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Tideglusib, a prospective alternative to nonoxynol-9 contraceptive

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ABSTRACT

Objectives: We aimed to evaluate the antifertility activity and vaginal irritation effects of tideglusib in vivo using rabbit models and to evaluate the cytotoxical effects of tideglusib to sperm, vaginal cells and vaginal bacteria (*L. acidophilus*) in vitro.

Study design: We treated female rabbits with vaginal tideglusib 1 mM, nonoxynol-9 (N-9) or vehicle control (Poloxamer 407). In experiment 1, we sacrificed females ($n = 6$ each) after 10 days of daily administration and assessed vaginal histological changes using Eckstein irritation score. In experiment 2, females ($n = 9$ each) received estradiol benzoate to induce ovulation 24 h prior to vaginal treatment followed by introduction of a fertile male. These females underwent necropsy at the 21st day to assess pregnancy status. In experiment 3, we used an HTM-TOX IVOS sperm motility analyzer and scanning electron microscopy (SEM) to evaluate the effect of tideglusib on human sperm samples. In experiment 4, we evaluated the effect of tideglusib on lactobacillus and vaginal cell growth in vitro.

Results: The total irritation score of tideglusib vs. N-9 was 3.4 ± 2.07 vs. 7.8 ± 3.82, $p<.05$. The pregnancy rate of tideglusib, N-9 and control group was 11.1%, 0% and 88.9%, respectively. Tideglusib exhibited a dose-dependent spermostatic/spermicidal activity, and the minimum effective concentrations of tideglusib and N-9 were 8.724 ± 3.047 μM and 219.75 ± 41.78 μM, respectively. SEM and transmission electron microscopy revealed acrosomal membrane impairments caused by tideglusib. Tideglusib was much less toxic to vaginal cells and *L. acidophilus* than N-9 in vitro.

Conclusions: Evaluation using rabbit models indicated that tideglusib is a prospective spermicidal contraceptive with low vaginal irritation effects.

Implications: Tideglusib or tideglusib analogues may be a contraceptive with perspective to replace N-9. It is possible for a spermicide to balance spermicidal activity and vaginal/cervical irritation effects very well.

1. Introduction

Safe contraception is an unmet need, resulting in health burdens. Almost 10% of fertile women in China have unintended pregnancy [1], and 51% of pregnancies annually in the United States [2] are unintended. In China, between 2005 and 2012, heterosexual transmission of human immunodeficiency virus (HIV) among infected women surged from 25.8% to 87.4%, with the majority of reported cases in the 20–39 age groups [3]. A woman's choice of contraception can affect her risks for both unintended pregnancy and sexually transmitted infections (STIs), especially HIV transmission during sexual contact with her infected partners. Unfortunately, most contraceptives do not protect women from HIV infection or other sexually transmitted diseases (STDs).

Spermicides, designed to prevent fertilization by killing or inactivating sperm through chemical toxic (most commonly nonoxynol-9, N-9) to sperm and a formulation, have been used alone or with barrier products [4,5], for example, condoms to prevent HIV infection [6]. However, the high incidence of unwanted pregnancy (Pearl index for ideal use: 18) [7] associated with spermicidal method is one of its disadvantages. Although N-9 had protective effects against STDs [8] and HIV [9], recent clinical trials showed that spermicide actually increased urinary tract infections among spermicide-exposed women [10], and frequent use of N-9 even increased the risks of rectal transmission of HIV and other STIs through genital lesions (mucosal

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inflammation) [11,12]. Thus, WHO considered spermicide a category 4 method (contraindicated) for women at high risk of HIV infection.

However, spermicides have important advantages. At present, young, urban women in China (north or southwest) prefer user-controlled methods [13]. Spermicides as readily available, over-the counter, woman-controlled contraceptive methods, highly acceptable to most women [14], may meet the need. Because spermicide use is not influenced by acceptability [15], spermicide may be considered as a route of administration for contraception for women, especially suitable for perimenopausal [16] and breastfeeding women [17].

The major challenges are to improve protective effects of spermicides and maintain the balance between contraceptive efficacy and disturbance to the vaginal environments [18]. Therefore, only spermicides with higher efficacy and fewer adverse effects than N-9 would be novel alternatives. We identified tideglusib (Fig. 2A, Cas 865854-05-3), a selective and irreversible non-ATP competitive inhibitor of glycogen synthase kinase 3β (GSK-3β) [19], as a promising compound by screening >7000 compounds. The objectives are to evaluate the antifertility activity and vaginal irritation effects of tideglusib in vivo and the cytotoxical effects to sperm, vaginal cells and vaginal bacteria (*L. acidophilus*) in vitro.

