



ASSOCIATING A HYPERTONIC SOLUTION WITH SPECIFIC PLANT PROCYANIDINS FOR THE TREATMENT OF GENITAL HERPES

Ravi Shrivastava*

VITROBIO Research Institute, ZAC de Lavaur 63500 Issoire, France

Email: vitrobio@orange.fr

Received on: 21/01/13 Revised on: 25/02/13 Accepted on: 27/02/13

ABSTRACT

Presence of free Herpes Simplex Virus (HSV) particles in the vaginal cavity and consequent continuous infection of new healthy cells delay the recovery from Genital herpes (GHSV). As the HSV envelop contains several surface proteins and since some plant tannins have a strong affinity for these proteins, we incorporated specific tannins into an osmotically active solution to evaluate clinical efficacy.

60 women having visible lesions of genital herpes were treated with RPEX (10ml per day) for 14 consecutive days. Product was administered daily into the vaginal cavity and the symptoms of GHSV were evaluated before treatment and on days 1 (2 h), 4, 7, and 14. Smears from GHSV lesions were also collected to evaluate the number of virus-loaded multinucleated giant cells using Tzanck test. Data were analyzed using CFR21 USFDA software and SAS9.1.3. statistical program.

The mean amount of free virus was diminished by nearly 17%, 44%, 65%, and 100% on days 1, 4, 7, and 14, respectively, with a corresponding reduction in the size of the lesions, and complete recovery on day 14.

HSV surface glycoprotein inhibitors incorporated into an osmotically active solution represents a new approach to treat GHSV.

Keywords: Genital herpes, plant procyanidins, virus glycoprotein inhibition.

INTRODUCTION

Genital herpes (GHSV) is a sexually transmitted disease caused by the double-stranded DNA herpes simplex virus Type 2 (HSV-2) and occasionally by Type 1 (HSV-1). The virus enters into the body through microscopic tears and remains dormant indefinitely. The virus is activated periodically under certain conditions of stress or pregnancy.¹ When the infected person has a herpes outbreak, the virus travels down the nerve fibers to the genital area (vagina or penis), enters into a few cells by attaching with specific cell membrane receptors such as nectin-1, HVEM or 3-O heparan sulfate, and then multiplies.² As the cell lyses liberate free virus particles onto the infected surface, these virions begin attacking adjacent new healthy cells, leading to the formation of blisters and inducing pain, inflammation, itching, vaginal dryness, turbid discharge, and abnormal vaginal flora due to alkaline pH. When the blisters rupture, an even greater amount of free HSV particles is liberated, attacking more new cells and maintaining the state of infection. Symptoms usually last between 15 and 30 days depending upon the functioning of the immune system of the infected person.³

Since this presence of a large amount of infective free virus particles on the infected area is the primary cause of maintaining and further developing the infection, any treatment strategy must be directed to inactivate and to stop new host cell infection. Unfortunately, in absence of any topical antiviral or topical virus entry-blocking drug, only symptomatic treatments are currently used to treat HSV-2 infections.⁴

The HSV envelop contains at least 10 virus encoded glycoproteins (GPs),⁵ and some of these GPs, presumably GPC, GPB, and GPD, interact with the host cell surface heparan sulfate receptors to trigger pH-dependent fusion of the viral envelop with the host cell plasma membrane.^{6,7} Due to the multiplicity of the HSV virus GPs and poor antigenicity, it is also extremely difficult to elaborate an efficient vaccine. Several approaches including subunit

vaccines, peptide vaccines, live virus vectors and DNA vaccine technology have been used to develop prophylactic and therapeutic vaccines but their efficacy remains limited.⁸ It is also postulated that HSV virus use certain topically available matrix metalloproteins (MMPs) for proteolytic degradation of host cell membrane and infection.

