubating for 4 h to complete the lentiviral transduction, the cells were washed with PBS and then incubated in cell culture medium for another 48 h. After incubation, the fluorescence intensity of GFP was observed with a confocal microscope.

2.6. Preparation of vaginal smear and detection of the rat estrous cycle

Female SD rats (Charles River Laboratories, Wilmington, MA) were anesthetized via isoflurane inhalation, and then affixed to a rodent operating table. A small cotton swab, moistened with saline, was placed at the vaginal orifice, gently inserted into the rat's vagina, slowly rotated, and then slowly removed. The mucus picked up by the small cotton swab was evenly spread onto a glass slide to make a vaginal smear. It was dried and then stained with hematoxylin and eosin (H&E). Under the microscope, we determined the estrous cycle of female rats by the type and proportion of the cells observed.

2.7. Rat mating experiment

Female rats in the estrous phase were randomly divided into a treatment group and a control group, with 6 rats in each group. A total volume of 0.3 mL of contraceptive gel was placed in a 1 mL syringe and administrated into the distal vagina. The Contragel® group received Contragel administration by the same route. The rats in the control group received blank carbomer gel as a placebo. After the gel was applied, the rats were continuously monitored for 2 h for any vaginal leaks. Female rats and fertile male SD rats were placed in the same cage for mating (1:1). The termination of mating was determined by the presence of sperm in vaginal lavage fluid or the occurrence of the vaginal plug.

2.8. Recording the frequency of rat mating

The male rats were randomly divided into a treatment group and a

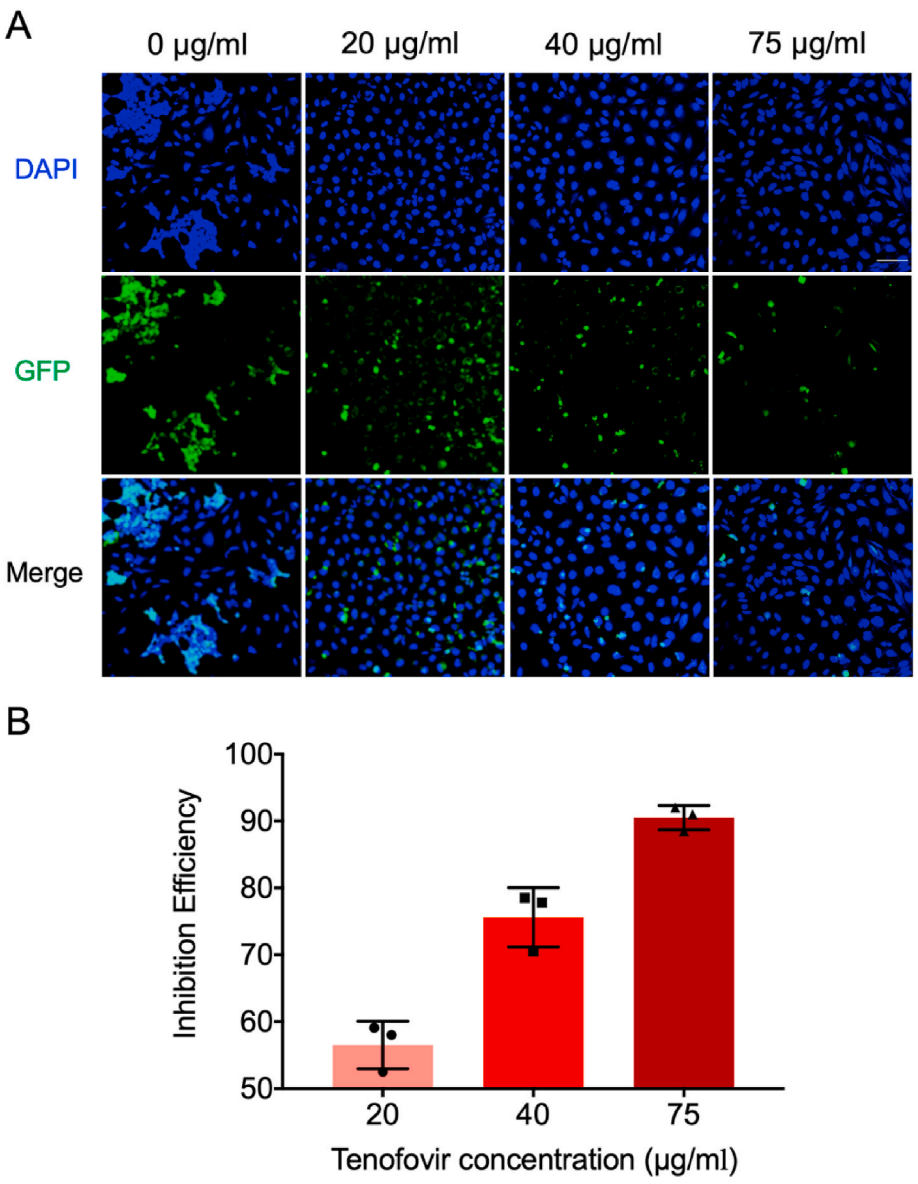

Fig. 3. Antiviral effects of the TGN gel. A. Representative fluorescence images of viral transfection under different concentrations of tenofovir. Scale bar, 100 μm. **B.** Quantitative analysis of the efficiency (%) of tenofovir inhibiting viral transfection.

