

Original Article

Effects of Morphine on Replication of Herpes Simplex Virus Type 1&2

Monavari SH^{1*}, Shamsi-Shahrabadi M¹

1. Department of Virology and Antimicrobial resistance research center, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

Abstract

Background and Aims: Several drugs are being used in treatment of HSV infection in human but still introducing an effective safe drug is desirable.

Methods: We investigated the inhibitory effect of morphine on replication of HSV in vitro.

Results: The results indicated that a concentration of up to 200 ug/ml morphine had a limited effect on Vero cell viability. At this concentration the growth of HSV was inhibited considerably and after the third passage in presence of morphine it was completely diminished. Presence of viral antigens in infected cells in presence of morphine by IF staining showed that after the first passage a small number of infected cells contained viral proteins and at the third passage no cells with viral antigen was observed.

Conclusion: This was confirmed by page and immunoblotting techniques. Electron microscopy observation in cellular section indicated that there was no virus present in treated cells as compared with control untreated infected cells.

Keywords: Herpes simplex virus; morphine; Cytotoxicity

Introduction

Herpes simplex virus type 1&2 belongs to Alpha-herpesvirinae subfamily of Herpesviridae family. HSV virus genome has a double strand DNA which codes over 70 gene products. HSV infection is the most common viral infections in human and causes an extended range of diseases (1, 4, and 5). There are several antiviral drugs which are effective against HSV infections that most of them inhibit viral DNA synthesis. Recently, physicians and researchers have faced with the problem of elongated treatment with Acyclovir due to formation of drug resistant mutants and

toxicity of the drug (6,8, 19, and 20). However, resistance has been reported, mainly among immunocompromised patients (prevalence around 5%) and particularly allogeneic bone marrow transplant patients (prevalence reaching 30%). Resistance to ACV is associated with mutations on one of the two viral enzymes involved in the ACV activation: thymidine kinase (TK) and DNA polymerase. In 95% of the cases, ACV resistance is associated with a mutation in the TK gene as this enzyme is not essential for viral replication, unlike viral DNA polymerase it seems that an alternative method of treatment HSV infection by using a new effective drug is in demand. It has been shown that Morphine has the potential in preventing HSV pathogenesis in certain lab animals. The main objective of this study was to determine the inhibitory effect of morphine on HSV replication and growth in vitro and to

*Corresponding author: Seyed Hamidreza Monavari, PhD, Department of Virology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
Tel/Fax: (+98) 21 88 60 22 05
Email: hrmonavari@yahoo.com

investigate its possible use for treatment of HSV infection.

Methods

Cell culture and virus

Vero cells were grown in disposable plastic dishes or in 24 well plates and incubated in Dulbeccos Modified Eagles (DMEM) supplemented with 8% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 µg/ml of streptomycin.

Herpes simplex virus type 1 (HSV-1) was isolated from a patient and identified by specific monoclonal anti HSV-1 antibodies.

Virus titration

Virus titration was performed by both TCID₅₀ and plaque assay procedures. For TCID₅₀ cells were grown in 24 wells tissue culture dishes and the test was performed according to the Reid & Muntch method. For plaque assay, monolayer of Vero cells were grown in 24 wells tissue culture dishes and were infected with serial dilutions of HSV virus. The infected monolayer were overlaid and incubated at 37°C for 72 hr. A second overlay containing 0.1% neutral red was used and plaques were counted after 12 hr.

Determination of morphine cytotoxicity

All samples were tested for the presence of HSV-1 DNA by Real Time PCR method (Rotor Gene 6000, Corbett Research, Australia). PCR was performed using specific Primers and probe for detection HSV-1 DNA by Real Time PCR. Primers and probe were designed by Primer3 plus online tools (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) from conserved domain of Glycoprotein B gene (Table 1). 10 µl of DNA was added to 10 µl of the reaction mixture containing 2 units TaqMan polymerase, 0.01% gelatin, 0.6 µM of each primer, 0.2 µM of probe, 200 µM of each deoxynucleotid triphosphate, 5 µl of reaction buffer (50 mM KCl, 10 mM tris-HCl, pH = 8.3) and 3 mM MgCl₂. Polymerase chain reaction was performed at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15s, 60°C for 40s and fluorescence detection on the channels Fam

