Menopause: The Journal of The North American Menopause Society
Vol. 27, No. 2, pp. 000-000
DOI: 10.1097/GME.0000000000001435

Biocompatibility and zinc release testing of a zinc-containing vaginal gel

Ferenc Fenyvesi, PhD,[1] Judit Váradi, PhD,[1] Pálma Fehér, PhD,[1] Ildikó Bácskay, PhD,[1] Miklós Vecsernyés, PhD,[1] Attila Sipos, MD,[2] and Peter Takacs, MD, PhD, MBA[3]

Abstract

Objective: To test the biocompatibility of a zinc-containing vaginal gel, evaluate its ability to release zinc, and to assess the transepithelial permeability of zinc on human vaginal epithelium.

Methods: The release and membrane diffusion of zinc from the vaginal gel was tested by a vertical Franz-diffusion cell system. The biocompatibility of the gel was tested on HaCaT cells and reconstructed human vaginal epithelium. MTT assay was used to detect cell viability. Lactate dehydrogenase (LDH) assay was used to access cytotoxicity. The permeability of zinc was tested on the reconstructed human vaginal epithelium. The integrity of the reconstructed human vaginal epithelium after the permeability experiments was measured by transepithelial electric resistance. Zinc levels were determined by inductively coupled plasma optical emission spectrometry.

Results: 20 μM zinc sulfate did not decrease cell viability during the 24 and 72-hour treatment. Similarly, cell viability did not decrease significantly after 60 minutes of incubation with the gel and no toxic compound released from the vaginal gel during the 120 minutes diffusion experiment. A total of 72-hour exposure to the zinc-containing vaginal gel showed no cytotoxicity using LDH assay. Using cellulose-acetate membranes, 24.6% of the zinc content of the gel was released and appeared in the acceptor phase after 15 minutes. Zinc had high permeability ($2.2 \pm 0.8 \times 10^{-5}$ cm/s) from the vaginal gel on reconstructed human vaginal epithelium.

Conclusions: The zinc-containing (20 μM) vaginal gel was not toxic. The release of zinc is rapid from the vaginal gel. Zinc permeated rapidly through the vaginal epithelial cell layers.

Key Words: Menopause – Permeability – Toxicity – Vaginal gel – Zinc.

Genitourinary syndrome of menopause (GSM) is a common condition affecting up to 40% of postmenopausal women.[1-7] Frequently, women with GSM present with vaginal dryness, burning, dysuria, urgency, itching, vagina pain, and dyspareunia.[5-7] The pathophysiology of GSM can be explained by the decline in estrogen levels caused by diminished ovarian function and natural aging.

Traditionally, the treatment option for GSM was vaginal estrogen supplementation, but many women are either unable to or are afraid to use hormones. There are several nonhormone treatment modalities for the management of GSM-related symptoms, especially for vaginal dryness—the most bothersome symptom. The 2013 position statement of The North American Menopause Society indicates that first-line therapies to alleviate symptoms of vulvovaginal atrophy should include nonhormone vaginal lubricants and moisturizers, and also regular sexual activity.[6] Although there are numerous vaginal moisturizers and lubricants commercially available, only a few have been tested in clinical trials. A recent analysis of 12 commonly used commercially available vaginal gel products revealed that most products do not comply with recommended standards, and three of the tested products were found to be substantially cytotoxic even at 1:100 dilutions.[8]

Recently an over-the-counter zinc-containing vaginal gel became commercially available for the alleviation of postmenopausal vaginal dryness.[9] The scientific foundation for the zinc-containing gel originates from previous investigations of the role of vaginal zinc supplementation on vaginal remodeling.[10,11] Takacs et al[10] have shown that in human vaginal smooth muscle cells, zinc has a beneficial effect on the production of extracellular components. Zinc sulfate 20 μM increased significantly the smooth muscle cells' tropoelastin production.[10] Earlier, animal studies have revealed that zinc plays a vital role in the vaginal extracellular matrix (ECM) composition. When rats were kept on a zinc-deficient diet, the histological and histochemical pictures of the rats'

Received April 16, 2019; revised and accepted July 8, 2019.

From the [1]Department of Pharmaceutical Technology, University of Debrecen, Egyetem tér 1, Hungary; [2]Department of Obstetrics and Gynecology, University of Debrecen; Egyetem tér 1, Hungary; and [3]Eastern Virginia Medical School Department of Obstetrics and Gynecology, Division of Female Pelvic Medicine and Reconstructive Surgery, Norfolk, VA.

Funding/support: GINOP-2.1.1-15-2016-00783 (Economic Development and Innovation Operational Program Grant of the European Union and Hungary).

Financial disclosure/conflicts of interest: Dr Takacs is a paid consultant for Fempharma LLC. The other authors have nothing to disclose.

Address correspondence to: Peter Takacs, MD, PhD, MBA, Eastern Virginia Medical School, Department of Obstetrics and Gynecology, Division of Female Pelvic Medicine and Reconstructive Surgery, 825 Fairfax Avenue, Suite 526, Norfolk, VA 23507-2007. E-mail: takacsp@evms.edu

vagina resembled those of ovariectomized animals.[12] The vaginal ECM could be regenerated with characteristics equivalent to juvenile rats with vaginal zinc replacement alone.[11]

The primary aim of this study was to test the biocompatibility of the zinc-containing vaginal gel, to evaluate the gel for its ability to release zinc, and to assess the transepithelial permeability of zinc on reconstructed human vaginal epithelium. We hypothesized that zinc is readily released from the gel and zinc can permeate efficiently through the vaginal epithelial layers.