control group, each consisting of six rats. The gel containing 1% nitroglycerin or the blank carbomer gel was applied to the surface of the rat penises in the treatment group or the control group, respectively. Five minutes after administration, the male rats were placed in mating cages alone to give them time to adapt to the new environment. After another 5 min, an adult female rat was added to each cage. The following indicators were recorded: (1) The incubation latency, which is the span of time from when the female rat is added to the cage to when the male mated with the female for the first time; (2) The number of erections, which is counted as the number of times the male rat pounced on the female rat, or the number of visible times that the male rat had penile erections (penis exposed over the foreskin) over a 20 min period.

2.9. Rats blood tests

After administration of the gel overnight, whole blood and serum from all female rats in the gel group and the control group were harvested for a biochemical combination test and a complete blood count.

2.10. Histology

This methodology was taken from our previous study. For immunohistochemistry, rat vagina were first cut into three parts (top, middle, down) for cryosection. Cryosections were fixed with 4% paraformaldehyde. Permeabilization and protein blocking were done with protein block solution (Dako, Carpinteria, CA, USA) containing 0.1% saponin (Sigma-Aldrich, St. Louis, MO, USA). Proteins of interest in the samples were targeted with the following primary antibodies after an overnight incubation at 4 °C: rabbit anti-Myeloperoxidase (MPO) (1:100; PA5-16672, Invitrogen, Carlsbad, CA, USA), rabbit anti-CD45 (1:100; ab10558, Abcam). Primary antibodies were conjugated with Alexa Fluor® 594 (1:200; ab150080, Abcam). DAPI (Life Technologies, Carlsbad, CA, USA) was used for nuclear staining. Images were taken with an Olympus epifluorescent microscope.

For H&E staining, sections were fixed in hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature, and then rinsed for 2 min in running water. The sections were then dipped in acid alcohol for 2s, in sodium bicarbonate (five dips), and in dehydrant (Richard-Allan Scientific, Kalamazoo, MI, USA) for 30 s. They were