2. Materials and methods

2.1. Reagents and cell lines

The bioactive compound library screened and tideglusib was purchased from Selleck Co., Ltd., (Houston, TX, USA). N-9 was obtained from Xi'an Ruilin Biotechnology Co., Ltd.2, (Shanxi, China). A Live/Dead sperm viability kit (L-7011) was purchased from Invitrogen, Life Technologies Ltd., (Paisley, UK). The cell counting kit-8 (CCK-8) was from Dojindo, Co. Ltd., Japan. Defined keratinocyte serum-free medium (GibcoTM10744–019) was from Thermo Fisher Scientific Inc., Waltham, MA, USA.

The human vaginal epithelial Vk2/E6E7 cell line (ATCC CRL-2616) was a gift from Professor Wenliang Zhou at Sun Yat-sen University, Guangzhou, China. The cells were cultured in keratinocyte serum-free medium supplemented with epidermal growth factor (0.01 ng/mL) and penicillin (100 U/mL) in the incubator at 37 °C with 5% CO_2.

2.2. Tideglusib-loaded vaginal gel

Add poloxamer 407 to distilled water with uniform stirring and store at 4°C until the polymer dissolved completely to prepare 20% poloxamer 407 (*w*/*v*) gels. Mix the prepared gel and tideglusib stock solution in DMSO with constant stirring to obtain a colorless transparent gel at 1 mM (approx. 100× minimum effective concentration [MEC]), as the highest solubility of tideglusib in the present gel was approximately 100× MEC (1 mM).

2.3. Antifertility and vaginal irritation effects of tideglusib gels

The animal use and welfare committee of SIPPR approved all experiments. We used breeding rabbits with proven fertility and divided the females into three groups receiving treatments as follows: vehicle only (Poloxamer 407, 20%), tideglusib gel at 1 mM (approx. 100× MEC) and N-9 gel at 40 mg/mL. We inserted a catheter-equipped 5-mL disposable syringe 8–9 cm into the orificium of rabbits to administer 2.5-mL gel intravaginally.

2.3.1. Experiment 1

After daily administration of the vaginal gels for 10 days, we euthanized the female rabbits ($n = 6$/group) and removed the vaginas and uteri. We longitudinally incised each rabbit vagina, observed the morphological changes in the tissue, and then fixed the tissues in PBS containing 10% formaldehyde at 25°C for 4 h, dehydrated in graded ethanol solutions and embedded in paraffin. We stained paraffin sections with hematoxylin and eosin (H&E) followed by observation under a light microscope (Nikon 80i, Japan) and assessments of the Eckstein irritation score [20].

2.3.2. Experiment 2

We hypodermically injected 0.1 mg/kg estradiol benzoate to induce ovulation (females, $n = 9$/group) 24 h prior to the vaginal treatment. Ten minutes after the gel administration, we allowed females to monogamously mate with fertile males. On day 21, we euthanized the females and conducted exploratory laparotomies to observe ovulation points, implantation sites and fetus and then calculated pregnancy rates.

2.4. Spermostatic/spermicidal effects of tideglusib on human sperm in vitro

The Institutional Ethics Committee of Shanghai Institute of Planned Parenthood Research (SIPPR, Shanghai, China) approved this study, and all participants provided the informed consent. According to established guidelines (WHO manual 5th edition, 2010), we analyzed human semen [21] using a HTM-TOX IVOS sperm motility analyzer (Hamilton Thorne Research, Beverly, MA, USA) to select samples with $>15 \times 10^6$ mL sperm counts and >70% motility with normal sperm morphology for subsequent assays. We collected the "swim-up" spermatozoa (WHO manual 5th edition, 5.4.2 procedure) [21] and diluted in pre-equilibrated Biggers–Whitten–Whittingham (BWW) medium to $10–20 \times 10^6$ cells/mL. To prepare mouse sperm, we euthanized male C57BL/6 mice and squeezed the cauda of the epididymis with tweezers to release the sperm [22]. We cultured the sperm in 1 mL prewarmed BWW medium (37 °C) for 15 min and diluted the removed suspension of uppermost sperm to $4–10 \times 10^6$ cells/mL.

2.4.1. Experiment 3

In this study, we defined the MEC of a compound as the lowest concentration that irreversibly immobilizes sperms within 20 s. Using a modified Sander–Cramer assay, we mixed the motile spermatozoa (5-10×106/mL) with BWW medium (v/v,1/1) containing diluted tideglusib for 20 s to determine the MEC. Then, we washed the immotile sperms twice and resuspended in fresh BWW medium to observe the revival at 37°C for 60 min.