METHODS

Proprietary zinc-containing vaginal gel

A commercially available over-the-counter zinc-containing vaginal gel (JUVIA vaginal gel; Fempharma Europe, LLC, Budapest, Hungary) was used for all the vaginal gel-related experiments. The key ingredients of the gel are water, hydroxyethyl cellulose, and zinc sulfate heptahydrate at a concentration of 20 μM, and lactic acid. The zinc containing vaginal gel is used in the clinic, for the alleviation of postmenopausal vaginal dryness. The gel is self-applied intravaginally via an applicator. Two milliliters of the gel is inserted into the vagina nightly for 2 consecutive weeks and once or twice a week after that as needed per the product package insert.

Chemicals

The chemical 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced Salt Solution (HBSS), phosphate-buffered saline (PBS), trypsin-EDTA, heat-inactivated fetal bovine serum (FBS), L-glutamine, nonessential amino acids solution, and penicillin-streptomycin were purchased from Sigma-Aldrich (St Louis, MI). The 96-well plates, 12-well plates, and culturing flasks were obtained from Corning (Corning, NY). All other reagents were purchased from Sigma-Aldrich (St Louis, MI).

Zinc in vitro release and membrane permeation of the vaginal gel

The release and membrane diffusion of zinc from the proprietary vaginal gel was tested by a vertical Franz-diffusion cell system (Hanson Microette TM Topical and Transdermal Diffusion Cell System). Vaginal gel samples (0.3 g) were placed as donor phase on a cellulose-acetate (CA) membrane (pore size 0.45 μm) in the diffusion system. The experiments were also done after the CA membrane was impregnated by isopropyl-myristate (IPM). The effective diffusion surface area was 1.767 cm^2, and 30% (v/v) ethanol was used as an acceptor phase. The receptor medium was maintained at $37 \pm 0.5^{\circ}C$ throughout the experiment. Experiments were run for 2 hours, and samples of 0.5 mL were taken from the acceptor phase at specific time points and replaced with fresh receiving medium. The zinc concentration of the samples was determined by elemental analysis.

Cell culture

Human skin keratinocytes (HaCaT, Cell Lines Service [CLS], Heidelberg, Germany) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acid, and 1% penicillin-streptomycin solution at 37°C in an incubator containing 5% CO_2.

Reconstructed human vaginal epithelium (SkinEthic HVE/S/5) was purchased from EPISKIN (Lyon, France). The vaginal epithelial cells were previously grown on membrane inserts, and the inserts were placed into the growth medium according to the manufacturer's instructions. After 24 hours of incubation, the permeability test was carried out on the reconstructed vaginal epithelium.

Biocompatibility tests

The biocompatibility of zinc sulfate and the proprietary zinc-containing vaginal gel was tested on HaCaT cells and the reconstructed human vaginal epithelium by different experimental set-ups. The MTT method (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used to detect cell viability. The HaCaT cells were seeded on a flat bottom 96-well tissue culture plate at a density of 5×10^3 cells/well and allowed to grow in a CO_2 incubator at 37°C for 4 days. Then the culture medium was removed, zinc sulfate test solutions were added, and the cells were incubated for a further 60 minutes or 12 hours. Zinc sulfate was dissolved in cell culture medium at the final concentration of 0.1, 1, 5, 10, 20, 40, and 60 μM. After removing the samples, the cells were washed with HBSS and incubated with HBSS containing 0.5 mg/mL MTT for an additional 3 hours at 37°C. The dark blue formazan crystals were dissolved in acidic isopropanol (isopropanol:1.0N hydrochloric acid 25:1). Absorbance was measured at 570 nm against a 690 nm reference with a FLUOstar OPTIMA Microplate Reader (BMG LABTECH, Offenburg, Germany). Cell viability was expressed as the percentage of the untreated control. 1% Triton X-100 (TX-100) was used as a positive control in the experiments.

The biocompatibility of the zinc-containing vaginal gel was further tested with samples collected from the in vitro zinc release experiments, after 15, 30, 60, 90, and 120-minute diffusion. The acceptor phase in these experiments was HBSS. The zinc samples were placed on HaCaT cells, cultured in 96-well tissue culture plates, and incubated for 60 minutes. After incubation, the cell viability was tested by MTT method as described earlier.