The HSV envelop GPs and MMPs are proteinacious in nature, and since certain plant procyanidins (PCDs) have a strong affinity for proteins, we searched specific plant PCDs having strong affinity for these particular proteins using *in vitro* methods as described by Shrivastava.⁹ The hypothesis was that the PCDs may bind to virus GPs, thereby inhibiting virus-cell interaction and new infection. As PCDs are big molecules, they cannot enter into the cells and are naturally eliminated through vaginal secretion. To enhance vaginal secretion, the PCDs were incorporated into an osmotically active, hypertonic solution, as described by Shrivastava,¹⁰ and the resulting solution was named RPEX. After initial *in vitro* selection of the best antiviral PCDs, a pilot clinical trial was conducted to evaluate the efficacy of RPEX in women with acute GHSV infection.

MATERIALS AND METHODS

Selection of anti-GHSV PCDs

Several tannin-rich plants went through extraction process, and the PCD-rich plant extracts were prepared as described by Khanal et al.¹¹ Extracts were atomized for drying and diluted in the culture medium or in the test product base before use. Vero cells were grown *in vitro* following the method published by Shrivastava.¹² The minimum tissue culture infective dose of HSV2 virus capable of killing 50% or 100% vero cells *in vitro* (TCID₅₀ or TCID₁₀₀) was determined. A fixed amount of PCD-rich plant extract (50 µg/ml) was prepared in Dulbecco's Modified Eagle's Medium (DMEM, PEE, France) and pre-incubated in a test tube with TCID₁₀₀ virus concentration for 1h at 37°C to allow PCD-virus GP interaction. HSV-sensitive vero cells were then infected with

the PCD–virus suspension and virus titer was measured. Active plant extracts were then associated with each other at half the concentration (25 µg/ml) and the PCD association (VB-PCDs) capable of neutralizing 90% to 100% virus growth was selected for the preparation of RPEX.

Preparation of test product batches

The test product containing 0.54% VB-PCDs in glycerol as an osmotically active solution (pH 4.5) was prepared as described by Shrivastava et al.¹³ This transparent and viscous solution was filled into 10ml low-density polyethylene tubes with a 4cm long canula for vaginal application.

Table 1: Distribution of External Genital Herpes Lesions in the Patients (n=60).

Total number of patients	Patients presenting outer skin lesions	1 outer genital herpes lesion	2 outer genital herpes lesion	>2 outer genital herpes lesions
60	42/60	16	17	9

Table 2: Number of Patients Rating the Sensation of Vaginal Itching, Redness, Pain, Dryness, Discharge and Presence of Blisters, on a 0 to 3 Scale (0 = No Symptom, 1= Mild, 2 = Moderate, 3 = Severe) at the Start of Treatment, 2h after 1st Product Application and on Days 4,7, and 14. The p-Values Indicate Statistical Significance Compared to the Before Treatment Values.

Parameter	Number of replies (n=60)				p-value
	None	Mild	Moderate	Severe	
Vaginal itching					
Day -1 before treatment	19	18	11	12	0.3430
+ 2h	28	21	8	3	<.0001
Day 4	47	11	2	0	<.0001
Day 7	60	0	0	0	.
Day 14	60	0	0	0	.
Vaginal redness					
Day -1 before treatment	07	18	14	21	0.0620
+ 2h	11	19	18	12	0.3430
Day 4	11	29	20	0	0.0174
Day 7	27	30	3	0	<.0001
Day 14	58	1	1	0	<.0001
Vaginal pain					
Day -1 before treatment	8	12	21	19	0.0620
+ 2h	13	8	32	7	<.0001
Day 4	29	12	13	06	<.0002
Day 7	36	13	09	02	<.0001
Day 14	55	4	1	0	<.0001
Vaginal dryness					
Day -1 before treatment	5	11	25	19	0.0015
+ 2h	14	28	18	0	0.0743
Day 4	17	34	09	0	0.0003
Day 7	20	40	0	0	0.0098
Day 14	57	3	0	0	<.0001
Vaginal discharge					
Day -1 before treatment	16	15	18	11	0.6295
+ 2h	21	10	20	9	0.0433
Day 4	36	12	8	4	<.0001
Day 7	42	15	1	2	<.0001
Day 14	51	9	0	0	<.0001
Presence of blisters					
Day -1 before treatment	04	18	16	22	0.0074
+ 2h	04	18	16	22	0.0074
Day 4	16	17	15	12	0.8174
Day 7	23	24	13	0	0.1572
Day 14	57	2	1	0	<.0001
Vaginal pH	Normal 3.6-4.6	Acidic <3.6	Slightly Alkaline 4.6 -7.0	Highly alkaline >7.0	p-value
Day -1 before treatment	10	2	31	17	<.0001
+ 2h	21	0	36	3	<.0001
Day 4	39	0	19	2	<.0001
Day 7	51	0	7	2	<.0001
Day 14	57	0	3	0	<.0001