To prepare SEM samples, we spread sperm on slides, fixed with 2.5% glutaraldehyde for 2 h, washed in PBS, fixed with 1% osmium tetroxide and then dehydrated using a graded acetone series. After critical point drying, we coated the samples with gold and observed under a field emission SEM (FEI Quanta FEG 250, Thermo Fisher Scientific Inc.). To prepare transmission electron microscopy (TEM) samples, we fixed human sperm precipitates, then washed, dehydrated and embedded them in EP812 for 48 h. We stained the sliced ultrathin sections and examined by TEM (Philips CM 120, Netherlands). We also used hypo-osmotic swelling (HOS) test (WHO Manual 2010, 2.6.3 procedure) [21] and flow cytometry (BD LSR II, BD Biosciences, San Jose, CA, USA) with a Live/Dead sperm viability kit to evaluate sperm viability and membrane integrity.

2.5. Cytotoxicity of tideglusib in vitro

2.5.1. Experiment 4

We cultured *L. acidophilus* (ATCC 4356) in De Man, Rogosa and Sharpe agar (MRS) medium (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 36 h and diluted to 1.0×10^6 CFU/mL and then incubated the diluted suspensions with N-9 or tideglusib in MRS medium. We then diluted the mixture and spread on the MRS agar plates before incubation under anaerobic condition at 37°C for 48 h.

We co-incubated Vk2/E6E7 cells (5×10^3 cells/well) with nurture medium containing compounds for 24 h and quantified the cell proliferation using a CCK-8 kit and a microplate reader (BioTek Instruments Inc., VT, USA) [23]. Then, we calculated according to the formula: (%)

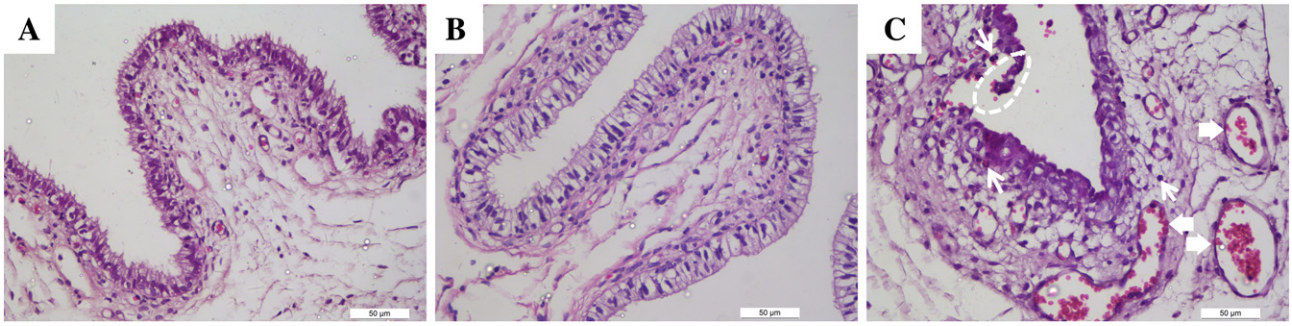

Fig. 1. Light microscopic changes in rabbit vagina. H&E staining of histological sections of rabbit vaginal mucosa after being consecutively exposed to gels for 10 days. Representative light micrographs of sections of rabbit vaginal tissue ($n = 6$) after a 10-day intravaginal administration of gel (A) alone, (B) gel with tideglusib or (C) gel with N-9. Note the intactness of vaginal epithelium and only a small number of vascular congestion in control and tideglusib-treated rabbit vaginal mucosa. The dashed circles indicate the ulceration of the epithelial cell layers. The thick arrows indicate vascular congestion, and thin arrows indicate increased leukocyte infiltration in the N-9 gel group (original magnification ×400). The detailed pathological scores were shown in Table 1.

Table 1
Histopathologic scores of rabbit vagina

Groups	Vaginal mucosal involvement score				Total scores
	Epithelial injury	Leukocyte infiltration	Vessel congestion	Interstitial edema	
Control	0 ± 0	0.8 ± 0.27	0.1 ± 0.22	0.5 ± 0.5	1.4 ± 0.82#
Tideglusib group	1 ± 1.06	1.1 ± 0.65	1 ± 0.61	0.3 ± 0.27	3.4 ± 2.07#
N-9 group	2 ± 1.58	1.6 ± 1.56	1.8 ± 0.84	2.4 ± 1.34	7.8 ± 3.82*

0 = no obvious change, 1 (minimal), 2 (mild), 3 (moderate) to 4 (marked irritation).
The data are presented as mean ± SD of three independent experiments; $n = 6$ rabbits in each group.
* $p<.05$ vs. blank control.
# $p<.05$ vs. N-9 group.