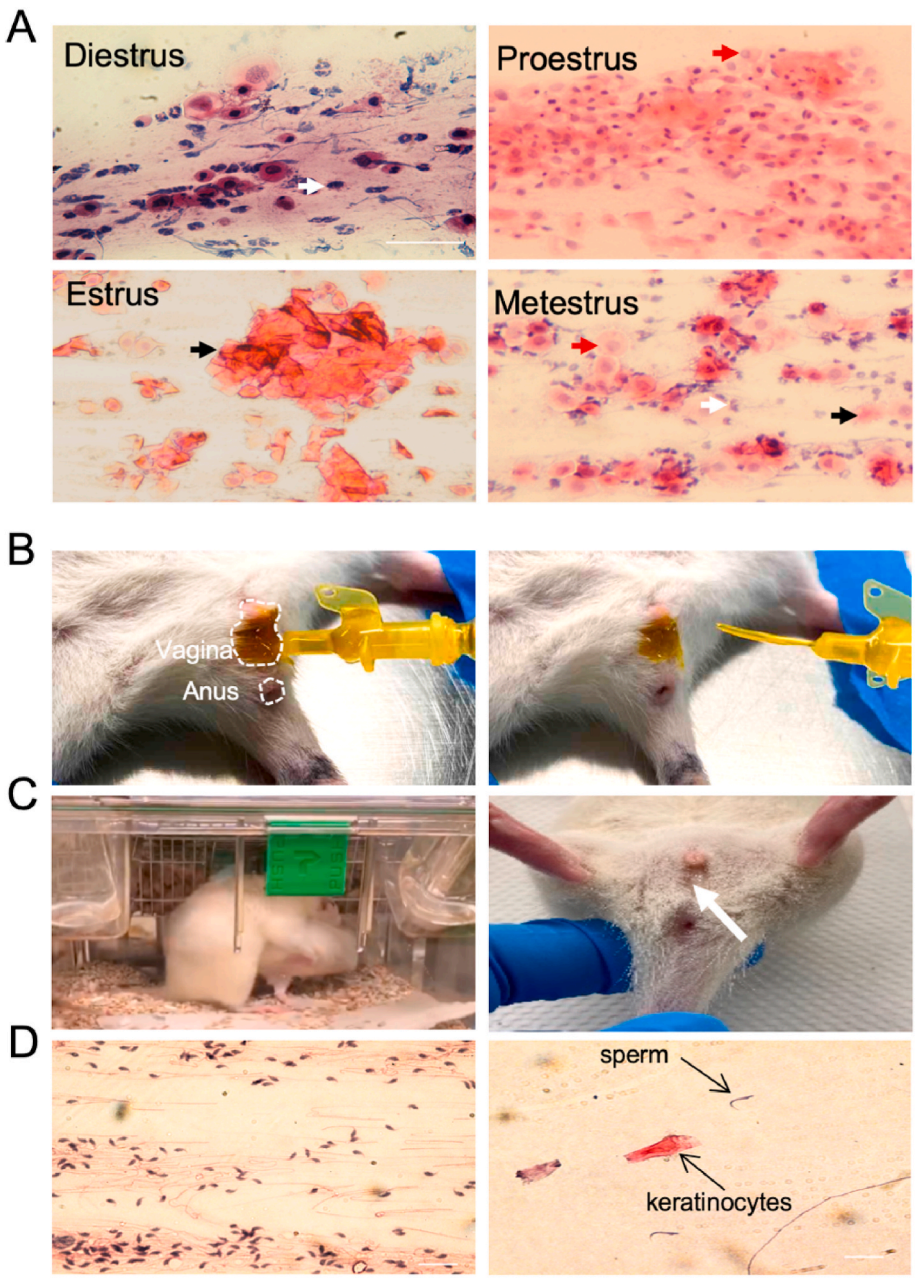

Fig. 4. Determination of estrus cycle and mating success. A. Representative H&E images of different estrus cycles in female rats. Red arrow indicates epithelial cell, black arrow indicates keratinized epithelial cell, white arrow represents leukocytes. Scale bar, 50 μm. **B.** Representative photos showing the intravaginal injection of the gel into female rats. **C.** Representative photo of mating behavior in rats and the observation of vaginal plugs (white arrow). **D.** Representative H&E images of morphology of standard rat sperm and rat sperm found in vaginal lavage fluid in female after mating. Scale bar, 50 μm.

subsequently submerged in eosin (Sigma-Aldrich, MO, USA) for 2 min and thoroughly washed in dehydrant and xylene (VWR, Radnor, PA, USA).

2.11. Statistical analysis

Statistical analysis were performed by Graphpad Prism 8 (GraphPad Software, California, USA). Cell numbers were counted using Image J software. All experiments were performed independently at least three times. Results are shown as means ± standard deviation (SD). Comparisons between any two groups were performed using the two-tailed, unpaired Student's *t*-test. Comparisons between more than two groups were performed using the one-way analysis of variance (ANOVA), followed by the post hoc Bonferroni test. We assume equal variance in all of the statistical analyses. Single, double, and triple asterisks represent $P < 0.05$, 0.01, and 0.001, respectively; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation and characterization of the TGN gel

To make a trifunctional contraceptive gel, three functional ingredients of gossypol-acetic acid, tenofovir and nitroglycerin are prescribed in the protocol (Fig. 1A), and the functions of the gel in decreasing sperm motility, antiviral activities and promoting libido and erection were evaluated (Fig. 1A). As shown in the SEM micrograph (Fig. 1B), we observed that the gel was in a compact and porous structure that is benefit for degradation and clearance in the vagina. As shown in the figure (Fig. 1C), a yellow trifunctional contraceptive gel was prepared. At room temperature, when settled at the bottom of a small glass vial that is subsequently turned upside down, the gel does not fall, demonstrating the required viscosity and rigidity for its application.

Rheology is an important parameter to study the way in which materials deform or flow in response to applied forces or stresses [45], and well-characterized rheological properties of the gel will definitely make