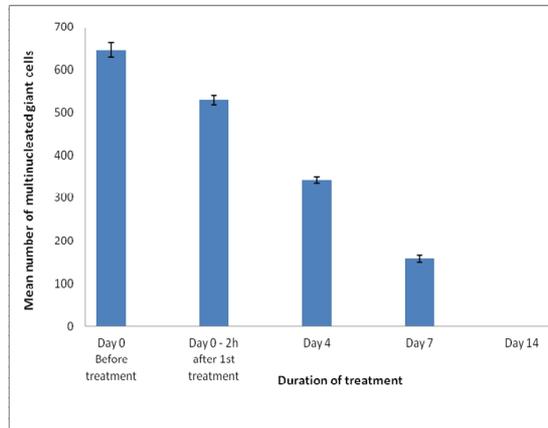


Fig.1: Number of virus-loaded multinucleated giant cells per field in virus-positive patients after Tzanck staining of the HSV lesion smears, compared to before treatment values in the same lesions (\pm SD).

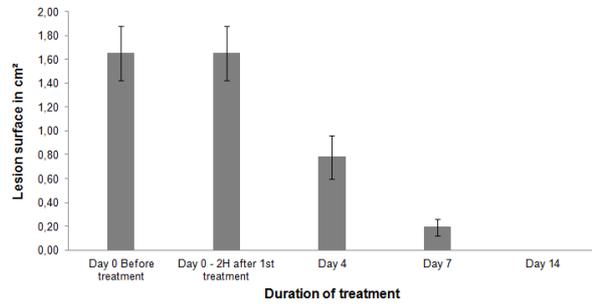


Fig. 2: Mean surface area of 60 lesions calculated by multiplying lesion length by width in cm² (\pm SD). Same lesions were photographed before treatment and on days 4 and 7. All selected lesions were completely healed on day 14.

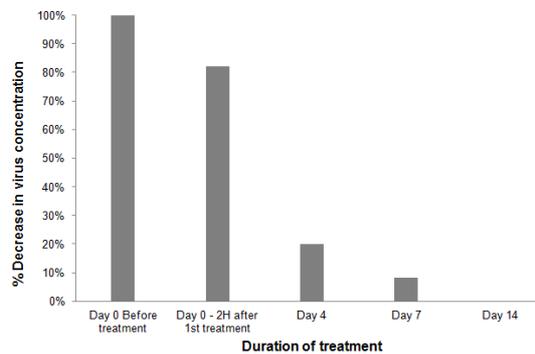


Fig. 3: Mean % decrease in the number of virus-loaded cells per field compared to the surface area of the lesions 2h after 1st treatment, and on days 4 and 7.

CLINICAL TRIAL

Location

An open label, single-arm, prospective, multicentric, pilot study was conducted by the Nexus Clinical Research Pvt Ltd in Mumbai, India, between 08-2009 and 02-2011.

Ethical aspects

The study protocol was approved by Institutional Review Board/ Independent Ethical committee agreed by the Indian Council of Medical research (ICMR) respecting GCP guidelines and following the principles laid down in the declaration of Helsinki and subsequent amendments. The investigative institute is authorized to conduct clinical trials and is regularly inspected by the regulatory authorities. It was decided to stop the treatment in case of any critical event.

Patients

Only women belonging to the 18-65 age group, diagnosed for recent (4-5 days) onset of genital herpes infection (inside or outside of vagina), not undergoing any treatment for genital herpes, willing to participate in the study (written informed consent) and to follow study protocol, were screened for initial selection.

Patients with known immunological disorders or known allergy to any of the ingredients of RPEX, having genital herpes for the last 6 days or more, having other genital infections, or not able to read or write were excluded.