The cytotoxicity and biocompatibility of the vaginal gel and zinc sulfate were also tested directly on HaCaT cells. For these experiments, the HaCaT cells were grown in 96-well plates for 4 days. The vaginal gel was diluted to 1:5, 1:10, and 1:100 with cell culture medium and placed to the monolayer of HaCaT cells. 20 μM zinc sulfate was also tested under the same conditions. The effect of the gel or zinc sulfate was detected after 24 and 72 hours by MTT test, as described earlier, and by lactate dehydrogenase (LDH) assay. For the LDH test, 1% FBS-containing cell culture medium was used. After 24 and 72 hours of incubation of cells with the dilutions

of the gel, the supernatants were collected and analyzed according to the manufacturer's instructions (Cytotoxicity Detection Kit [LDH]; Sigma-Aldrich, St Louis, MI). The absorbance of the formed dye was measured at 492 nm with a FLUOstar OPTIMA Microplate Reader (BMG LABTECH, Offenburg, Germany). TX-100 was used for the perfect release of LDH from the cells (high control), whereas untreated cells were used to measure the baseline level of LDH in the supernatant (low control). Cytotoxicity was calculated by the following equation according to the manufacturer's instructions:

$$Cytotoxicity\,(\%) = \frac{Sample\ value - low\ cntrol}{High\ value - low\ cntrol} \times 100$$

In addition, the biocompatibility of the zinc sulfate and the zinc-containing vaginal gel was also tested on the reconstructed human vaginal epithelium (SkinEthic HVE/S/5). After the permeability experiments, zinc solutions were removed from the apical compartment of the inserts and replaced by growth medium. The inserts were then placed into 12-well plates containing 0.5 mg/mL MTT dissolved in HBSS. The inserts containing vaginal gel from the permeability experiment were placed into 12-well plates containing 0.5 mg/mL MTT dye, but the gel was not removed from the epithelium because of its viscous properties. Inserts were incubated overnight at 37°C, and the resulted formazan dye was measured as described earlier. A positive control was 2% TX-100 treatment for 2 hours applied to the vaginal epithelium.

Transepithelial permeability test of zinc

The permeability of zinc was tested on the reconstructed human vaginal epithelium (SkinEthic HVE/S/5) grown on porous membrane inserts. Zinc permeability was measured using zinc sulfate solution in growth medium at 20 μM final concentration or the proprietary zinc-containing vaginal gel alone. The experiment started by placing the zinc test solutions or the vaginal gel into the donor chamber of the inserts. After 40, 80, and 140 minutes samples were taken from the acceptor solution, and the zinc concentration was determined by elemental analysis. With the formula below the apparent permeability coefficients were calculated:

$$P_{app} = dQ/dt(1/C_0A)$$

P_{app}: apparent permeability coefficient (cm/s)
dQ/dt: permeability rate of substances (mol/s)
C_0: initial concentration of the substances in the apical chamber (mol/mL)
A: surface area of the membrane (cm^2).

Transepithelial electric resistance measurement

To test the integrity of the reconstructed human vaginal epithelium after the permeability experiments, transepithelial electric resistance (TEER) measurement was used. This method was applied only on those inserts which were treated with zinc sulfate solutions in the permeability experiments. The inserts, treated with the vaginal gel in the permeability measurements, were not tested by TEER, because the gel disturbs the precise measurements with the TEER-testing electrodes. The TEER values of the inserts with cells were corrected by the values of blank inserts, and the results were multiplied by the surface of the insert membrane (0.5 cm^2). The results were expressed as Ωcm^2.

Quantitative zinc measurement by elemental analysis

Sample pretreatment

Samples collected from the in vitro zinc release studies, and the permeability tests were stored in Eppendorf tubes and transferred without loss into glass beakers by 2.00 mL of concentrated nitric acid (65% m/m; Merck) before the sample preparation steps. Beakers were placed on an electric heater plate and were heated until dryness, and another 4.00 mL of 65% (m/m) nitric acid was added to wet digest the samples for the complete oxidization and mineralization. They were heated again until dryness and transferred without loss into plastic test tubes with a screw cup where they were diluted up to 10.00 mL with 0.1 M nitric acid prepared in ultrapure water (MilliQ, Millipore System, Merck, Darmstadt, Germany). The purity of acids was verified by digesting blank samples containing only the chemicals, but no samples. All samples were kept in polypropylene tubes in which they were diluted and stored at 4°C in a refrigerator until measurement.

Elemental analysis

The elemental analysis of the pretreated membrane samples was carried out by inductively coupled plasma optical emission spectrometry (ICP-OES 5100, Agilent Technologies, Santa Clara, CA). The measurements were conducted in synchronous vertical dual view (SVDV) mode, gaining intensity data from the axial and radial view, simultaneously (Table 1). Automatic sample introduction was applied (SPS 4, Agilent Technologies, Santa Clara, CA) and the samples were measured in a completely randomized design. The zinc concentration was quantitatively determined from the samples next to a 5-point calibration. Calibration solutions were

TABLE 1. *The elemental analysis was carried out by inductively coupled plasma optical emission spectrometry (ICP-OES) by applying the following operating parameters*

ICP-OES operating parameters	
Repetition	3
Pump speed	15 r.p.m.
Uptake time	20 s, fast pump
Rinse time	30 s, fast pump
Read time	10 s
RF energy	1.20 kW
Stabilization time	7 s
View mode	SVDV
View height	0 mm
Nebulizer gas	0.70 L/min
Plasma gas	12.0 L/min

RF, radio frequency; SVDV, synchronous vertical dual view.