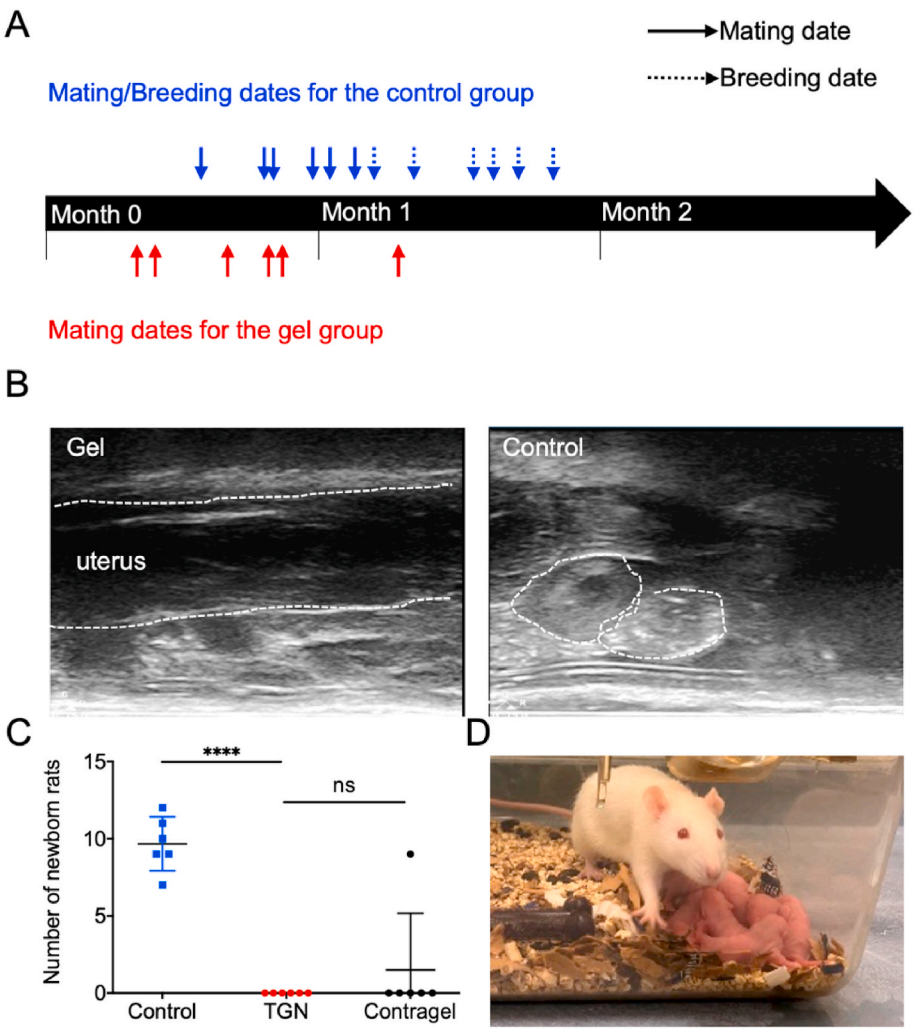

Fig. 5. The contraceptive effects of the TGN gel. A. Timeline of the mating assessment. **B.** Representative ultrasound images of the uterus of female rats in the gel group and control group (fetuses were highlighted in circles). **C.** Quantification of the number of newborn rats in the TGN gel group, Contragel® group and control group (all groups passed the mating assessment) (n = 6). **D.** Representative photo of newborn rats in the Contragel® group.

sense to the feeling or quality. The dependence of the gel's viscosity on shear rate, a key rheological property, is shown (Fig. 1D). These are the steady shear viscosities calculated from the torque measured after 30 min of continuous shear. The gel exhibited shear thinning behavior over the range of shear rates studied, with the viscosity reduced by about two orders of magnitude as the shear rate was increased from 0.01 to 1000 s. The slope of the curve was relatively stable, indicating that the gel was a non-Newtonian fluid. In addition, the strain amplitude sweep tests were first performed to identify the linear viscoelastic region (LVE) for each sample and then frequency sweep tests were performed only within the LVE. In both tests, storage modulus (G') is higher than loss modulus (G'') (Fig. 1E and F), revealing a gel-like behavior of the TGN gel. Interestingly, we also found that the gel at pH = 4.0 and pH = 6.0 showed higher storage modulus than gel at pH = 8.0 (Fig. 1E and F), which reflected the strength of the gel. Similarly, the gel at pH = 4.0 and pH = 6.0 also had higher loss modulus when compared to the other group (Fig. 1E and F), demonstrating better viscoelasticity of the gel in acidic environment. The rheological properties of the gel demonstrate that it would be a good sexual lubricant when its pH is adjusted to 4.5 (similar to intravaginal environment), which will help enhancing the quality of intercourse for both male and female. Moreover, when the gel reaches the distal vagina, it will not flow out of the vagina on its own. Instead, unless mechanically forced out, it will stay in place, which is important if it is to be used as a contraceptive gel in the vagina.

3.2. Spermicidal effects of the TGN gel on pig sperms

The gossypol acetate infused carbomer gel had a significant inhibitory effect on pig sperm (Fig. 2). Under the microscope, we observed that when sperm cells made contact with the gel, they lost their forward-moving motility (Fig. 2A), which demonstrated that the gel not only had an inhibiting effect on sperm, but also had a spermicidal effect. Moreover, the spermicidal intensity of the gel increased with higher concentrations of gossypol acetate, showing a dose-dependent relationship (Fig. 2B). When we compared it with the blank control group without gossypol acetate, the difference was statistically significant ($P < 0.05$).

3.3. In vitro safety of the gel to vaginal epithelial cells

The results of the cck8 cytotoxicity assay were shown (Fig. 2C). After two time points, 2 h and 6 h of incubation with the gel, the viability of VK2/E6E7 cells remained stable. The CC50 value confirmed that the working concentration of the gel in vagina (approximately 2 mg/ml) is significantly less than the concentration (6.975 mg/ml) that could cause 50% cytotoxicity (Fig. 2D). Verified with fluorescence microscopy, the Live/Dead assay yielded large areas of live cells (green) and few dead cells (red) (Fig. 2E). While the proportion of dead cells increased with the concentration of the gel in the medium, it had a minimal effect on