=[(AC − Ab) − (AS − Ab)]/(AC − Ab) × 100, where AS, AC and Ab are the average OD of the experimental, control and blank wells, respectively.

2.6. Statistical analysis

Statistical analysis was performed with the Graphpad prism software using one-way ANOVA (HOS, cytotoxic effects) and nonpaired Student's *t* tests (MEC) as appropriate. The analysis of ovulation points, histopathologic scores of rabbit vagina (one-way ANOVA analysis) and implantation sites, and survived fetus (Kruskal–Wallis test) was performed with SPSS software. Quantitative data were expressed as mean ± SD. $p<.05$ was considered statistically significant.

3. Results

3.1. Pathological changes in rabbit vagina

Consecutive intravaginal exposure of rabbits to tideglusib for 10 days did not result in significant microscopic abnormalities of vagina tissues. The light microscopy examination revealed intact vaginal epithelium, lack of leukocyte influx and slight vascular congestion in the representative vaginal sections of rabbits receiving gel alone (Fig. 1A) or gel with tideglusib (Fig. 1B). However, ulceration of the epithelial cell layers, vascular congestion, submucosal edema and increased leukocyte infiltration (Fig. 1C) were prominent in N-9 group (a positive control). Accordingly, the total pathological score of tideglusib group (3.4 ± 2.07) was lower than N-9 (7.8 ± 3.82) ($p<.05$) but not significantly different from negative control (1.4 ± 0.82), as shown in Table 1.

3.2. Contraceptive efficacy of vaginal tideglusib in rabbits

No significant difference regarding ovulation rates was observed among the three groups except the implantation rates and pregnancy rates (Table 2). In contrast, both active treatments significantly reduced implantation sites (tideglusib 0.3 ± 1, N-9 0) compared to control (3.1 ± 2.4) and prevented pregnancy [tideglusib 1/9 (11.1%), N-9 0/9 (0%), control females 8/9 (88.9%)] ($p<.01$). Although intravaginal administration of tideglusib in rabbits effectively reduced pregnancy rate, N-9 appears better in contraceptive efficacy in their present formulations.

3.3. Novel spermostatic/spermicidal effects of tideglusib on human sperm

Both tideglusib and N-9 exhibited immobilizing activity in a dose-dependent manner (Fig. 2C–F′). The mean MECs of tideglusib (Fig. 2B–B′) were 8.724 ± 3.047 μM ($n = 4$, human) and 15.50 ± 4.107 μM ($n = 5$, mouse), which were lower than those of N-9 at 219.75 ± 41.78 μM ($n = 4$, human) and 310.80 ± 81.44 μM ($n = 5$, mouse). Tideglusib was more effective than N-9 in vitro. No revival of human

Table 2
Comparison of the contraceptive activity of tideglusib gel and N-9 gel in rabbits ($n = 9$)

Groups	Number of animals	Ovulation points	Implantation sites	Pregnancy	Survived fetus	Pregnancy rates (%)
Control	9	5.3 ± 2.5	3.1 ± 2.4	8	28	88.9
Tideglusib group	9	4.1 ± 1.9	0.3 ± 1.0**	1	3	11.1
N-9 group	9	4.8 ± 2.3	0.0 ± 0.0**	0	0	0

The data shown are the mean from three independent experiments and are presented as mean ± SD of three determinations.
** $p<.01$.