Selection of patients

After initial screening of 89 patients, 64 subjects were recruited for the study. As 4 subjects did not turn up for

follow-up visits, this study includes data obtained from 60 patients.

Efficacy and safety evaluation

After recruitment, the medical history of each patient was recorded in the observation diary. Each patient was asked to fill in the herpes symptom checklist in the diary, evaluating the sensation of itching, redness of vaginal mucosa, pain, vaginal dryness, discharge, pH and presence of sores or blisters on the vaginal mucosa. The intensity of symptoms was rated using a 0 to 3 scoring scale where 0 indicated no symptoms, 1 mild, 2 moderate, and 3 severe symptoms for each parameter recorded. The number and size of external lesions, if any, was also recorded. Although all external lesions were treated, 60 clearly visible lesions were selected in 42 patients and noted in the diary to measure the product's effect on the lesion healing. The size of the lesion was measured by taking photographs at each time point at a fixed distance and in identical fashion, and by measuring its length and width at each time point as suggested by Little et al.¹⁴ Recordings were made before first treatment application and on days 4, 7, and 14, either by the investigator in collaboration with the patient or by the patient herself.

Smears were collected from these 60 selected lesions before first treatment application as well as on days 4, 7, and 14. Smears were stained with Tzanck stain by the same technician for all patients and at each time point, to quantify the number of virus-infected multinucleated giant cells following the method described by Gupta et al.¹⁵

Statistical methods used for data analysis

Clinical data were analyzed using CFR 21 USFDA software and SAS 9.1.3. statistical program. The Wilcoxon Sign Rank test was used when the normality assumption was false. Descriptive Statistics i.e. mean Standard Deviation (SD), minimum and maximum frequency distribution were used for the analysis of the demographic details, clinical evaluation, and medical history, and laboratory parameters. If the p-value was greater than 0.05, the results were considered to be not significant.

RESULTS

Population analyses

Among the 64 women initially enrolled in the study, 4 did not turn up for follow-up visits and were considered drop-outs. The participants had a mean age of 45.85 years (± 10.78 years) and a mean body weight of 64.73 (± 12.63) kg. All 60 patients who completed the study had a positive Tzanck test indicating GHSV infection before the start of treatment, and cultures identified the organism as HSV.

Presence of lesions

As shown in **Table 1**, 42/60 patients had visible outer genital herpes lesions. Among these 42 subjects, 16 had only one lesion while 28 had two or more lesions.

Presence of virus-infected cells in the vaginal smears

As shown in **Fig. 1**, as early as 2h after the first application of RPEX, the mean number of multinucleated giant cells was 529 (± 11.32) compared to 645 (± 17.58) before treatment.

On day 4, the virus was absent in 4 subjects and the average number of multinucleated giant cells with virus had dropped to 341 (± 7.34) in 56 virus-positive patients. These results show a reduction of 47.13% in virus-infected cells within 4 days of start of treatment.

The mean number of infected cells was further reduced to 158 (± 8.77) on day 7 in 51 virus-positive smears, and all smears were virus-negative on day 14. These results show that RPEX treatment progressively reduces the number of virus-infected cells over the treatment period.

Effect on lesion healing

On day 1, the mean surface area of the 60 lesions was 1.65 (± 0.23) cm². Mean surface was reduced to 0.780 (± 0.18) cm² on day 4 and 0.191 (± 0.07) cm² on day 7. All lesions were completely healed on day 14 (**Fig. 2**).

Virus concentration compared to lesion surface

The amount of samples collected to quantify virus concentration (multinucleated giant cells) was always constant despite the fact that the size of the lesion started decreasing right after first RPEX application. Mean reduction in virus-loaded cells per field compared to the mean surface area of the lesion was 17.98% after 2h, 80.18% on day 4 and 91.80% on day 7 (**Fig. 3**).