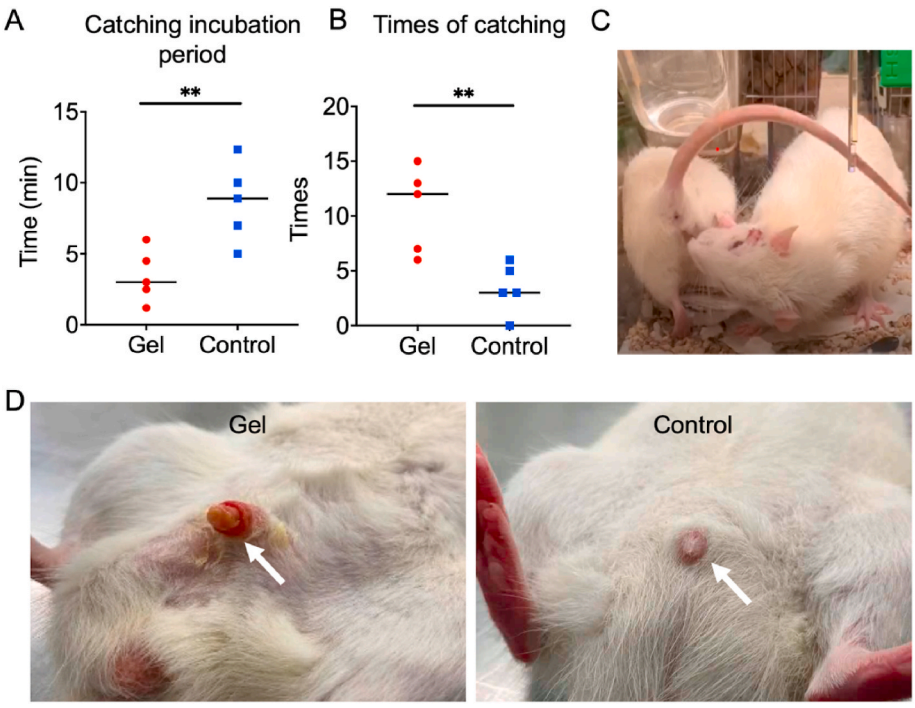

Fig. 6. Improvement of libido and male erectile function after gel application. A. Quantification of catching incubation period in both groups. **B.** Quantification of the times of catching in both groups (monitored during the first 20 min after the males and females were place in the same cage). **C.** Representative photo of mating behavior of males (right) and females (left). **D.** Representative photos of the erectile function of male rats penis compared to the control.

cell viability at low concentrations. At lower concentrations, the rate of increase in cell death was also relatively slow, showing safety and translation feasibility.

3.4. Inhibitory effect of TGN gel on viral infection

After having undergone GFP-lentiviral transduction, VK2/E6E7 cells expressed different levels of fluorescence intensity (Fig. 3A). We counted the percentage of cells expressing GFP as a fraction of the total number of cells, and used this to measure the degree viral infection. We took the degree of infection of the blank group (without gel) as the negative control and calculated the inhibitory efficiency of gel dilutions containing different concentrations of tenofovir on viral transduction (Fig. 3B). The curve suggested that within a certain range, as the concentration increased, the inhibitory efficiency also increased significantly, which indicated the potential of the gel to become a drug release system. This system is likely able to interfere with the viral infection process by releasing functional agents, and has the potential to prevent STD transmission during sexual intercourse.

3.5. Estrus cycle detection is used for determination of mating

Following spermicidal and anti-virus effect, we moved forward to the contraceptive effects of our gel. We performed vaginal smears of female rats at different stages of the physiological cycle and determined the rat estrous cycle based on the proportion of the observed cell types (Fig. 4A). If a large number of leukocytes and a small number of nucleated epithelial cells were observed under the microscope, then the rats were in the diestrus stage, which was not suitable for mating experiments. If a large number of elliptical nucleated epithelial cells were observed, accompanied by a small number of keratinocytes, then they were in the proestrus stage. If irregular keratinized epithelial cells, nucleated epithelial cells, and leukocytes were observed, and their proportions were equal, they were in the metestrus stage. When we observed a large number of irregularly shaped keratinocytes, they were in the estrus stage and ready for mating. Female rats at the estrus stage were selected as experimental subjects and had gel inserted in their vaginas. We observed that the gel continued to exist stably in the vagina for 2 h which proved the feasibility of its application. Rat sperm was obtained from the seminal vesicles of male rats. Through sperm smears, we determined the morphology of rat sperm under the microscope (Fig. 4D). Male and female rats were placed in the same cage for mating (Fig. 4C). The observation of a vaginal plug indicated successful mating (Fig. 4C). In addition, we collected vaginal lavage fluid and found male rat sperm (which had similar morphology as observed *in vitro*) (Fig. 4D), which further proved the successful mating of rats.

3.6. Evaluation of contraception effect of the TGN gel

Following estrus cycle detection, rats in the estrus phase were selected for mating experiments. Before mating, the gel was evenly applied in the rats' vaginas (Fig. 4B), which effectively inhibited sperm motility after mating. After 22 days after successful mating, there was no pregnancy recorded in the gel group (Fig. 5A). By contrast, the rats in the control group all completed normal delivery within one trimester of pregnancy after mating (Fig. 5A). A We used ultrasound imaging to monitor their pregnancies after successfully mating. In the female rats that were given the gel, the ultrasound images of the uterus did not show any pregnancy, while in the control group, the ultrasound images showed the existence of fetuses (Fig. 5B). A commercial product Contragel® was also applied to rats as a comparison and we did not observe any pregnancy cases in the gel group but observed one pregnancy case in the Contragel® group after successful mating (Fig. 5C and D). This comparison indicated the effective contraceptive function of the gel.