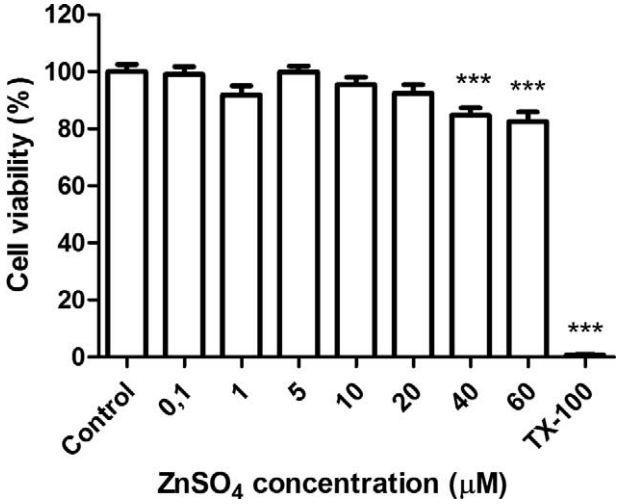

FIG. 1. Effect of zinc on HaCaT cell viability as assessed by MTT assay after 60 minutes of treatment. Zinc sulfate ($ZnSO_4$) 40 and 60 μM, and 1% Triton X-100 ([TX-100)] as a positive control) decreased ($P < 0.001$) cell viability significantly (n = 6). ***Statistically significant at $P < 0.001$.

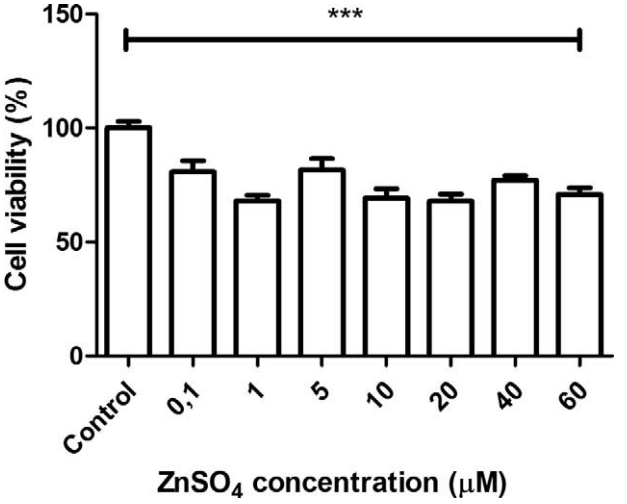

FIG. 2. Effect of zinc sulfate ($ZnSO_4$) on HaCaT cell viability after 12 hours of treatment as assessed by MTT assay. Cell viability decreased significantly at each concentration ($P < 0.001$), but no dose-dependent effect could be revealed (n = 6). ***Statistically significant at $P < 0.001$.

diluted from a multielement standard of 1000 mg/L (ICP standard IV, Merck, Darmstadt, Germany) with 0.1 M nitric acid in ultrapure water.

Statistical analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) and presented as means ± SD. Comparison of two groups was performed by unpaired *t* test, whereas more than two groups were compared by using analysis of variance. Differences were considered significant at $P < 0.05$.

RESULTS

Biocompatibility tests

The biocompatibility of zinc sulfate and the zinc-containing vaginal gel was tested on HaCaT cells and reconstructed human vaginal epithelium. Zinc sulfate did not decrease cell viability up to 20 μM concentration (92.3 ± 3%), compared with the untreated control (100%) ($P > 0.05$), whereas the positive control (TX-100) drastically decreased cell viability (0.68 ± 0.15%) during the 60-minute treatment ($P < 0.001$). Zinc sulfate in 40 and 60 μM concentration significantly decreased ($P < 0.001$) the cell viability to 84.8 ± 2.6% and 82.6 ± 3.3%, respectively (Fig. 1). Incubation of the HaCaT cells with zinc sulfate for 12 hours showed a more pronounced effect on the cell viability, but a dose-dependent effect was not apparent (Fig. 2).

The biocompatibility of the vaginal gel was further tested in combination with the diffusion method. The gel was placed on CA membranes, and the acceptor medium was HBSS. Samples from the acceptor buffer were collected at defined intervals and placed on HaCaT cells. No toxic compound released from the vaginal gel during the 120-minute diffusion experiment, and cell viability did not decrease significantly ($P > 0.05$) (Fig. 3).

In vitro zinc release from the vaginal gel

The release and bioavailability of zinc from the vaginal gel was tested with the in vitro Franz-diffusion system. In these experiments, the vaginal gel was placed on CA and IPM-impregnated CA membranes (donor phase), and the zinc concentration was measured on the other side of the membranes (acceptor phase) at specified time points. Using CA membranes, 24.6% of the zinc content of the gel released and appeared in the acceptor phase after 15 minutes (Fig. 4.). The impregnation of the CA membranes with lipophilic IPM decreased significantly ($P < 0.05$) the Zn membrane diffusion, with only 8.1% of the gel zinc content measured in the

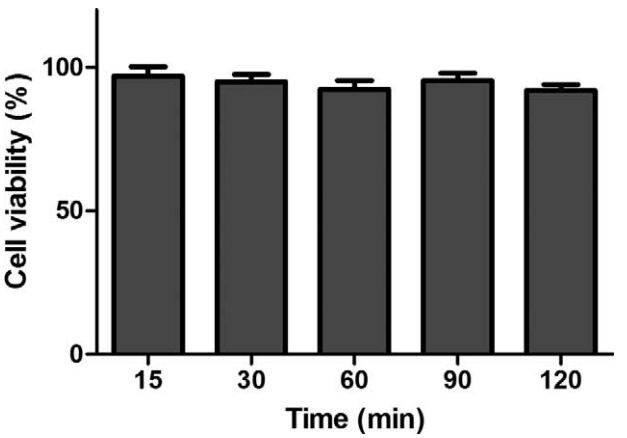

FIG. 3. Biocompatibility investigation of the proprietary zinc-containing vaginal gel on HaCaT cells. The biocompatibility of the zinc-containing vaginal gel was further tested with samples collected from the in vitro zinc release experiments, after 15, 30, 60, 90, and 120-minute diffusion. The acceptor phase in these experiments was Hank's Balanced Salt Solution. The zinc samples were placed on HaCaT cells, cultured in 96-well tissue culture plates, and incubated for 60 minutes. After incubation cell viability was tested by MTT method. No significant decrease in cell viability could be observed ($P > 0.05$) (n = 6).

