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ORIGINAL ARTICLE

In vitro studies of the antiherpetic effect of photodynamic therapy

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Abstract The number of viral infection cases in the Department of Gynecology and Obstetrics has tended to increase over last few years. Viruses form herpesvirus and cytomegalovirus families are associated with an increased risk for recurrent pregnancy loss. Photodynamic therapy (PDT) is a promising new approach to treat viral infections in which viral particles are inactivated. It exhibits great therapeutic potential, particularly among this group of patients. This study examined the use of PDT to treat herpesvirus infection (HVI) using an in vitro model. In this study, we used the Vero cell lineage as a suitable model of HVI, strains of HSV-1 (strain VR-3) and HSV-2 (strain MS) obtained from The National Virus Collection (London, UK), the photosensitizer Fotoditazine (Veta-Grand, Russia), an AFS physiotherapeutic device (Polironic Corporation, Russia). Laser light irradiation and the photosensitizer had different cytotoxic effects on the Vero cell cultures depending on the doses used. The optimal laser light and photosensitizer doses were determined. PDT had an antiviral effect on an in vitro model of HVI in cell culture. PDT has been shown to be effective treatment for HVI in vitro, leading to a reliable decrease of viral titer.

Keywords Herpes simplex virus · Photodynamic therapy · Photosensitizer · Fotoditazine · Recurrent pregnancy loss

Introduction

Herpes simplex virus (HSV) is a DNA virus known to cause primary genital infection. HSV may lead to chronic recurrent infections characterized by recurrent clinical course due to permanent viral presence in the body [1–3]. HSV remains a major worldwide health problem affecting women of reproductive age. This virus can be transmitted via asymptomatic viral shedding. More than 90 % of the population worldwide is infected with HSV, and 20 % of infected individuals exhibit

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clinical manifestations [4]. HSV infection is nearly incurable due to resistance of the virus to antiviral therapy [5, 6].

Long-term viral persistence contributes to immunodeficiency, which is characterized by decreased interferon and cytokine production [1, 7]. Weakened immunity results in HSV reactivation, which leads to generalized infection, affecting the placenta and fetus in pregnant infected women. Therefore, herpesvirus infection (HVI) is considered to be a major cause of spontaneous abortion and antenatal fetal death [8].

Recent studies have shown that HVI is a leading cause of obstetric and perinatal complications and that it may contribute to recurrent pregnancy loss. Viral infections during pregnancy may cause infertility, premature delivery, spontaneous abortion, antenatal fetal death, as well as developmental defects and prenatal infections, which manifest during the postnatal period [9]. Herpes simplex virus-2 (HSV-2) and cytomegalovirus infections are the most common causes of pregnancy-associated pathologies, miscarriage, and recurrent pregnancy loss [10, 11]. Although standard HVI therapy has been successfully used to prevent recurrent pregnancy loss, the incidence of HVI remains high in the Gynecology and Obstetrics Department, suggesting the need of a new safe and effective therapy [7, 12].

Currently, many physicians treat chronic HVI using nonpharmaceutical treatments. Photodynamic therapy (PDT) represents a potentially useful method for the treatment of chronic forms of HVI. It is a sophisticated method based on photochemical reactions that cause cell damage. It has been widely used to treat cancer [13] and other pathologies involving hyperproliferation and/or neovascularization of tissues, such as atherosclerosis of the aorta and large arteries, nonmalignant prostate hyperplasia, chronic wounds, trophic ulcers, cervical or vulval dysplasia, and pointed condylomas [14–18]. Recent studies have focused on the potential antimicrobial application of PDT and have demonstrated its efficacy in the treatment of bacterial and fungal infections [19–21]. Strong evidence of an antiviral effect of PDT has also been reported. Some studies have shown that membrane viruses lose their infectivity after treatment with a photosensitizer and red light irradiation, demonstrating the potential use of PDT to treat viral infections. In addition, Yin H et al. assessed the photoinactivation of a cell-free HIV by hematoporphyrin monomethyl ether and found that all of the tested virus particles were completely responsive to HMME-PDT [22]. Another research group investigated the effectiveness of PDT against HIV [23, 24]. Further, recent study examined the effects of PDT against human papillomavirus, reporting positive experimental and clinical results.