3.7. Improvement of male libido and erectile function by TGN gel

Coinciding with the contraception, the libido and male erectile function of rats was enhanced, which adds more actions to the gel. In a separate animal experiment, the gel was applied to the penis of male rats to determine its effect on male libido and erectile function. After application, the male and female rats were in the same cage (Fig. 6C).

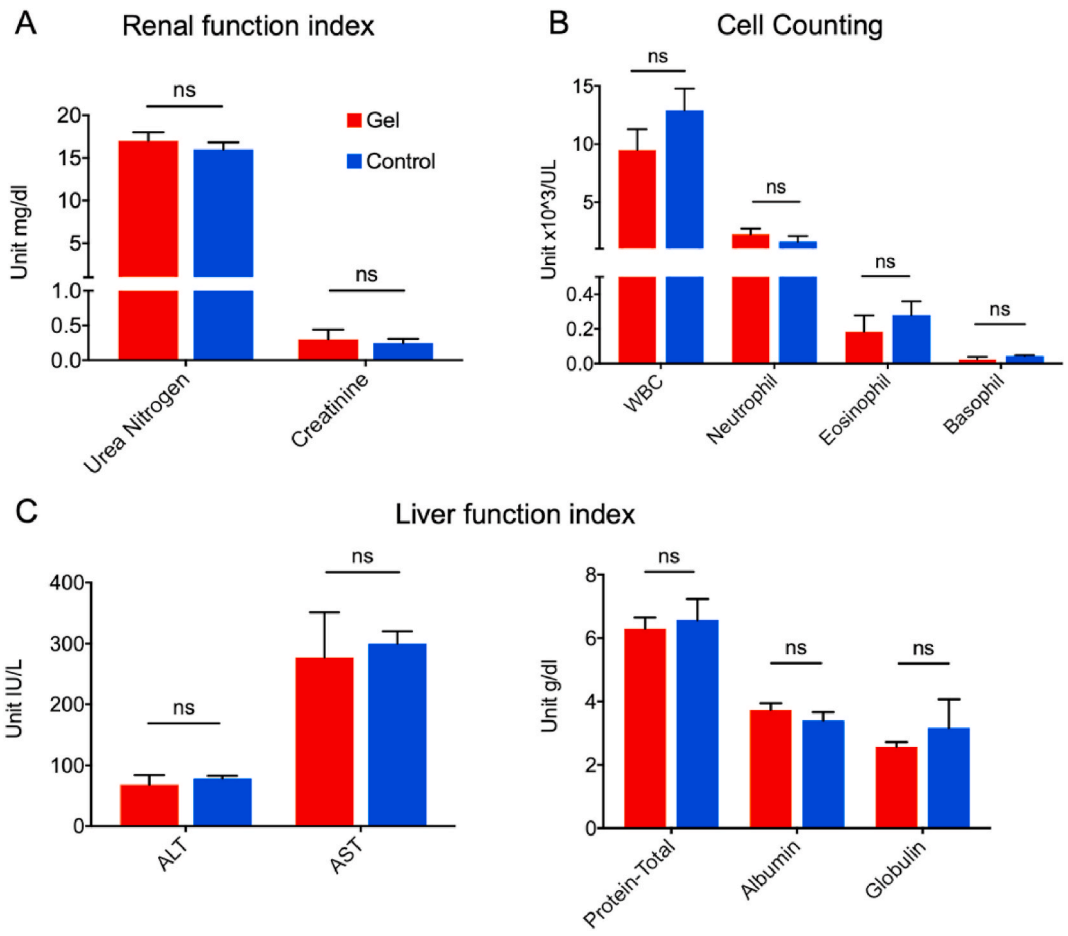

Fig. 7. Toxicity studies on the TGN gel. A. Quantative analysis of renal function indices in rat serum in both groups. **B.** Quantative analysis of inflammatory response indices in rat whole blood in both groups. **C.** Quantative analysis of liver function indices in rat serum in both groups. (ALT: Alanine aminotransferase; AST: Aspartate aminotransferase).

Our gel, containing 1% nitroglycerin, helped the male penis become fully erect (exposed over the foreskin) (Fig. 6D). There was visible difference between the signs of erection and non-erection in rats. In addition, the use of our gel significantly shortened the incubation period for male rats (amount of time the male took to mate once placed in a cage with a female), and increased the number of times male rats mated with females, compared with the rats without gel application (Fig. 6A and B), indicating the enhancement of male libido. $P < 0.05$.

