PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 593–597 (1980)

Topical Zinc in the Treatment of Mice Infected Intravaginally with Herpes Genitalis Virus (40922)

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Abstract. The course of the disease, HSV-2, inoculated intravaginally in immature mice was ascertained by studying the incidence of vaginitis, encephalitis, and mortality rate. The therapeutic efficacy of various zinc containing medications administered topically 4 hr after infection was studied. It was found that intravaginally applied zinc medicated collagen sponge containing 50 μg zinc sulfate ($7H_2O$) in acetate buffer, pH 5.5, significantly improved the course of disease and was significantly more effective than zinc administered intravaginally in a cream base. A solution of zinc sulfate administered as a douche was as effective as the comparable concentration of zinc applied through resilient collagen sponge. We conclude that topical administration of zinc through resilient collagen sponge or as a douche used shortly after the infection significantly inhibits the course of the herpes genitalis infection in the mouse.

Antiviral activity of zinc has been noted by several authors (1–7). Falke (3) showed that zinc added to tissue cultures of kidney cells infected with HSV-1 blocked the formation of giant cells induced by the virus. In 1975, Gordon *et al.* (5) showed 95% inhibition of the HSV-2 growth in the presence of 0.1 m*M* zinc. Studies by Schlomai *et al.* (6) and Gupta and Rapp (7) confirmed the antiviral effect of zinc in *in vitro* systems, but each group postulated different mechanisms of inhibition. These studies arrived at two clinically important conclusions, namely that inhibition of HSV-2 by Zn^{2+} is an irreversible phenomenon and that it is specific to the virus. DNA of eucaryotic cells, serving as substrate for the virus growth, was not affected by Zn^{2+} at that concentration (5, 6).

Our own studies (8, 9) reproduced the *in vitro* inhibitory effect of Zn^{2+} on HSV-2 replication. We also showed that, in an animal model of herpetic vaginal infection using virgin Balb/c mice, collagen sponges soaked with a solution of zinc sulfate in acetate buffer and introduced into the vagina retarded the onset and severity of encephalitis and significantly reduced the mortality rate encountered with insertion of sponges soaked in the acetate buffer solution.

In order to effectively use zinc in the prophylaxis and treatment of HSV-2 infection, the optimal physical form of zinc administration should be investigated. This report summarizes observations on zinc administered intravaginally in a solution as a douche, applied in a collagen sponge, or delivered in a vaginal cream.

Materials and Methods. Mouse model of herpes genitalis. Animals. One hundred and thirty 6-week-old virgin Balb/c mice (Charles River Breeding Laboratories, Wilmington, Mass.) were inoculated intravaginally with HSV-2 suspension (Strain 333) in 10% minimal essential medium, 0.1 ml of a 1:5 dilution of the stock suspension. Prior to inoculation the vagina was douched with normal saline and the cervix was swabbed with a sterile cotton-tipped applicator. The virus inoculum was delivered by a plastic catheter PE-20 on a calibrated Hamilton syringe. The virus inoculum was defined by previous titration in this animal model to produce LD_{50} in untreated 6-week-old females.

Viral inoculum. The HSV-2 (Strain 333) was grown on fibroblasts derived from human embryonic tonsil (HET-E, courtesy of E. Russel Alexander, University of Washington, Seattle) and was maintained in Eagle's minimal essential medium (MEM)

0037-9727/80/080593-05$01.00/0

supplemented with Eagle's nonessential amino acids, 100 U penicillin/ml, and 100 μg of streptomycin/ml (Gibco, New York). For cell culture, the MEM was supplemented with 2% fetal calf serum (Flow Laboratories). The supernatant from the viral infected HET-E was harvested 24 hr after the development of 4+ cytopathic effect. After centrifugation at 5000 rpm, the supernatant yielded 1×10^7 plaque forming units/ml (PFU/ml), as assayed on HET-E. Aliquots of this stock suspension of HSV-2 were stored at −95°C, then thawed just prior to use.

Evaluation of the course of the disease. The course of HSV-2 infection was evaluated by three criteria:

1. Vaginitis. The magnitude of the signs of the inflammation was subjectively scored as 1+ to 4+.

1+ Vaginal and vulvar mild edema or erythema
2+ Moderate edema and erythema
3+ Moderate to severe edema and erythema, vaginal exudate, perivulvar hair loss
4+ Severe edema and erythema accompanied by vaginal exudate and perivulvar hair loss

From our previous studies (9), we learned that vaginitis scored 4+ is irreversible and diagnosed with the greatest accuracy. For this reason, we present in Fig. 1 the incidence of 4+ vaginitis only.