PDT is known to stimulate the immune system; however, it is necessary to increase the current understanding of the immune response following antiherpetic PDT. In this study, we aimed to evaluate whether PDT is effective as an antiviral therapy in HVI by examining its antiherpetic effect in vitro. In future studies, we aim to establish a scientific basis for clinical application of antiherpetic PDT in women with recurrent miscarriage.

Materials and methods

Cell culture Vero cells represent one of the most adequate in vitro models for studying HVI due to their high affinity to HSV [25, 26]. The Vero cell lineage was isolated from kidney epithelial cells extracted from an African green monkey. Vero cells are commonly used as host cells for growing viruses. Considering this information, the Vero cell lineage was considered appropriate for use as an in vitro model in this study. Cells were cultured in sterile 96-well flat-bottom plates (Costar, USA). Then, 10,000–50,000 cells containing 5 or 7.5 % embryonic calf serum (HyClone, USA) were added to the plates after resuspension in DMEM without glutamine (PanEco, Russia). The culture of cells was reseeded every 3–4 days.

Viruses Strains of HSV-1 (strain VR-3) and HSV-2 (strain MS) were obtained from The National Virus Collection (London, UK). Prior to the use in our experiments, all strains of viruses were passaged 5–7 times in cultured Vero cells.

Photosensitizer The photosensitizer Fotoditazine (Veta-Grand, Russia) is part of a new generation of optimized photosensitizers that contain chlorine E_6 as the major component. It was registered in Russian Federation in 2003. In our study we used a sterile solutions of Fotoditazine at concentrations of 0, 10, 50, 100, and 200 μg/mL dissolved in saline (0.9 % NaCl) that were prepared prior to each experiment.

Laser light source For the laser light, an AFS physiotherapeutic device (Polironic Corporation, Russia), with a wavelength of $\lambda = 662$ nm and an exit power of 180 mWt was used. The technical parameters for PDT were all follows: density of power = 0.06 Wt/cm^2, diameter of light spot = 2 cm, area of the beam = 3.14 cm^2, and irradiation times = 5, 10, and 30 ss and 1 and 3 min. Calculation of the energy in joules was performed using the following equations:

$$\begin{gathered} \mathrm{P\ density\ of\ power}\left(\mathrm{Wt/cm^2}\right) = \mathrm{Pexit\ power(Wt)/S\ area\ of\ the\ beam}\left(\mathrm{cm^2}\right); \\ \mathrm{S} = \pi \cdot \mathrm{d^2 diameter\ of\ light\ spot}\left(\mathrm{cm^2}\right)/4; \\ \mathrm{W}\left(\mathrm{J/cm^2}\right) = \mathrm{Pdensity\ of\ power} \cdot \mathrm{t\ irradiation\ time(s)}, \end{gathered}$$

The experiments were moderated with different energy doses of red visible light (0.285, 0.57, 1.8, 3.42, and 10.62 J/cm^2).

The cell samples were irradiated in test tubes individually with the laser light at doses of 0.285, 0.57, 1.8, 3.42, and 10.62 J/cm^2. After irradiation, all samples were plated in cell-culture plates and incubated at 37 °C in an atmosphere containing 5 % CO_2 for 48 h. Cell viability was estimated