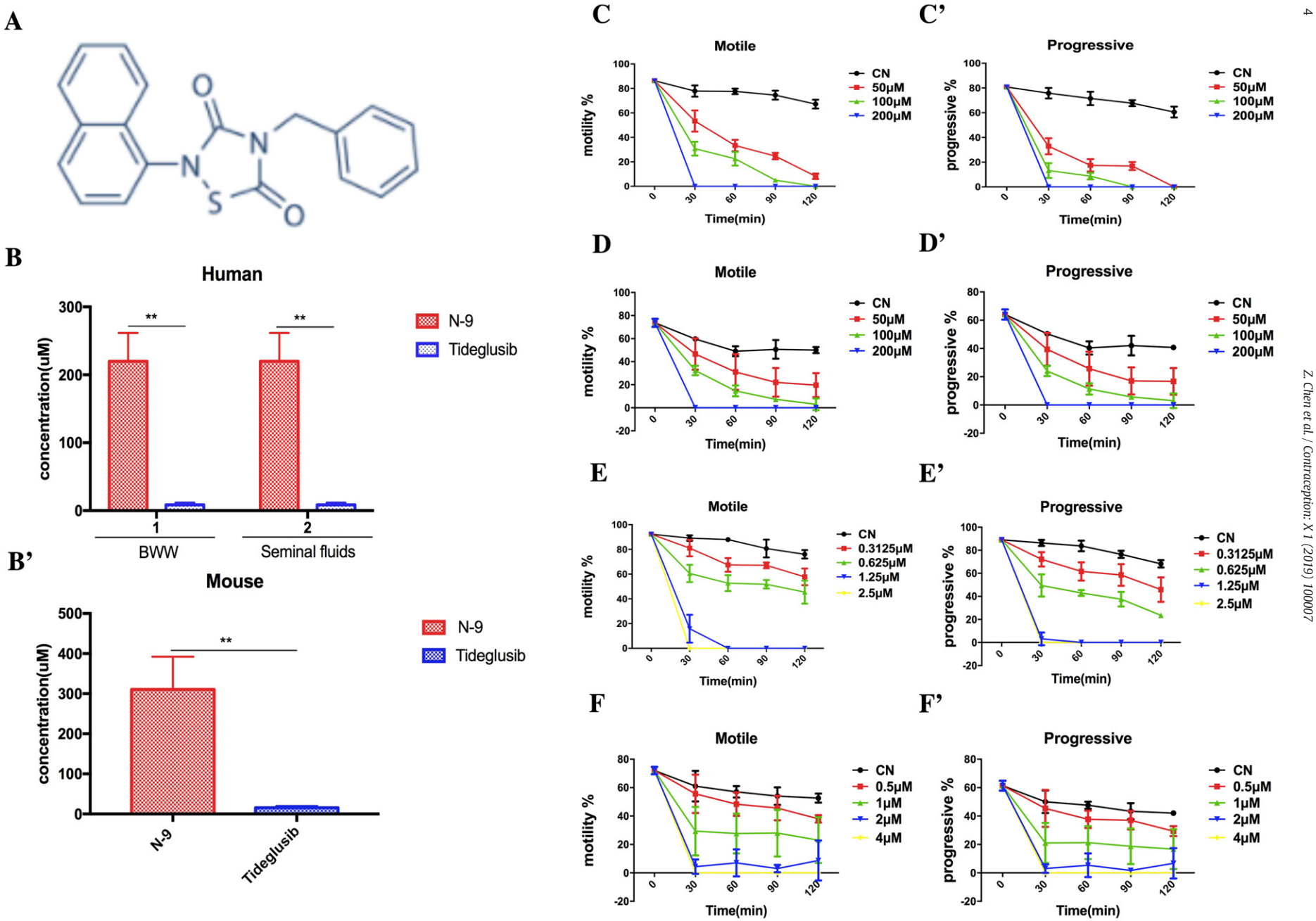

Fig. 2. Novel spermostatic/spermicidal activity of tideglusib in vitro. Spermostatic/spermicidal effects of (C, C′, D and D′) N-9 and (E, E′, F, and F′) tideglusib on human and mouse sperm in vitro. (A) Structure of tideglusib. (B) Measured MECs of N-9 and tideglusib in human and (B′) mouse spermatozoa within 20 s were compared. (B) Effect of human seminal fluids on spermostatic/spermicidal activity of tideglusib and N-9. **p<.01. (C, E) Time- and dose-dependent effects of compounds on human sperm motility, (C′, E′) progressive motility, (D, F) mouse sperm motility and (D′, F′) progressive motility. Motility (%): percentage of motile sperm (A+B+C%); progressive motility (%): percentage of forward spermatozoa (A+B%); CN: blank control. The data were presented as mean±SD.