(Green), for HSV-1 at 60°C. Before starting the runs, the tubes were kept at 50°C for 2 minutes for activation of UNG. Standard precautions were taken to avoid sample-to-sample contamination and PCR product carryover. As positive controls, we used DNA extracted from HSV-1 KOS Strain, infected Vero cells and a mixture without DNA template was used as negative control. Cytotoxicity was evaluated by the neutral red staining (NRS) method and examined by the growth inhibition of Vero cells. Briefly; Vero cells were grown in 24-well plates. After formation of complete monolayer, the culture medium was replaced by similar medium containing various concentration of morphine. After 4 days incubation at 4°C, 100 unit of 120 µg/ml of neutral red was added to each cell monolayer and incubated for 3 hr at 37°C. After that the cells were washed with 0.5% formaldehyde containing 1 % Cacho. The cells were extracted in citrate-ethanol buffer for 1 hr at 4°C. The absorbency of extracted solution was measured at 550 wave length.

Virus purification

For animal immunization, purified virus was required. HSV was purified on sucrose cushion followed by sucrose gradient centrifugation. Discontinuous sucrose gradient was formed by successive layering of 50, 40, 30, 20% sucrose (made up in O.OIM Tris buffer). These gradients were then centrifuged at 18000 rpm/min for 2hr using TST28.38 rotor. The virus band was suspended in a small volume of 0.01 M Tris buffer and frozen at -20°C.

Preparation of specific antiserum

Purified virus was mixed with Freund's adjuvant and used to inoculate female New Zealand white rabbits. Three injections were given at weekly intervals and the last booster was given three weeks later. Rabbits were bled 10 days after the last injection and sera were separated and kept at 70°C until used.

Immunofluorescent and Acridian Orange Staining

Immunofluorescent and Acridian Orange techniques were used in order to recognize viral antigens in infected cells. For immunofluorescent, Cells were grown in monolayer on glass cover slip in 24-wells

tissue culture dish and infected with HSV. Sixteen hr post infection, cover slips were removed, washed in PBS and fixed in acetone at 4 Co. They were stained indirectly with fluorescing conjugated anti-rabbit IgG using specific viral antiserum. Acridine orange staining was used to detect intracellular viral DNA. Infected cells in monolayer on cover slips were fixed in camoys fixative for 5 min. cells were dehydrated in successive serial dilutions of 95, 80, 70 and 50% ethanol. They were rinsed in phosphate buffer containing 0.01% acridine orange in phosphate buffer for 3 min. After treatment with differentiating solution, stained cells were examined in a uv microscope.

Analysis of viral proteins by SDS-PAGE and Immunoblotting

Viral proteins in infected cells treated with morphine were analyzed by SDS-PAGE and viral antigens were detected by Immunoblotting techniques. Infected cells were harvested 24 hr postinfection. The cells were scraped off the bottles and centrifuged for 10 min at low speed. The pellet was lysed by addition of equal volume of lysis mix and boiled. Samples were electrophoreses on 10% polyacrylamid gel according to the method of Lamella. Purified viruses were mixed with equal volume of lysis mix buffer, boiled for 5 min and similarly applied to the gel. After completion of electrophoresis the gels were fixed and stained with Commassie blue. For immunoblotting the protein bands in acrylamide gel were blotted onto nitrocellulose paper using a dry blotter at 35 mA for 1 hr. The blotted proteins were treated with specific antiserum to HSV and stained with anti-rabbit peroxidase conjugate using tetra methyl banzedine (TMB) as substrate.

Electron Microscopy

Cells in monolayer were infected with HSV and fixed in 3% glutaraldehyde at 4 Co for 3 hr. then post fixed in 1 % osmium tetroxide. After dehydration in serial dilutions of ethanol, they were embedded in araldite. Sections were cut, stained with uranyl acetate followed by lead citrate. Negative staining of virus particles was performed using purified virus and 1 % PTA.