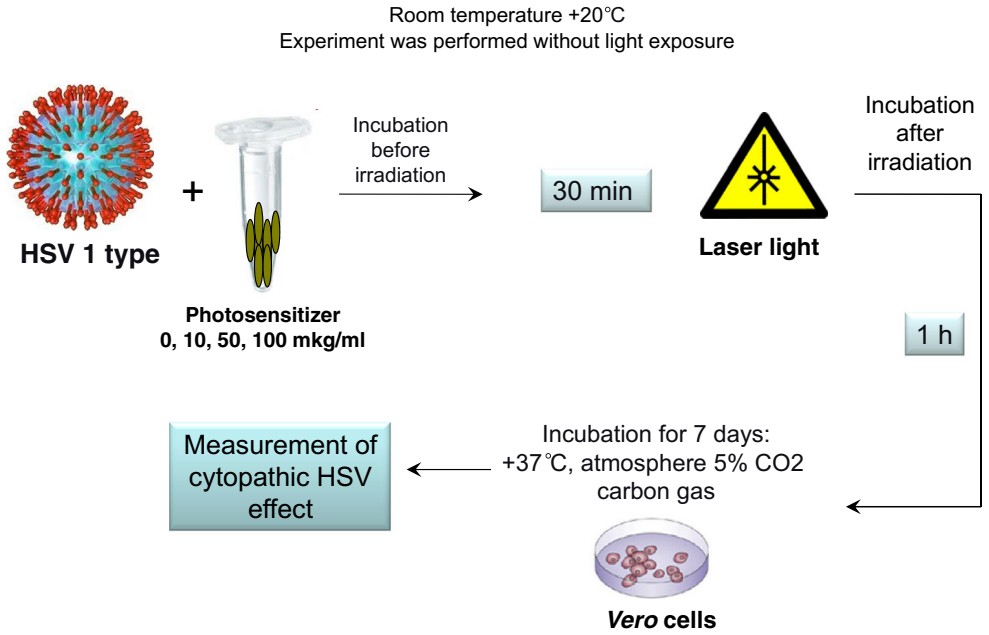

Fig. 1 Antiviral effect of photosensitizer «Fotoditazine» on HSV - 1 and HSV - 2

visually using a light microscope. Then, we determined the optimal dose of photosensitizer for which changes in cell viability were not significant. The procedure included a preparation of solutions with photosensitizer at concentrations of 0, 10, 50, and 100 μg/mL. A 200 μL volume of each solution was added to the cultured cells. After incubation with the photosensitizer for 30 min in the dark, all samples were irradiated with laser light. Irradiation was administered at doses of 0.285, 1.8, and, 3.42 J/cm^2. The distance from the test tube to the laser light source was 0 cm. After irradiation, the cells were incubated, similar to the previous experiment. Cell viability was estimated visually using a light microscope.

For the second stage, we studied the effect of the photosensitizer Fotoditazine on HSV-1 virus (Fig. 1). First, a solution containing HSV-1 was added to solutions containing the photosensitizer at concentrations of 0, 10, 25, 50, 100, and 200 μg/mL. One group of samples was immediately irradiated at the optimal dose (1.8 J/cm^2), and another group was irradiated after 30 min of incubation. A third group was irradiated after 1.5 h of incubation. Immediately after irradiation, one group of samples was added to Vero cell cultures for 24 h, whereas other two groups were incubated first for 1 or 3 h before addition to Vero cell cultures (time exposure = 24 h). Then, DMEM culture medium containing 2 % fetal calf serum was added to the cells, and they were incubated at 37 °C in a 5 % CO_2 atmosphere for 7 days. Inhibition of virus activity after PDT of HSV-1 and -2 was determined based on the inhibition of viral cytopathic activity in cultured cells, measured as the cytopathic dose (lg TCD50/mL).

To estimate an immediate effect of PDT on HSV-1 in vitro (Fig. 2) Vero cells were infected with HSV-1, and then the optimal concentration of photosensitizer (10 μg/mL) was added. The cells were irradiated with an optimized dose of laser light (1.8 J/cm^2). The time of incubation before and after irradiation was 30 min.

The complex effect of laser light and the photosensitizer on HSV-1-infected cultured cells was determined based on the