Herpes symptom checklist parameters

A marked and progressive reduction was observed in genital herpes symptoms such as itching sensation, pain, vaginal discharge, redness and the presence of blisters on the vaginal mucosa. The intensity of most of these symptoms was decreased just 2h after the 1st product application. A statistically significant reduction in all those GHSV symptoms was observed, and most of the patients had only mild symptoms, on day 7 (**Table 2**). All patients noted a significant increase in vaginal discharge about 10 minutes following each product application, which lasted for about 2-

3h. The discharge was transparent, watery, and easily absorbed with a simple cotton protection. The vaginal pH values were highly alkaline in 50/60 patients before the start of treatment, but returned towards the normal acidic range during the first 7 days of treatment. Almost all patients were relieved from symptoms of genital herpes between days 10 and 14.

Other observations

No cases of any adverse reaction, toxicity, local irritation or allergic type of reaction were noticed in any of the patients.

DISCUSSION

Genital herpes is a very common gynecological infection in women, each outbreak causing severe discomfort for an average 3-6 week period.^{16,17} After initial activation, virus particles attack a few superficial cells in and around the genital area, multiply without any severe symptomatic manifestation, and liberate a huge quantity of free virus particles on the infected surface. The virions infect new healthy cells, multiply and shed into the lesion or in the vaginal cavity as multinucleated giant cells.¹⁸ Cell lysis liberates many more new virus particles which maintain the infection over a long period of time. Therefore, any treatment strategy for GHSV infection must be directed at reducing the amount of the free surface virus. However, all the currently available antiviral drugs are intracellular virus growth inhibitors and have no effects on the free virus particles. All the existing or future new antiviral drugs, such as inhibitors of nucleosides, neuraminidase, TAT, reverse transcriptase, and integrase, target intracellular virus growth without affecting the free virus particles present on the surface of the lesion or in the genital cavity.^{19,20}

The results of this study clearly show that the use of specific PCDs capable of binding with virus surface GPs is highly effective in neutralizing free HSV particles and thereby preventing further cell infection. Such an approach should normally eliminate almost all virus particles within a day or two, but PCDs have no effect on intracellular virus reserves which continue emptying into the lesion during the first few days of the infection. The activated virus travels the nerve cells at a speed of about 1 mm per day, and continues infecting new cells, which explains why a minimum 7 to 10-day treatment is essential to eliminate the virus entirely from the lesions.

The specific PCDs were incorporated into a hypertonic glycerol solution to obtain RPEX. The presence of an osmotically active film over the genital mucosa or peripheral genital lesion surface will mechanically attract the hypotonic liquid from the inner parts of the mucosa, thereby cleaning all the tannin-virus conjugated molecules as well as other contaminants. The transparent and watery vaginal discharge observed during the first 2-3h of RPEX use may be related to the osmotic properties of the test product. As the free virus particles are continuously eliminated from the lesions and the vaginal cavity, further progression of the infection is stopped, and consequently the symptoms of genital herpes are also progressively reduced. The test product's pH of 4.0 to 4.5 helps normalize the alkaline pH of the vaginal cavity and restore normal vaginal flora. The absence of any systemic reaction and the rapidity of onset of the therapeutic effects are attributable to the drug's purely mechanical and topical mode of action on the surface of the vaginal cavity.

CONCLUSIONS

The results of this study clearly show that the topical application of specific virus GP-neutralizing plant tannins in an osmotically active solution represents a highly promising new scientific approach to treat GHSV.

ACKNOWLEDGEMENTS

This research was entirely supported by VITROBIO Research Institute, ZAC de Lavour, 63500, Issoire, without any other sponsor or funding source.