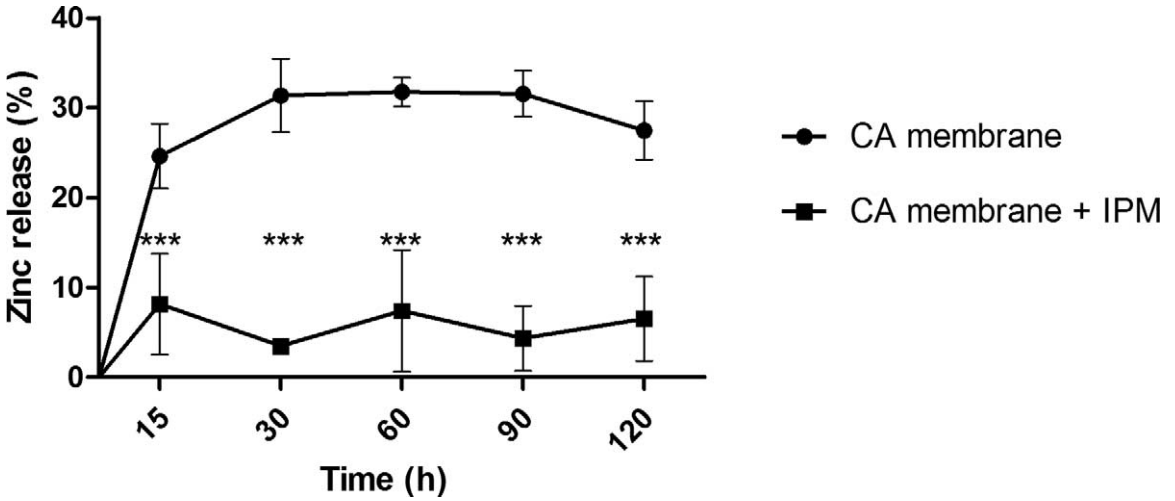

FIG. 4. In vitro zinc release from the vaginal gel using cellulose-acetate (CA) or isopropyl-myristate (IPM)-impregnated CA membranes in Franz-diffusion cell system. Vaginal gel samples were placed as donor phase on a CA or IPM-impregnated membrane in the diffusion system. The acceptor phase was 30% (v/v) ethanol. Experiments were run for 2 hours; samples of 0.5 mL were taken from the acceptor phase at specific time points and replaced with fresh receiving medium. The zinc concentration of the samples was determined by elemental analysis. The time of liberation did not affect significantly the liberated amount of zinc through CA or CA membrane with IPM, whereas the impregnation of the CA membrane with IPM had significant lowering effect on zinc release ($P < 0.001$) at each time points of the experiment. ***Statistically significant at $P < 0.001$.

acceptor phase after 15 minutes. The difference can be explained by the lipophilic character of IPM, the impregnation made the CA membrane lipophilic, and less water-soluble zinc was able to penetrate through the membrane. Despite the lipophilic impregnation, the zinc was able to permeate the membrane. The release of zinc is fast from the vaginal gel and within 30 minutes reaches the maximum level in the in vitro release experiments, by diffusion through the membranes.

Transepithelial permeability test of zinc and cell viability

The bioavailability of zinc from the vaginal gel was tested on the in vitro reconstructed human vaginal epithelium. Zinc-containing vaginal gel and zinc sulfate dissolved in cell culture medium were used in the donor compartment of the membrane inserts. Samples were taken from the acceptor chamber, the permeated amount of zinc was determined, and the apparent permeability of the zinc was calculated. Zinc had high permeability from both the zinc sulfate solution and the vaginal gel ($4.17 \pm 1.13 \times 10^{-5}$ and $2.2 \pm 0.8 \times 10^{-5}$ cm/s, respectively), and there was no significant difference between the zinc permeability values ($P > 0.05$). Permeability values of the zinc on reconstructed human vaginal epithelium show fast permeation through the vaginal epithelial cell layers. This model consists of six cell layers according to the manufacturer's certification, but the permeation of zinc was not inhibited. There was no significant difference between the zinc permeability from the zinc sulfate solution or the vaginal gel. It means that the zinc can be freely liberated from the gel and absorbed through the vaginal epithelium without significant toxicity according to the following biocompatibility results (Fig. 5).

After the permeability test, TEER values of the vaginal epithelium were recorded in the case of zinc sulfate-treated cell layers. TEER values of these epithelial layers were $63 \pm 0.5\ \Omega cm^2$, whereas TEER decreased to $32\ \Omega cm^2$ after 2 hours of 2% TX-100 treatment. After the permeability experiments, the viability of reconstructed human vaginal epithelium was tested. Two per cent TX-100 had significant toxicity ($P < 0.001$), whereas 20 μM zinc sulfate or the zinc-containing vaginal gel had no toxic effect on the epithelial cell layers after 140 minutes incubation of the permeability experiments (Fig. 6).