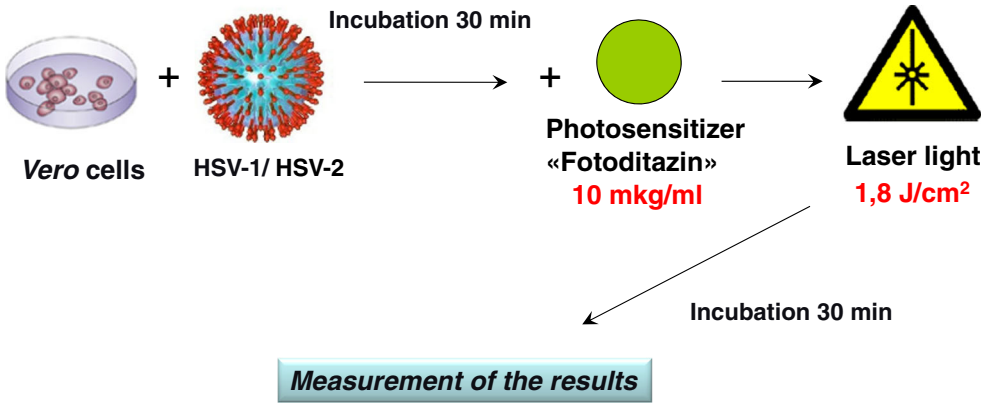

Fig. 2 The immediate action of PDT on HSV-1 and HSV-2 in vitro

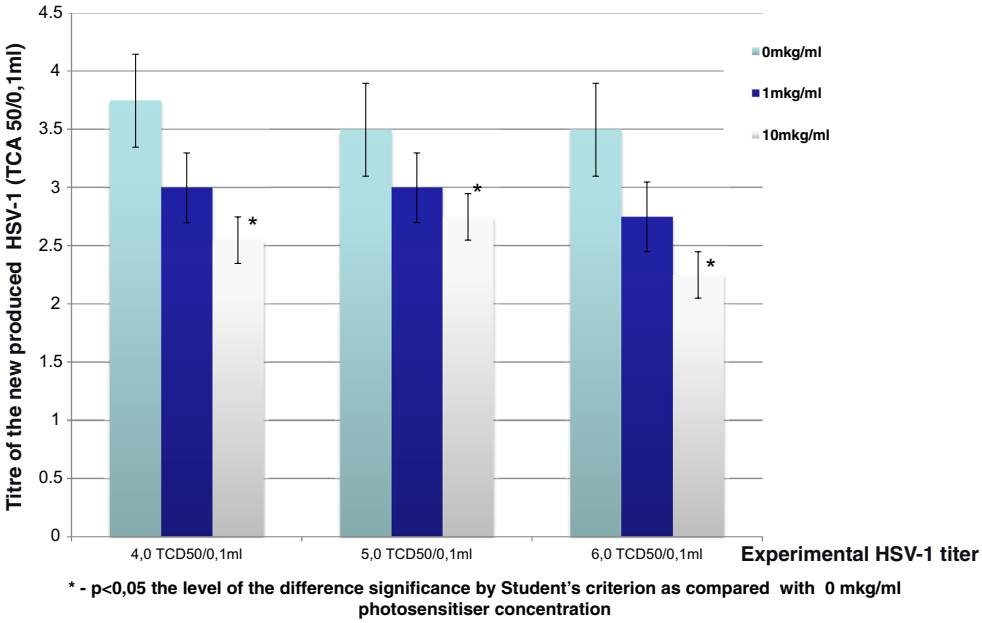

Fig. 3 Proliferation and cell death as a result of cytotoxic PDT effect with photosensitizer «Fotoditazine» in vitro HVI model

effect of PDT on the titer of newly produced virus, termed "viral posterity" (Fig. 3).

Analysis Data were obtained by analyses of cell cultures under a luminous microscope (Optica Fluoa, Italy) at 200–400× magnification. The Reid and Mench method was used to determine the viral titers [27]. Viral cytotoxicity was determined using the MTT test with vital colorant [28]. Statistical analyses were conducted using widely accepted methods for variational statistics.

Results

We measured the cytotoxicity caused by the laser light and the effects of various doses of the photosensitizer (Fig. 4a, b) on