2. Encephalitis/paralysis. Animals are observed daily for signs of encephalitis including lethargy, lack of responsiveness to tactile stimulation and paralysis, usually of the hind limbs.

3. Mortality. Deaths are recorded daily.

Treatment regimens. Three procedures of applying zinc were tested—solution administered intravaginally as douche, same solution soaked into the matrix of the collagen sponge, and finally in the form of zinc cream. Every zinc treatment was compared to the appropriate control group, treated with the same method but without zinc. The treated experimental groups were then compared with nontreated animals (sham controls), which were handled as the

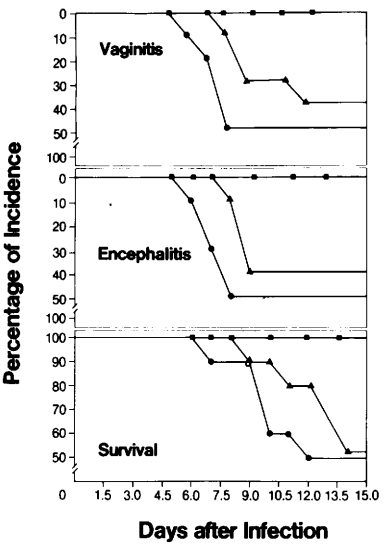

FIG. 1. The effect of topical intravaginal administration of zinc through collagen sponge or in cream on the course of herpes genitalis infection in mouse model. ○, Mice treated with collagen sponge soaked in acetate buffer; ▲, mice treated with cream containing zinc; ■, mice treated with collagen sponge containing zinc in acetate buffer. Treatment started 4 hr after infection and continued with daily administrations for 8 days. There were 10 animals, 6 weeks old, in each group.

treated ones to simulate the treatment procedure. This included restraint of the animals and insertion of forceps.

The treatment was started 4 hr after the inoculum. It was repeated daily for a total of 8 days. Spontaneous expulsion of inserted collagen sponges occurred in less than 5% of the animals. The animals were observed daily for signs of vaginitis, encephalitis, and deaths up to 14 days after infection.

Collagen sponge was reconstituted by Chvapil's procedure (10) and was tanned with glutaraldehyde. The final sponge had high resilience and fluid binding capacity (42 ml/g of collagen sponge, dry weight).

Collagen sponge tampons (CS). Cylinders (3 × 10 mm) soaked in 0.025 *M* acetate buffer, pH 5.5, containing 0.1 *M* zinc sulfate ($ZnSO_4 \cdot 7H_2O$), were inserted intravaginally with forceps at defined time intervals following infection. We established that there is 50 ± 5 μg zinc sulfate,

heptahydrate, retained in each sponge which corresponds to 11 μg elemental zinc. In other studies, we already reported the nontoxicity of zinc medicated collagen in intravaginal administration (11, 12).

Douching with zinc in acetate solution. Four hours after the mice were infected, each mouse was douched with 0.5 ml of zinc solution in acetate buffer or with acetate buffer alone. There was 14.4 μg $ZnSO_4 \cdot 7H_2O$/douche. The solution was administered through a 5-ml plastic syringe with an 18-gauge needle adapted with 5-mm piece of polyethylene catheter tubing PE-20. Animals were douched daily for 8 days.

Zinc cream (ZnCr) Zinc cream was prepared by homogenizing 1.0 *M* zinc sulfate in 0.025 *M* acetate buffer, pH 5.5, with hydrophilic ointment, USP (1:10 v/v). The final cream contained 0.1 *M* zinc sulfate of which 15 μl (35 μg $ZnSO_4 \cdot 7H_2O$) was administered from Day 2 through Day 8 using a Hamilton syringe with attached catheter. In the control group, zinc in the cream was replaced with 0.9% sodium chloride in the same acetate buffer.

Statistical analysis. Survival analyses were performed by plotting a survival curve for each group (for encephalitis and level 4 vaginitis "survival" is defined as the absence of the condition) by Gehan's modification of the Wilcoxon test (13). This test results in a statistic—a *Z* value—which was used to investigate the significance of difference in survival.

Results. The effects of collagen sponge containing zinc in acetate buffer (ZnCS) and cream containing zinc (ZnCr) treatments as compared with control group treated with collagen sponge soaked in acetate buffer (AcDS) are shown in Fig. 1. The data show that daily zinc treatment, started 4 hr after inoculation, provided complete protection against the infection whereas the application of zinc in cream or collagen sponge soaked only in acetate buffer was associated with approximately a 50% incidence of signs of virus infection. A statistically significant difference ($P < 0.01$) was found in all three parameters of the disease between ZnCS on one side and ZnCr and AcCS on the other side. No statistical difference in either of the parameters studied was found between treatment with zinc cream (ZnCr) and cream alone (data not shown). Infected mice treated with acetate soaked collagen sponge showed the same course of infection as sham controls (data not shown).