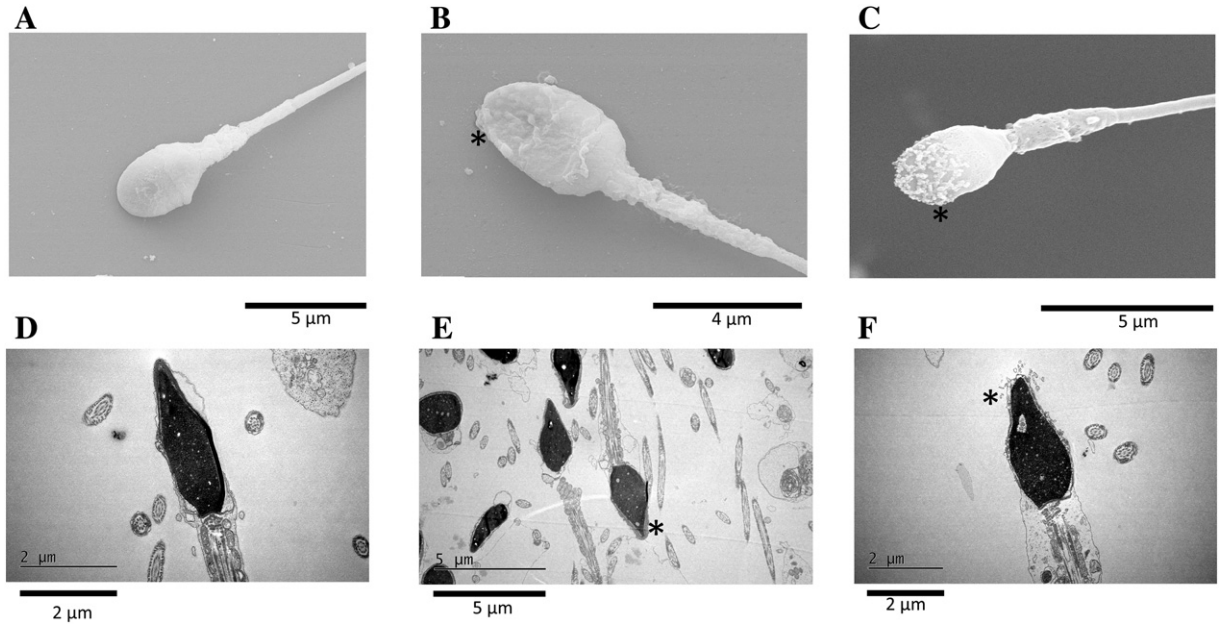

Fig. 3. Ultrastructural impairments of human sperm. (A–C) Typical damages to human sperm membrane caused by tideglusib were observed using SEM and (D–F) TEM. (A, D) Intact human spermatozoa and (B, C, E, F) membrane damage after treatment with 10 μM tideglusib for 20 s. * indicates typical membrane impairment.

sperm motility was observed in sperm revival tests when tideglusib was washed out.

The combination of the SEM and TEM analysis revealed considerable damage to the acrosomal and sperm membrane of middle piece after tideglusib treatments (Fig. 3). Intact sperms (Fig. 3A, D) had a smooth membrane surrounding the oval nucleus, whereas the damaged sperms exhibited dissolution, distortion and detachment of the acrosomal cap membrane (Fig. 3E, F) and blebbing of the acrosomal membrane (Fig. 3B, C) as well. Expansion and separation of the acrosomal cap membrane from the nucleus, vesiculation of the acrosomal membrane and disintegration of the plasma membrane after treatments were also revealed by TEM (Fig. 3E, F).

N-9 and tideglusib treatments for 20 s at their MECs decreased HOS responsiveness of human sperm from 83% to 7% and 17%, respectively (Fig. 4A–D), which suggested the overall loss of sperm membrane integrity after tideglusib treatment (**$p<.01$). Treatments with different concentration of tideglusib resulted in different sperm mortality (1.25 μM 60 min 10.8%, 10 μM 20 s 97.4%; Fig. 4G, H). Because dying moribund sperms were double-stained [24], the subpopulation between the quadrant regions of SYBR-14 +/propidium iodide (PI) − and SYBR-14 −/PI + featured dying sperms after treatment.

3.4. Cytotoxicity of tideglusib to L. acidophilus and vaginal cells

The inhibition (%) on *L. acidophilus* by DMSO, 100× MEC of tideglusib, 100× MEC of N-9 and commercialized N-9 was 17%, 27%, 82% and 100%, respectively. Tideglusib was less toxic than N-9 to *L. acidophilus* ($p<.01$) (Fig. 5A.) As assessed in vivo, tideglusib was much less toxic to vaginal cells than N-9 (Fig. 5B) in vitro.

4. Discussion

The in vivo assays in rabbits suggested that tideglusib had antifertility activity through decreasing implantation sites, survived fetus and pregnancy rate (Table 2). However, the tideglusib gels at 100× MEC did not completely protect the females from pregnancy. There were limitations in the present research that may account for the incomplete contraceptive efficacy. In the present study, the solubility of tideglusib was limited, and 100× MEC was the highest dose at which tideglusib could be completed dissolved in the basal formulation. A proper preparation of tideglusib may help to make preparations of higher doses to achieve complete contraceptive efficacy.