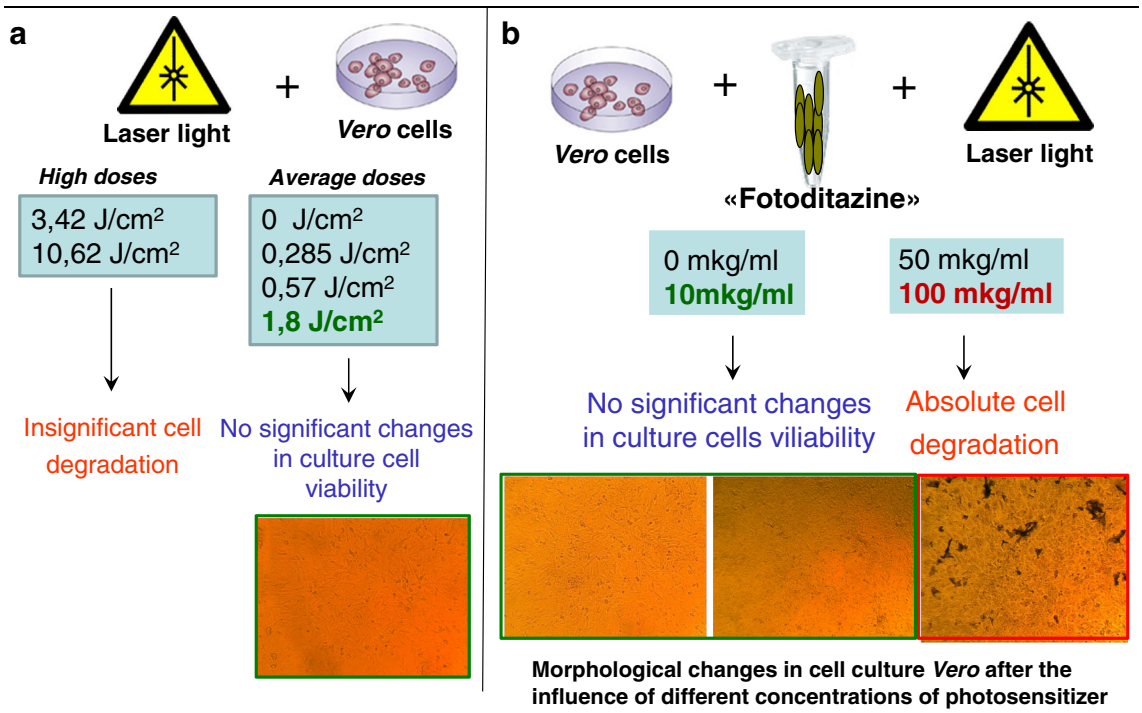

Fig. 4 Estimation the cytotoxity of laser light and evaluation the effects of various doses of the photosensitizer. Scheme of experiment. **a**. Cytotoxic effect of laser light **b**. Effects of various doses of the photosensitizer

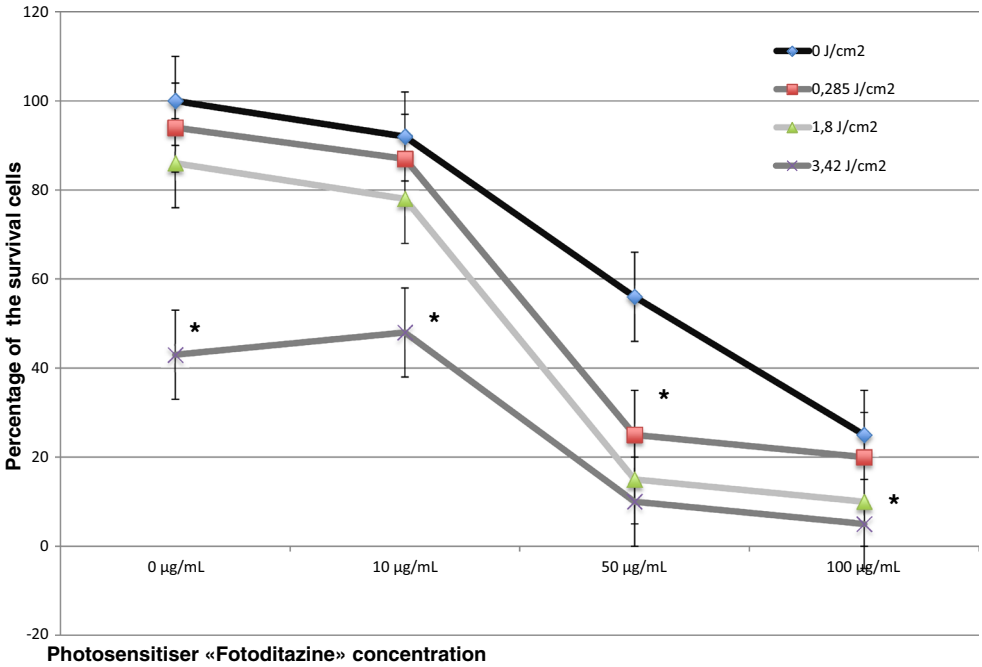

Fig. 5 The toxic effect of photosensitizer «Fotoditazine» in the complex with laser light on the culture cells

* - p<0,05 the level of the difference significance by Student's criterion as compared with culture cells non irradiated with laser light

cultured cells. In addition, we determined the optimal doses of laser light and photosensitizer that did not cause significant observable changes in cell viability.