3.8. Biosafety studies on the TGN gel application in vivo

Determined by complete blood count test, the liver and kidney function indexes of the female rats exposed to the gel overnight were not significantly different from those of the control group, indicating that the gel had not been metabolized by the rats' liver and kidneys, or that it had no effect on liver and kidney function (Fig. 7A and C). The number and type of leukocytes in the experimental group were not significantly different from those in the control group, which proved that the use of the gel did not cause acute or chronic inflammation in the rats (Fig. 7B). Furthermore, after repeated local application (5 times in 10 h) of the TGN gel to rat's vagina, similar numbers of $CD45^+$ leukocytes or myeloperoxidase (MPO) positive neutrophils were found in the vagina at each position (top, middle, down) between the TGN gel and the control group, indicating minimal immunogenicity of the gel to the vagina (Fig. 8A and B). Also, H&E images showed no significant differences in leukocyte numbers between the groups. Thus, the use of the gel in rats is safe.

4. Discussion

Currently, marketed contraceptive gel products do not have high efficacy rates [4]. Gossypol, extracted from cotton, has been used to make oral contraceptives for men, and gossypol derivatives, especially gossypol acetate, have been shown to effectively reduce sperm activity *in vitro* [21–23]. However, it was shown to damage the seminiferous epithelium and possibly lead to lifelong male infertility in oral administration [46,47]. This fact has inspired us to turn internal medication into external medication to minimal the side effects and develop the idea of designing a new type of contraceptive gel with gossypol acetate as the main component. *In vitro* experiments showed that our contraceptive gel's spermicidal efficacy is clearly correlated to the concentration of gossypol acetate used.

We chose carbomer as the base material for fabricating the gel mainly because the thickening effect of carbomer is related to the pH value [41–43]. Specifically, this material can form a gel at pH 4.5, which is the same as the normal pH value in the vaginal environment. Thus, this property can effectively prevent the imbalance of the vaginal flora, or the destruction of the vaginal microecological balance, caused by pH changes. At present, there are many studies devoted to developing contraceptive tools that have multiple functions during sexual intercourse [48]. Among them, the prevention of STDs has always been the focus of attention. Most STDs are caused by viruses invading the reproductive organs locally, including HIV and Herpes Simplex Virus (HSV) [49,50]. Therefore, we use hydrogel as a topical drug release system, and infuse it with FDA-approved tenofovir fumarate. *In vitro* experiments proved that under the effect of tenofovir, the degree of viral