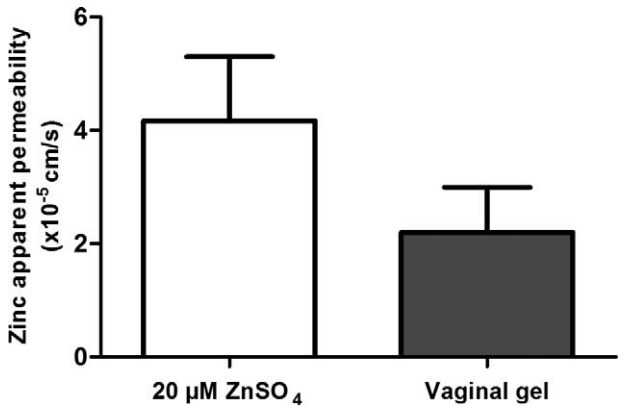

FIG. 5. Transepithelial permeability test of zinc on reconstructed human vaginal epithelium grown on porous membrane inserts. Zinc permeability was measured using zinc sulfate ($ZnSO_4$) solution in growth medium at 20 μM final concentration or the proprietary zinc-containing vaginal gel alone. The experiment started by placing the zinc test solutions or the vaginal gel into the donor chamber of the inserts. After 40, 80, and 140 minutes samples were taken from the acceptor solution, and the zinc concentration was determined by elemental analysis. Zinc permeability was not significantly different, measured from the zinc sulfate solution or the vaginal gel ($P > 0.05$). Values are presented as the mean apparent permeability values ± SD (n = 3).

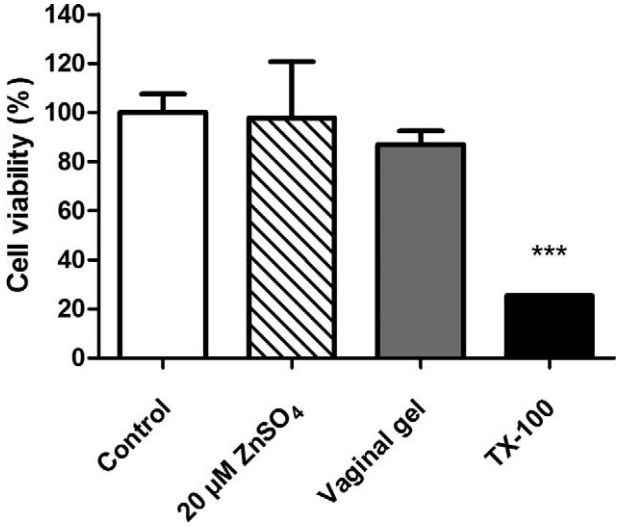

FIG. 6. Effect of 2% Triton X-100 (TX-100), 20 μM zinc sulfate ($ZnSO_4$), or the zinc-containing vaginal gel on the viability of reconstructed human vaginal epithelium as measured by MTT assay. TX-100 was used as a positive control. Values are presented as means ± SD (n = 3). ***Statistically significant at $P < 0.001$.

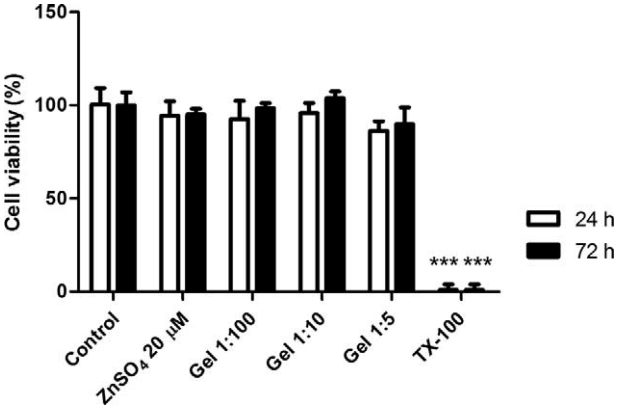

FIG. 8. Effect of zinc-containing vaginal gel and 20 μM zinc sulfate on HaCaT cell viability as assessed by MTT assay after 24 and 72 hours of treatment. The vaginal gel was diluted to 1:5, 1:10, and 1:100 with cell culture medium. 1% Triton X-100 ([TX-100] as a positive control) decreased cell viability significantly ($P < 0.001$), but neither dilution of zinc-containing vaginal gel or 20 μM zinc sulfate affected cell viability (n = 6). ***Statistically significant at $P < 0.001$.

To test the cytotoxic effect of the vaginal gel and 20 μM zinc sulfate over a longer time course, we have exposed HaCaT cells to zinc-containing vaginal gel or zinc sulfate over 72 hours. The gel or 20 μM zinc sulfate showed no toxicity at all tested dilutions using LDH assay (Fig. 7). In addition, cell proliferation was not affected either after 24 or 72 hours of exposure (Fig. 8).