The maximum dose of laser light that caused no significant changes in cell cultures was 1.8 J/cm^2. Cell degradation was observed at the other experimental doses of laser light (3.42 and 10.62 J/cm^2).

We found that 50 and 100 μg/mL concentrations of the photosensitizer caused complete cell degradation, indicating that these concentrations were cytotoxic. The doses of laser light tested did not influence cell viability in this assay. The visible changes to cultured cells exposed to different doses of laser light were similar at photosensitizer concentrations of 50 or 100 μg/mL. A

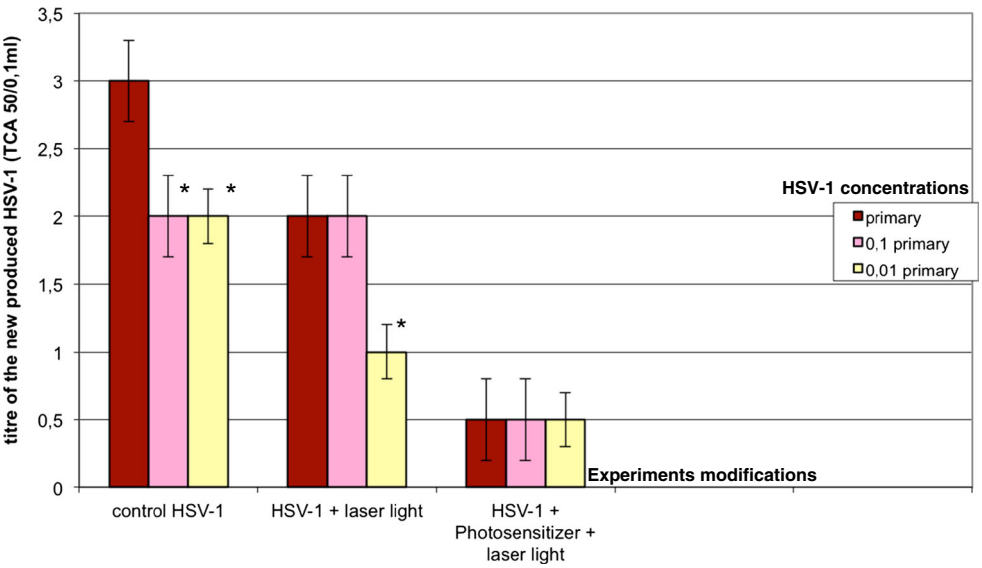

Fig. 6 Cytotoxic effect of PDT with photosensitizer «Fotoditazine» on in vitro model of HSV-1 infection by measuring cell proliferation and death

*** -p <0,05 the level of the difference significance by Student's criterion as compared with control group (primary HSV-1 concentration)**

**** -p <0,05 the level of the difference significance by Student's criterion as compared with control group (HSV-1 concentration 0,01)**

photosensitizer concentration of 10 μg/mL resulted in minor changes to the cultured cells (Fig. 5). Therefore, we selected this photosensitizer concentration together with an optimal experimental dose of irradiation for subsequent experiments.

Based on the results of these experiments, we determined that the optimal concentration of photosensitizer was less than 50 μg/mL and the optimal laser light dose was 1.8 J/cm^2. The following optimal conditions for the photodynamic inhibition of HVI were selected: photosensitizer—10 μg/mL; laser light—1.8 J/cm^2.

The optimal experimental conditions for virus inhibition included a time limit of incubation before and after irradiation of less than 30 min. Photosensitizer doses of 50 μg/mL or more led to decreases in HSV-1 viral titer of 1000-fold or greater (1.5–2.5 orders of magnitude) (Fig. 6).

Thus, the optimal conditions for evaluating the antiherpetic effect of PDT in vitro were as follows: 10 μg/mL photosensitizer (the cytotoxicity at this concentration was 25 %), 1.8 J/cm^2 laser light irradiation for 30 s (the cytotoxicity at this dose was 25 %) and less than 30 min incubation before and after irradiation.