As there is a correlation between humans and rabbits in the irritation potential of vaginal gels [25], the vaginal irritation tests and histopathological scores (Table 1) support the potential use of tideglusib in humans in future. The vaginal cytotoxicity tests using Vk2/E6E7 cells (Fig. 5B) also support the prospects of tideglusib as a contraceptive agent compared with N-9. However, there are still limitations in the present assays. The present irritation tests and histopathological scores could not reflect the irritations of higher doses that may acquire complete contraception.

In vitro tests showed that tideglusib was a better spermicide than N-9 compared their MECs. The spermicidal potency of tideglusib was higher or comparable to that of many reported nondetergent spermicides such as DSE-37 [26,27], c-butyrolactone derivatives [28] and N, N′-dithiobisphthalimide [29]. Compared with N-9, tideglusib did not significantly affect the growth of *L. acidophilus* even at 100× MEC (Fig. 5A). These data suggested that tideglusib might be an alternative to N-9 considering its less disturbing effects to vaginal environments.

The highly "selective" toxicity of tideglusib to sperms would facilitate the discovery of novel contraceptive agents and male contraceptive targets. The novel structure of heterocyclic thiadiazolidinones provides a template to produce and test active derivatives of tideglusib, with the aim of identifying novel and druggable candidates. Identification and characterization of molecular "targets" of tideglusib on the sperm membrane would also facilitate the discovery of male contraceptive targets.

To the best of our knowledge, the rapid in vitro spermostatic activity of tideglusib identified in this study is a novel finding. Tideglusib is the first non-ATP competitive, irreversible inhibitor of GSK-3β with a novel structure derived from a small heterocyclic thiadiazolidinone [30]. Our data suggest that the rapid spermicidal activity of tideglusib on sperms may be derived from its detergent-like activity (partition coefficient [ClogP 5.21]) rather than its GSK-3β inhibitory activity, although the GSK-3β inhibitory activity is essential for most all of its known applications [31]. We observed that ATP-competitive GSK-3β inhibitors did not have spermostatic effects (data not shown). According to previous hypotheses, inhibition of Wnt/GSK-3β signaling would increase sperm motility [32,33], which is contrary to the observed spermostatic activity of tideglusib. GSK-3α, rather than GSK-3β, is the