DISCUSSION

Nonhormone treatment options for GSM symptoms include lubricants (water, silicone, or oil-based) and moisturizers. Regular use of nonhormone, long-acting vaginal moisturizing agents can significantly improve symptoms of vaginal dryness and decrease vaginal pH, but not as effectively as hormonal treatments.[13-15] Although several lubricants and moisturizers are commercially available over the counter, very few clinical trials have been performed to show their safety and efficacy.[13-16] Dezzutti et al[16] examined the safety of personal moisturizers and lubricants, and these investigators found that many water-based gels are hyperosmolar and may lead to epithelial cell damage and toxicity. An analysis by Cunha et al[8] on 12 commonly used commercially available vaginal gel products revealed that most products do not comply with recommended standards and three of the tested products were found to have substantial cytotoxicity even at 1:100 dilutions.

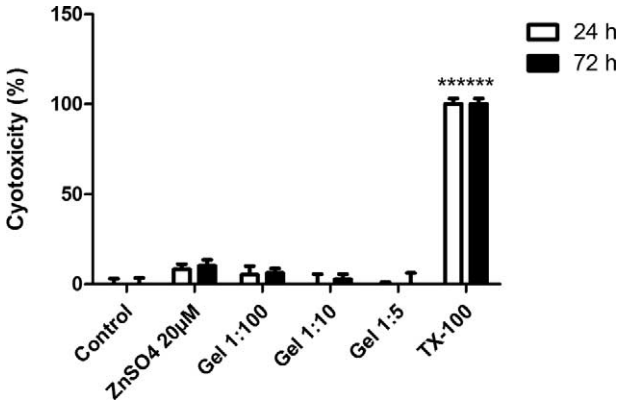

FIG. 7. The cytotoxicity of the zinc-containing vaginal gel and 20 μM zinc sulfate was tested on HaCaT cells by lactate dehydrogenase (LDH) assay after 24 and 72 hours of treatment. The vaginal gel was diluted to 1:5, 1:10, and 1:100 with cell culture medium and placed on HACaT cell monolayers in 96-well plate. 1% Triton X-100 ([TX-100] as a positive control) had significant ($P < 0.001$) toxicity, but neither dilution of zinc-containing vaginal gel or 20 μM zinc sulfate showed toxicity (n = 6). ***Statistically significant at $P < 0.001$.

Our biocompatibility tests results indicate that the 20 μM zinc-containing vaginal gel is nontoxic to vaginal epithelial cells. These findings are in line with our prior experiments with human vaginal smooth muscle cells and in vivo rat experiments.[10,11] Previously, we have shown that zinc sulfate at a concentration of 20 μM is not toxic to human vaginal smooth muscle cells.[10] Rat experiments utilizing 20 μM zinc sulfate-containing vaginal suppositories revealed no evidence of toxicity or inflammation at either the mucosal or submucosal level.[11] Previous research indicates that vaginal application of zinc at much higher concentrations than that found in the currently tested gel might cause epithelial disruption. Bourne et al[17] found that the application of different zinc (acetate, sulfate gluconate, and chloride) solutions to the vaginal mucosa of rats in much higher concentration (100-200 mM) than used in our experiments has the potential to cause vaginal epithelial disruption. We have found no evidence of epithelial disruption after application of the zinc-containing vaginal gel or similar low concentration of zinc sulfate solution by measuring the TEER. In addition, microscopic analysis of the vaginal epithelium of rats after 2 weeks of intravaginal application of 20 μM zinc sulfate-containing

suppositories did not cause epithelial disruption.[11] The daily intravaginal application of a zinc-containing gel (14 mM zinc acetate dihydrate) in monkeys neither affected the viability of lactobacilli or *Candida albicans*, nor enhanced vaginal herpes simplex virus-2 infection.[18] Kenney et al[18] concluded based on their extensive monkey experiments that a modified zinc acetate-containing gel is safe. In a rabbit vaginal irritation study 1% zinc acetate solution did not irritate or cause ulceration of rabbit vaginal epithelium.[19] These in vitro and in vivo animal experiments support the findings of a recent human pilot trial with the currently tested zinc-containing gel which revealed no evidence of vaginal inflammation or infection after using the gel daily for 2 weeks.[9]

Although zinc is present in the vaginal gel, it is imperative to test for its release from the gel. The release and bioavailability of zinc from the vaginal gel was tested with the in vitro Franz-diffusion system. We have found that the release of zinc was fast from the vaginal gel and within 30 minutes reached the maximum level in the in vitro release experiments, by diffusion through the membranes. The fast release of the zinc from the gel is an important attribute of the gel. The zinc concentration of the gel is similar to high upper normal values of zinc in the vaginal tissue from healthy premenopausal women. The rapid release of zinc from the gel allows the zinc to be available for biological action, penetration through the vaginal wall, and potentially reaching the submucosal layers.

Permeability values of zinc on reconstructed human vaginal epithelium showed fast permeation through the vaginal epithelial cell layers. Our vaginal epithelial model consisted of six cell layers according to the manufacturer's certification, but the permeation of zinc was not inhibited because we found no significant difference between the zinc permeability between the zinc sulfate solution and the vaginal gel. Based on our experiments zinc can be freely liberated from the gel and absorbed through the vaginal epithelium without significant toxicity. Similar to our findings, Houston et al[20] found that from a zinc sulfate (0.25 M)-containing hydrogel that the zinc readily permeated the vaginal epithelium in pigs. The amount of zinc permeating the vaginal epithelium was comparatively very large, and zinc permeated the vaginal epithelium to a much greater extent compared with both epidermal and buccal epithelium.[20]

There are several limitations to our study including the short testing period of 72 hours. Longer exposure to zinc or to the vaginal gel might have caused cytotoxicity. In addition, we have used only two different types of cells in our current experiments (keratinocytes and vaginal epithelial cells), but not endothelial cells. Vascular damage through endothelial cell disruption may be a different mechanism for toxicity which was not tested in our experiments.