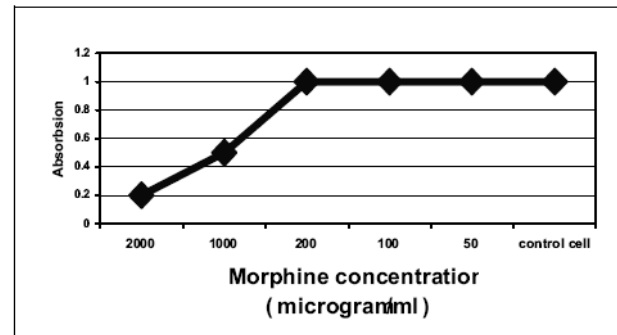


Fig. 1. Effect of morphine on viability of Vero cells.

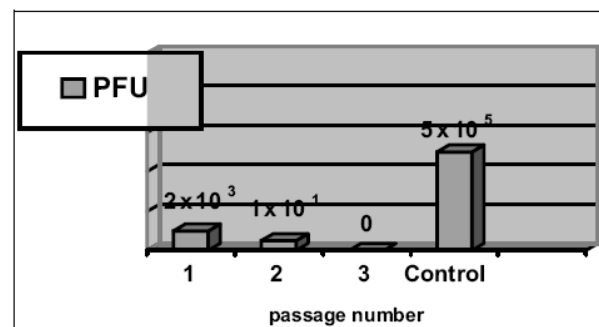


Fig. 2. Effect of morphine on virus yield in infected Vero cells.

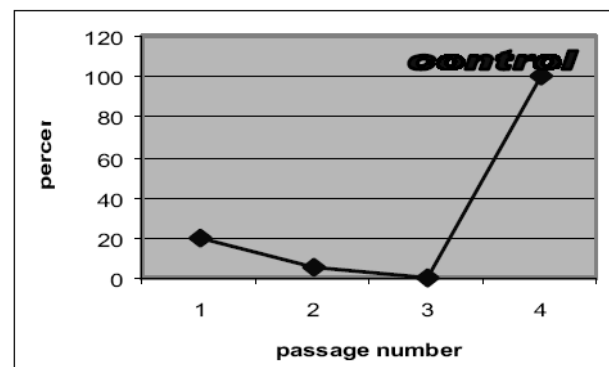


Fig. 3. Percent of infected cells containing HSV antigen after different passages
Cells were infect with the harvest of morphine treated infected cells at different passages infect cells were stained with IF and percentage was counted.

Results

The optimum concentration of morphine which was nontoxic for Vero cells was determined using neutral red uptake in presence of various amount of morphine (Fig. 1). It was found that concentration of 200 µg/ml was nontoxic to Vero cells. The concentration used for HSV infected cells did not exceed 150 µg/ml.

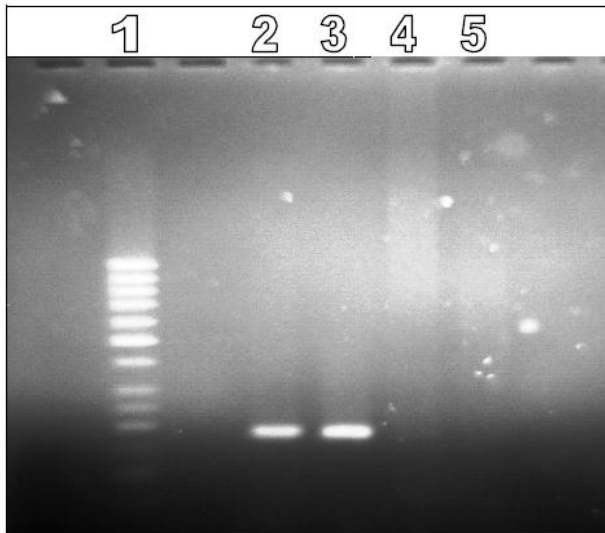


Fig. 4. Effect of Morphine on Viral DNA synthesis Lane-2: Positive control, Lane-3: first passage, Lane-4: third passage, lane-5: Negative control respectively.