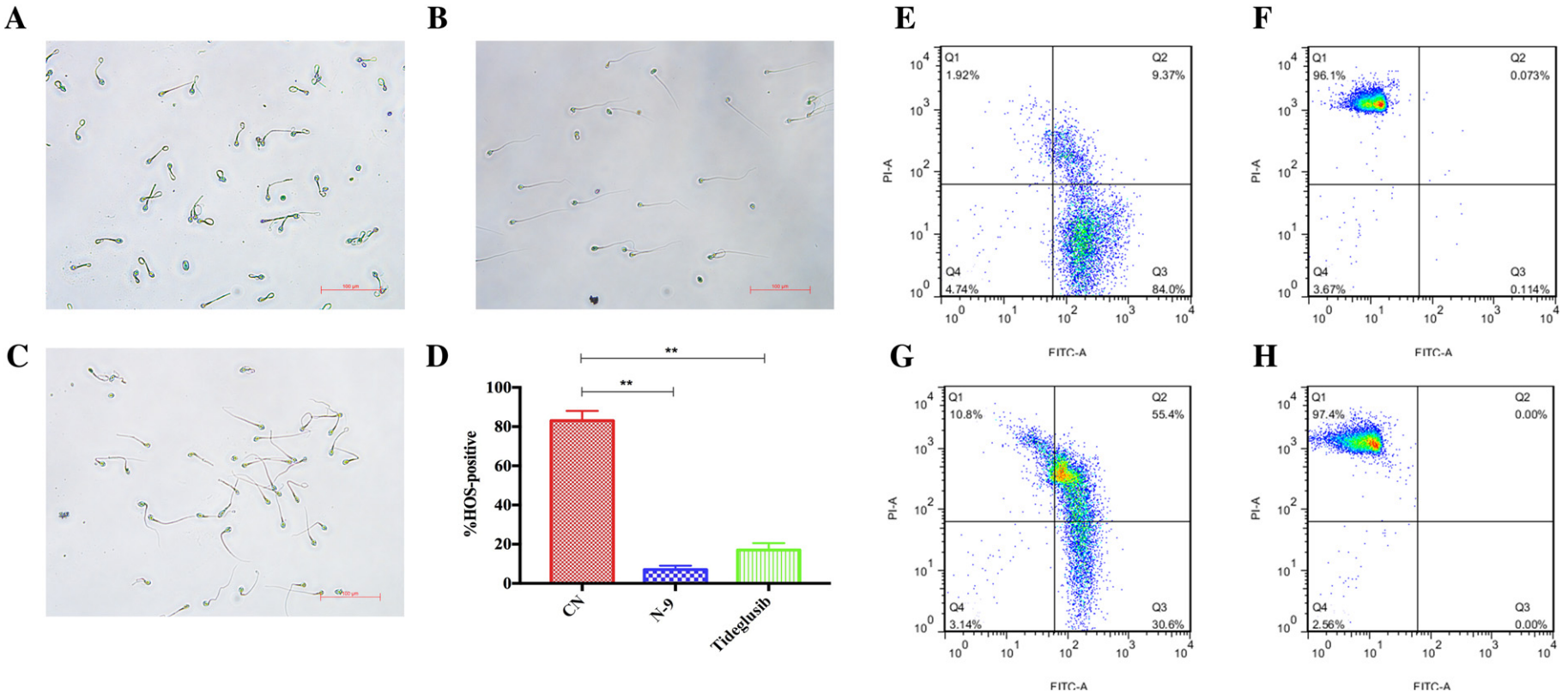

Fig. 4. Impairment of sperm membrane integrity by tideglusib. (A–D) Evaluation of sperm membrane integrity using HOS assays and (E–H) SYBR-14/PI staining. HOS assays of (A) intact (blank control), (B) N-9 treatment (at 1× MEC, approx. 260 μM for 20 s) and (C) tideglusib treatment (10 μM for 20 s) on human sperm. (D) Percentage of HOS-positive sperms after treatment with N-9 or tideglusib at MECs for 20 s or blank control. Data are expressed as percentage (%) of total sperm counts. CN: blank control; **p<.01. (E–H) SYBR-14/PI staining and flow cytometry analysis. (E) Blank control. (F) N-9 treatment (260 μM) for 20 s. (G) Tideglusib treatment (1.25 μM) for 60 min. (H) Tideglusib treatment (10 μM) for 20 s.

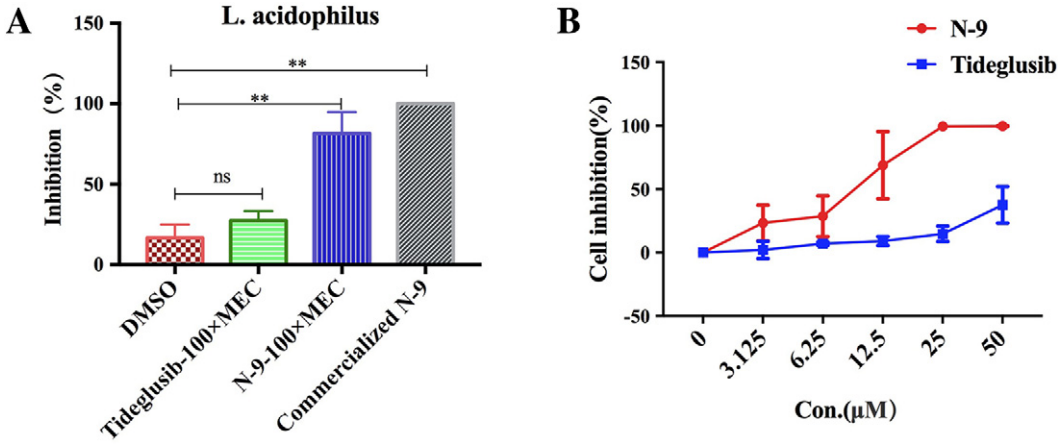

Fig. 5. Cytotoxicity of tideglusib to vaginal cells and *L. acidophilus*. Cytotoxic effects of tideglusib and N-9 on Lactobacillus acidophilus (A, $n = 3$) and vaginal cells (B, $n = 3$). (A) The inhibition (%) on *L. acidophilus* colonies after treatment with DMSO (17%), 100× MEC of tideglusib (27%), 100× MEC of N-9 (82%), commercialized N-9 (100%). (B) Inhibition (%) of the compounds on VK2/E6E7 cell proliferation after incubation for 24 h. **p<.01, ns: p>.05.

major GSK-3 subtype in mouse sperm [34], and the targeted disruption of GSK-3α in males affects sperm motility and causes male infertility [33]. Therefore, whether tideglusib inhibits sperm motility by inhibiting GSK-3α is worth further investigation.

Tideglusib or its derivatives, as an alternative to N-9, may be prepared in variant contraceptive preparations or products such as condoms for further assessment. The final success of a spermicide with high activity and low irritation may encourage the repeated use by women without the risks of increased susceptibility to HIV infection and other STDs, especially for those in whom hormonal products may be contraindicated.

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