Effect of ZnCS vs douching with zinc solution. We studied the effect of comparable concentrations of zinc sulfate solution administered either topically as a douche or soaked into intravaginally inserted collagen sponge.

The results summarized in Fig. 2 were tested for significance. It was found that all three parameters of the course of the disease were significantly inhibited by treatment of mice, either with zinc medicated collagen sponge or with douching with zinc solution ($P < 0.001$). These two groups differ significantly from both control groups, treated with either acetate solution alone or sham treated. No significant difference was found between either the two zinc treated groups or the two control groups.

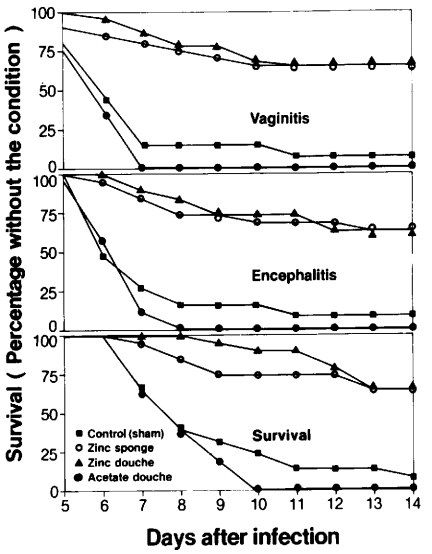

FIG. 2. The effect of topical intravaginal administration of zinc solution through collagen sponge or douche on the course of herpes genitalis infection in mouse model. The efficacy of two treatment regimens was compared with appropriate controls treated by acetate buffer douche or sham treated. There were 20 mice, 6 weeks old, in each group.

Discussion. Only a few studies deal with the *in vivo* effect of zinc on the herpes genitalis infection. Tennican *et al.* (8) reported a reduction of the incidence of encephalitis and mortality in immature mice with vaginal herpes infection by topical use of zinc medicated collagen sponges. Recently the same group has shown that only topical intravaginal zinc was effective, while systematic administration of zinc did not inhibit the course of the infection (9). Other investigators (14) used zinc together with other factors (ultrasound, urea, and tannic acid) in male patients with herpes genitalis; this makes it rather difficult to ascertain the actual role of zinc as a treatment modality.

The results of this study indicate the effectiveness of the treatment of vaginal herpes genitalis by zinc mediated sponge containing 50 μg zinc sulfate, heptahydrate. The acidic pH (pH 5.5) of the sponge by itself does not affect the virulence of this infection. We assume that the multiple rugated surface of vaginal mucosal lining, organized in longitudinal folds, should first be slightly distended by the resilient collagen sponge to flatten the folds. As a result, the infection residing randomly at the irregular surface or the inside of epithelial cells would become available to the treatment. Although we have no information regarding the rate of Zn^{2+} transport across the cell membrane, we assume that this is a rather fast process judging by our studies with the kinetics of zinc accumulation in granulocytes or macrophages (15).

We present evidence that zinc medicated collagen sponge is a more effective method of delivering therapeutic doses of zinc to the virus than cream (ointment) as a vehicle for zinc. Still, various medications which are applied intravaginally are administered in the form of creams. We assume that just the opposite explanation as suggested above for the effectiveness of ZnCS is valid to explain lower therapeutic efficiency of zinc cream. The ointment does not penetrate to the folds of crypts of the complex vaginal wall surface. It is also possible that zinc does not diffuse so quickly from the cream and is therefore not available for interaction with HSV-2.

The solution with zinc applied in daily douches was equally effective as zinc medicated collagen sponge. Our experiments suggest a minimal possibility that, by douching only 4 hr after inoculating the virus intravaginally, we washed out the virus, thus diluting the inoculum. Similar douching with acetate buffer alone did not affect the course of infection. The zinc douche method has the risk of disseminating herpes infected material onto adjacent normal tissues. The douche method also would not provide sustained local levels of the medication for eradication of residual virus. Although the zinc douche may not be practical for human clinical use, its efficacy in our animal model supports the effectiveness of bioavailable zinc delivered by the collagen sponge tampon for herpes simplex genitalis.

While in this study we initiated the various treatment procedures 4 hr after inoculation of the virus, other results will document that zinc treatment starting 18 hr after inoculation still effectively inhibited the propagation of the virus infection in the mouse (Tennican, Carl, Thies, and Chvapil, unpublished data).

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Received March 17, 1980. P.S.E.B.M. 1980, Vol. 164.