We discovered that the use of laser light and photosensitizer together may consistently decrease viral titer by more than two orders of magnitude (100–1000 times).

Discussion

Phototherapy is a new promising treatment for HVI. Our in vitro experiment revealed that the use of PDT with the photosensitizer Fotoditazine was effective for the treatment of HSV-infected culture cells.

Our in vitro model of HVI is of practical importance because it provides evidence on the antiviral effect of the PDT on HSV. Our results establish a basis for future study of the antiviral mechanisms of PDT both in vitro and in vivo, as well as for the development of novel treatments for patients with chronic forms of HVI and recurrent pregnancy loss. Moreover, this approach can be applied, either separately or as part of a complex treatment, to treat chronic forms of HVI. In future studies, we aim to investigate the mechanisms that influence the interactions of PDT with the immune system. Skillful future application of these principles may be used to eliminate HSV in women who experience miscarriages and recurrent pregnancy loss. We anticipate that the use of PDT will result in improved pregnancy outcomes in this category of patients.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Kim ID, Chang HS, Hwang KJ (2012) Herpes simplex virus 2 infection rate and necessity of screening during pregnancy: a clinical and seroepidemiologic study. Yonsei Med J 53(2):401–407. doi:10.3349/ymj.2012.53.2.401
2. Arenas Y, Monro S, Shi G, Mandel A, McFarland S, Lilge L (2013) Photodynamic inactivation of Staphylococcus aureus and methicillin-resistant Staphylococcus aureus with Ru(II)-based type I/type II photosensitizers. Photodiagn Photodyn Ther 10(4):615–625. doi:10.1016/j.pdpdt.2013.07.001
3. Nafee N, Youssef A, El-Gowelli H, Asem H, Kandil S (2013) Antibiotic-free nanotherapeutics: hypericin nanoparticles thereof for improved in vitro and in vivo antimicrobial photodynamic therapy and wound healing. Int J Pharm 454(1):249–258. doi:10.1016/j.ijpharm.2013.06.067
4. Wilson SS, Fakioglu E, Herold BC (2009) Novel approaches in fighting herpes simplex virus infections. Expert Rev Anti-Infect Ther 7(5):559–568. doi:10.1586/eri.09.34
5. Kovalchuk LV, Gankovskaya LV, Gankovskaya OA, Lavrov VF (2007) Herpes simplex virus: treatment with antimicrobial peptides. Adv Exp Med Biol 601:369–376
6. Gankovskaia OA, Koval'chuk LV, Gankovskaia LV, Lavrov VF, Romanovskaia VV, Kartashov DD, Fenzeleva VA (2008) Role of Toll-like receptors and defensins in antimicrobial protection of urogenital tract in females. Zh Mikrobiol Epidemiol Immunobiol 1:46–50
7. Juarranz A, Jaen P, Sanz-Rodriguez F, Cuevas J, Gonzalez S (2008) Photodynamic therapy of cancer. Basic principles and applications. Clin Transl Oncol 10(3):148–154
8. Shurshalina AV, Veryasov VN, Sukhikh GT (2001) The ratio of cytokine levels in genital herpes during various phases of infection. Bull Exp Biol Med 132(1):660–662
9. Anzivino E, Fioriti D, Mischietelli M et al (2009) Herpes simplex virus infection in pregnancy and in neonate: status of art of epidemiology, diagnosis, therapy and prevention. Virol J 6:40
10. Gardiner DW, Lunn DP, Goehring LS, Chiang YW, Cook C, Osterrieder N, McCue P, Del Piero F, Hussey SB, Hussey GS (2012) Strain impact on equine herpesvirus type 1 (EHV-1) abortion models: viral loads in fetal and placental tissues and foals. Vaccine 30(46):6564–6572. doi:10.1016/j.vaccine.2012.08.046
11. Gankovskaia OA, Bakhareva IV, Gankovskaia LV, Somova OI, Zverev VV (2009) Study of expression of TLR9, NF-kappaB, TNFalpha genes in cells of cervical canal mucosa in pregnant women with herpesvirus infection. Zh Mikrobiol Epidemiol Immunobiol 2:61–64
12. Matthews YJ, Damian DL (2010) Topical photodynamic therapy is immunosuppressive in humans. Br J \Dermatol 162:637–641. doi:10.1111/j.1365-2133.2009.09562.x
13. Kasermann F, Kempf C (1998) Buckministerfullerene and photodynamic inactivation of viruses. Rev Med Virol 8:143–151
14. Tan DH, Murphy K, Shah P, Walmsley SL (2013) Herpes simplex virus type 2 and HIV disease progression: a systematic review of observational studies. BMC Infect Dis 13(1):502
15. Jones CA (2009) Vertical transmission of genital herpes. prevention and treatment options. Drugs 69(4):421–434. doi:10.2165/00003495-200969040-00003
16. Vibholm L, Reinert LS, Sogaard OS et al (2012) Antiviral and immunological effects of tenofovir microbicide in vaginal herpes simplex virus 2 infection. AIDS Res Hum Retrovir 28(11):1–8. doi:10.1089/AID.2012.0078
17. Moor AC, Wagenaars-van-Gompel AE, Brand A, Dubbelman MA, VanSteveninck J (1997) Primary targets for fotoinactivation of vesicular stomatitis virus by AlPcS4 or Pc4 and red light. J Photochem Photobiol 65(3):465–470