REFERENCES

1. Money D, Steben M. Society of Obstetricians and Gynaecologists of Canada. SOGC clinical practice guidelines: Guidelines for the management of herpes simplex virus in pregnancy. Number 208, June 2008. *Int J Gynaecol Obstet.* 2009; 104(2):167-171. PMID: 19241496
2. Connolly SA, Jackson JO, Jardetzky TS, Longnecker R. Fusing structure and function: a structural view of the herpesvirus entry machinery. *Nat Rev Microbiol.* 2011; 9(5):369-381. Epub 2011 Apr 11. PMID: 21478902
3. Brugha R, Keersmaekers K, Renton A, Meheus A. Genital herpes infection: a review. *Int J Epidemiol.* 1997; 26(4): 698-709. PMID: 9279600.
4. Hamuy R, Berman B. Topical antiviral agents for herpes simplex virus infections. *Drugs Today (Barc).* 1998; 34(12):1013-1025. PMID: 14743269
5. Akhtar J, Shukla D. Viral entry mechanisms: cellular and viral mediators of herpes simplex virus entry. *FEBS J.* 2009; 276(24): 7228-7236. PMID: 19878306
6. Rajcáni J, Vojvodová A. The role of herpes simplex virus glycoproteins in the virus replication cycle. *Acta Virol.* 1998; 42(2):103-118. PMID: 9770079.
7. Spear PG, Manoj S, Yoon M, Jogger CR, Zago A, Myscofski D. Different receptors binding to distinct interfaces on herpes simplex virus gD can trigger events leading to cell fusion and viral entry. *Virology* 2006; 344(1):17-2. PMID: 16364731.
8. Ramachandran S, Kinchington PR. Potential prophylactic and therapeutic vaccines for HSV infections. *Curr Pharm Des.* 2007; 13(19):1965-1973. PMID: 17627530.
9. Shrivastava R. A new therapeutic approach to neutralize throat surface proteases and virus glycoproteins simultaneously for the treatment of influenza virus infection. *International J Virol.* 2011. ISSN 1816-4900 / DOI: 10.3923/ijv.2011.
10. Shrivastava R. Non-solid composition for local application. Patent PCT/FR99/01340, International publication N°WO 00/74668 A1 on 14/12/2000.
11. Khanal RC, Howard LR, Brownmiller CR, Prior RL. Influence of extrusion processing on procyanidin composition and total anthocyanin contents of blueberry pomace. *J Food Sci.* 2009; 74(2): H52-58. PMID: 19323751.
12. Shrivastava R. Clinical evidence to demonstrate that simultaneous growth of epithelial and fibroblast cells is essential for deep wound healing. *Diabetes Res Clin Pract.* 2011; 92-99
13. Shrivastava R. New synergistic compositions for the treatment of topical viral infections. Patent PCT/EP2010/050236, International publication N° WO 2011/082835 A1 on 14/07/2011.
14. Little C, McDonald J, Jenkins MG, McCarron P. An overview of techniques used to measure wound area and volume. 2009; 18(6):250-253.
15. Gupta LK, Singhi MK. Tzanck smear: A useful diagnostic tool. *Indian J Dermatol Venereol Leprol [serial online]* 2005 [cited 2011 Feb 15]; 71:295-299. PMID: 19661849.
16. Hossain A, Bakir TF, Siddiqui MA, Khawaja MM, De-Silva S, Sengupta BS, et al. Genital herpes simplex virus infections: laboratory confirmation in diverse patient groups. *Int J Gynaecol Obstet.* 1989; 29(1):51-56. PMID: 2566530.
17. Tanzi P, Lojaco A, Tarantini M, Faden D. Herpes gestationis. *Int J Gynaecol Obstet.* 1994; 45(1): 47-49. PMID: 7913059
18. Rimdusit P, Yoosook C, Srivanboon S, Sirimongkolkasem R, Pumeechockchai W. Prevalence of genital herpes simplex infection and abnormal vaginal cytology in late pregnancy in asymptomatic patients. *Int J Gynaecol Obstet.* 1989; 30(3): 231-236. PMID: 2575048.
19. Lingappa JR, Celum C. Clinical and therapeutic issues for herpes simplex virus-2 and HIV co-infection. *Drugs* 2007; 67(2):155-174.
20. Littler E, Oberg B. Achievements and challenges in antiviral drug discovery. *Antivir Chem Chemother.* 2005; 16(3):155-168. PMID: 16004079

QUICK RESPONSE CODE 	ISSN (Online) : 2277-4572 Website http://www.jpsionline.com
---	---

How to cite this article:

Ravi Shrivastava. Associating a hypertonic solution with specific plant Procyanidins for the treatment of genital herpes. *J Pharm Sci Innov.* 2013; 2(1): 47-51.