CONCLUSIONS

In summary, based on our experiments, we have concluded that the zinc-containing (20 μM) vaginal gel was not toxic to the vaginal epithelium. The release of zinc is rapid from the vaginal gel, and permeability values of the zinc on reconstructed human vaginal epithelium showed fast permeation through the vaginal epithelial cell layers. Because zinc can be freely liberated from the gel and absorbed through the vaginal epithelium, it may reach the submucosal part of the vagina and could potentially affect not just the epithelium but the deeper layers of the vaginal wall.

REFERENCES

1. Weber MA, Limpens J, Roovers JP. Assessment of vaginal atrophy: a review. *Int Urogynecol J* 2015;26:15-28.
2. Bachmann GA, Nevadunsky NS. Diagnosis and treatment of atrophic vaginitis. *Am Fam Physician* 2000;61:3090-3096.
3. Levine KB, Williams RE, Hartmann KE. Vulvovaginal atrophy is strongly associated with female sexual dysfunction among sexually active postmenopausal women. *Menopause* 2008;15 (4 Pt 1):661-666.
4. Nappi RE, Kokot-Kierepa M. Women's voices in the menopause: results from an international survey on vaginal atrophy. *Maturitas* 2010;67: 233-238.
5. Gandhi J, Chen A, Dagur G, et al. Genitourinary syndrome of menopause: an overview of clinical manifestations, pathophysiology, etiology, evaluation, and management. *Am J Obstet Gynecol* 2016; 215:704-711.
6. Management of symptomatic vulvovaginal atrophy: 2013 position statement of The North American Menopause Society. *Menopause* 2013;20:888-902.
7. Portman DJ, Gass ML, Panel VATCC. Genitourinary syndrome of menopause: new terminology for vulvovaginal atrophy from the International Society for the Study of Women's Sexual Health and The North American Menopause Society. *Menopause* 2014;21:1063-1068.
8. Cunha AR, Machado RM, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, das Neves J, Palmeira-de-Oliveira R. Characterization of commercially available vaginal lubricants: a safety perspective. *Pharmaceutics* 2014;6:530-542.
9. Takacs P, Koza B, Erdodi B, Jakab A, Larson K, Poka R. Zinc-containing vaginal moisturizer gel improves postmenopausal vulvovaginal symptoms: a pilot study. *J Menopausal Med* 2019;25:63-68.
10. Takacs P, Zhang Y, Candiotti K, Jaramillo S, Medina CA. Effects of PPAR-delta agonist and zinc on vaginal smooth muscle cells collagen and tropoelastin production. *Int Urogynecol J* 2012;23:1775-1779.
11. Takacs P, Jaramillo S, Zhang Y, et al. The effects of PPARδ agonist and zinc on ovariectomized rats' vagina. *Female Pelvic Med Reconstr Surg* 2013;19:126-131.
12. Taneja SK, Kaur R. Pathology of ovary, uterus, vagina and gonadotrophs of female mice fed on Zn-deficient diet. *Indian J Exp Biol* 1990;28:1058-1065.
13. Lee YK, Chung HH, Kim JW, Park NH, Song YS, Kang SB. Vaginal pH-balanced gel for the control of atrophic vaginitis among breast cancer survivors: a randomized controlled trial. *Obstet Gynecol* 2011;117: 922-927.
14. Bygdeman M, Swahn ML. Replens versus dienoestrol cream in the symptomatic treatment of vaginal atrophy in postmenopausal women. *Maturitas* 1996;23:259-263.
15. Nachtigall LE. Comparative study: Replens versus local estrogen in menopausal women. *Fertil Steril* 1994;61:178-180.
16. Dezzutti CS, Brown ER, Moncla B, et al. Is wetter better? An evaluation of over-the-counter personal lubricants for safety and anti-HIV-1 activity. *PLoS One* 2012;7:e48328.
17. Bourne N, Stegall R, Montano R, Meador M, Stanberry LR, Milligan GN. Efficacy and toxicity of zinc salts as candidate topical microbicides against vaginal herpes simplex virus type 2 infection. *Antimicrob Agents Chemother* 2005;49:1181-1183.
18. Kenney J, Rodríguez A, Kizima L, et al. A modified zinc acetate gel, a potential nonantiretroviral microbicide, is safe and effective against simian-human immunodeficiency virus and herpes simplex virus 2 infection in vivo. *Antimicrob Agents Chemother* 2013;57:4001-4009.
19. Fahim MS, Wang M. Zinc acetate and lyophilized aloe barbadensis as vaginal contraceptive. *Contraception* 1996;53:231-236.
20. Houston DMJ, Robins B, Bugert JJ, Denyer SP, Heard CM. In vitro permeation and biological activity of punicalagin and zinc (II) across skin and mucous membranes prone to Herpes simplex virus infection. *Eur J Pharm Sci* 2017;96:99-106.