18. Malik Z, Hanania J, Nitzan Y (1990) Bacterial effect of photoactivated porphyrins—an alternative approach to antimicrobal drugs. J Photochem Photobiol B Biology 5: 281–293
19. Yang YG, Zou XB, Zhao H, Zhang YJ, Li HJ (2012) Photodynamic therapy of condyloma acuminata in pregnant women. Chin Med J 125(16):2925–2928
20. Calvino-Fernández M, García-Fresnadillo D, Benito-Martínez S, McNicholl AG, Calvet X, Gisbert JP, Parra-Cid T (2013) Helicobacter pylori inactivation and virulence gene damage using a supported sensitiser for photodynamic therapy. Eur J Med Chem 68:284–290. doi:10.1016/j.ejmech.2013.07.023
21. Kashiwabuchi RT, Khan Y, Carvalho FR, Hirai F, Campos MS, McDonnell PJ (2012) Antimicrobial susceptibility of photodynamic therapy (UVA/riboflavin) against Staphylococcus aureus. Arq Bras Oftalmol 75(6):423–426
22. Yin H, Li Y, Zheng Y, Ye X, Zheng L, Li C, Xue Z, Yin H, Li Y, Zheng Y, Ye X, Zheng L, Li C, Xue Z (2012) Photoinactivation of cell-free human immunodeficiency virus by hematoporphyrin monomethyl ether. Lasers Med Sci 27(5):943–950. doi:10.1007/s10103-011-1013-z
23. Xu J, Xiang L, Chen J, He Q, Li Q, Li J, Wang J (2013) The combination treatment using CO_2 laser and photodynamic therapy for HIV seropositive men with intraanal warts. Photodiagn Photodyn Ther 10(2):186–193. doi:10.1016/j.pdpdt.2012.11.005
24. Abe H, Yamada-Ohnishi Y, Hirayama J, Owada T, Ikeda H, Ikebuchi K (2000) Elimination of both cell-free and cell-associated HIV infectivity in plasma by a filtration/methylene blue photoinactivation system. Transfusion 40(9):1081–1087
25. Kelly BJ, Bauerfeind R, Binz A, Sodeik B, Laimbacher AS, Fraefel C, Diefenbach RJ (2014) The interaction of the HSV-1 tegument proteins pUL36 and pUL37 is essential for secondary envelopment during viral egress. Virology 454–455:67–77
26. Sauter MM, Gauger JJ, Brandt CR (2014) Oligonucleotides designed to inhibit TLR9 block Herpes simplex virus type 1 infection at multiple steps. Antiviral Res 109C:83–96. doi:10.1016/j.antiviral.2014.06.015
27. Glants S. Medical and biological statistics. M.: Praktika, 1998; P. 459.
28. Bajgar R, Kolarova H, Bolek L, Binder S, Pizova K, Hanakova A (2014) High oxygen partial pressure increases photodynamic effect on HeLa cell lines in the presence of chloraluminium phthalocyanine. Anticancer Res 34(8):4095–4099