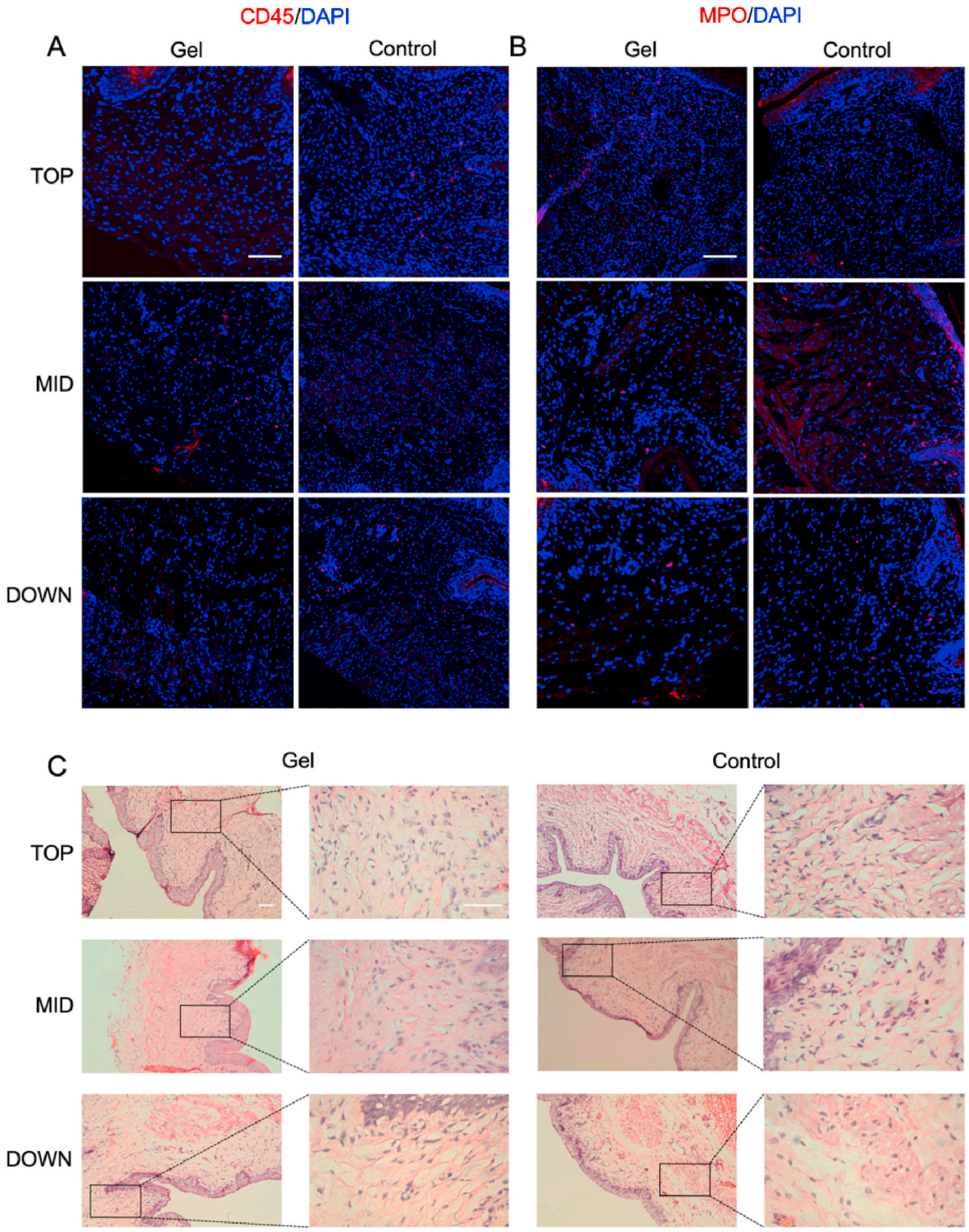

Fig. 8. Biocompatibility and immunogenicity studies on the TGN gel. A. Representative fluorescence images of inflammatory response (leukocytes) indicated by $CD45^{+}$ at different positions (top, middle, down) on the vagina in both groups. Scale bar, 100 μm. **B.** Representative fluorescence images of inflammatory response (neutrophils) indicated by MPO at different positions (top, middle, down) on the vagina in both groups. Scale bar, 100 μm. **C.** Representative H&E images of inflammatory response indicated by leukocytes at different positions (top, middle, down) on the vagina in both groups. Scale bar, 100 μm.

infection in human cervical cells was significantly reduced.

Considering the quality of sexual intercourse, we use nitroglycerin as another topical agent. This medicine can locally improve blood flow through the cGMP mechanism after transdermal diffusion. Therefore, nitroglycerin is added to the gel to improve the erection of male rats. After local application on the male genitals, the gel has the function of keeping the penis fully erect during the sexual intercourse, which could potentially improve the quality of human intercourse activity as well. Notably, in addition to enhancing erection function physiologically, the application of our TGN gel also led to significant improvement on the libido of male rats which can be simply defined as sexual desire. Increased libido was indicated by the shortening of incubation period for

male rats (from the timepoint when a male rat was placed in a cage with a female, to the timepoint when he started to mate), as well as increased number of times that male rats mated with females. In clinics, erectile dysfunction and decreased libido are independent concepts but they have some overlap to some extent [51]. Decreased libido plays a causative role in some cases of ED, but most cases of ED are primarily vasculogenic [51]. Decreased libido may be the result of androgen deficiency, but it could also be psychogenic [51]. Therefore, we performed different experiments to examine the libido and erection respectively.

Regarding to biosafety of the gel, we first used *in vitro* cell study to proof the nontoxicity of the gel to vaginal epithelial cells at the timepoint of 2 h which was long enough, in our views, to mimic human intercourse activity. Then, for *in vivo* study, we conducted complete blood count test to ensure the normal function of liver and kidney after gel application to female rats. Regarding to immunogenicity which could possibly occur in biomaterials transplantation, we combined blood cell counting with histological evaluation, including IHC and H&E stainings. The numbers of leukocytes and neutrophils showed non-significant differences between gel group and control in all three approaches, indicating minimal inflammatory response in the rats both systematically and locally after gel application. Remarkably, in the histology study, rats received repeat gel application to the vagina (5 times in 10 h), which mimicked frequent human intercourse activity in real life. And still, rats' vagina did not show any inflammation. Generally, we took practical use into consideration in both *in vitro* and *in vivo* study and made the nontoxicity and biosafety of our TGN gel very solid.

There are several limitations to our study. First, the inhibitory effect on STDs was not verified in animal models, but only by *in vitro* lentiviral transduction, which cannot replicate the exact conditions present during *in vivo* viral infection. What is more, the improvement of sexual quality cannot be quantified or even evaluated since no clinical trials were conducted. But what we can confirm is that the application of the gel enhances the male libido and erection, which lessens the risk of male erectile dysfunction, which would otherwise decrease the quality of sexual intercourse.

To summarize, the TGN contraceptive gel we created yielded higher contraceptive success rates than those on the market, and was formulated with the added benefits of protecting against STDs and improving male erectile function during sexual intercourse. Combining three FDA-approved and marketed agents together, our trifunctional contraceptive gel has great potential for translational, off-the-shelf applications.

CRediT authorship contribution statement

Junlang Li: Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing. **Sichen Zhang:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing. **Dashuai Zhu:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing. **Xuan Mei:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing. **Zhenzhen Wang:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing. **Xiao Cheng:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing. **Zhenhua Li:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing. **Shaowei Wang:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing. **Ke Cheng:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Software, Validation, Writing - review & editing, Supervision.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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All authors have contributed to the writing of this manuscript. All authors have approved the final version of this manuscript. Rheology study was completed with the help of Dr. Haotian Zheng and his research group at North Carolina State University. Final grammatical edits were completed by Jhon Cores, Ph.D.

Ethical statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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