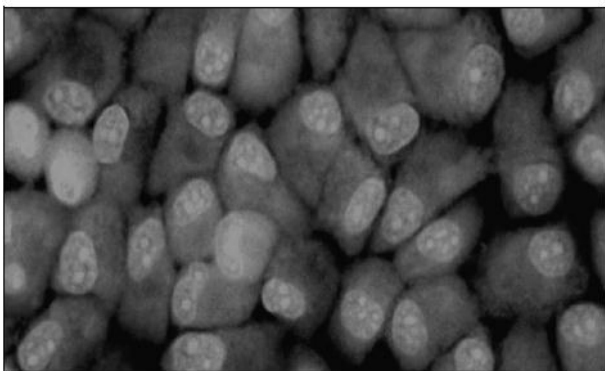


Fig. 5a. Effect of Morphine on Viral DNA synthesis Lane-2: Positive control, Lane-3: first passage, Lane-4: third passage, lane-5: Negative control respectively.

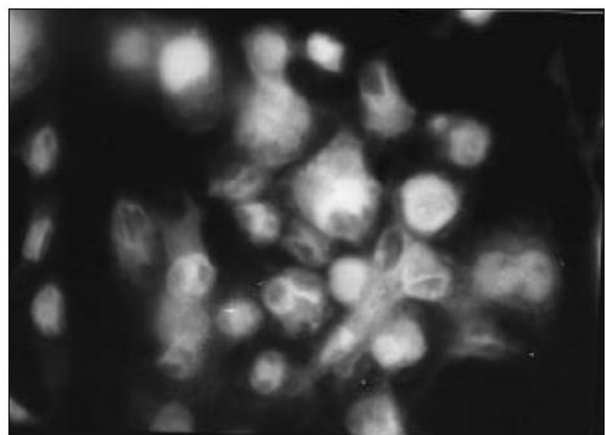


Fig. 5b. Morphine treated infected cells in presence of Naloxone showing inclusion bodies.

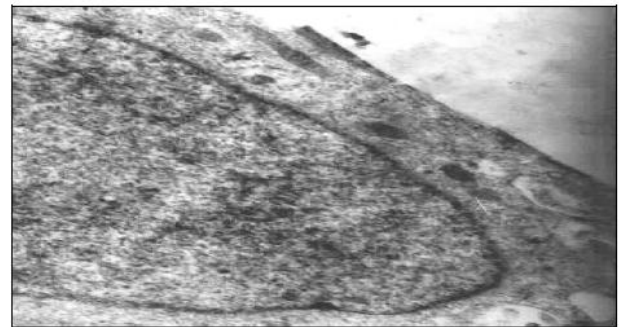


Fig. 6a. Electron micrograph of morphine treated infected cells showing no HSV-2 virus particles.

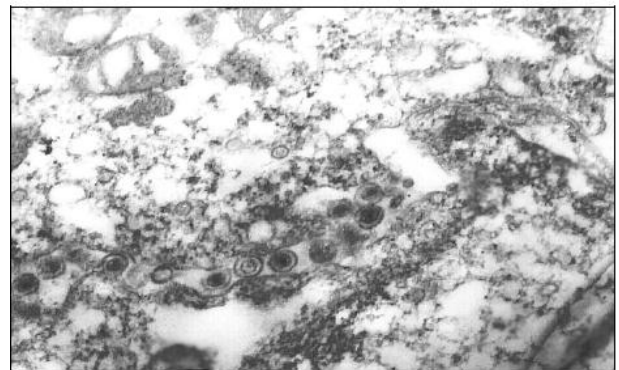


Fig. 6b. Electron micrograph of morphine treated infected cells in presence of Naloxone showing HSV-2 virus particles.

Effect of morphine on virus yield

Antiviral effects of morphine was determined by addition of various amounts of morphine (below the toxic dose) to the HSV infected cells after the time of adsorption and the amount of infectious virus produced after 48 hrs was determined by both TCID50 and plaque assay methods.

As it is shown in Fig. 2, the titer of virus produced in presence of morphine was reduced 2 logs but the virus growth was not inhibited completely. After the third passage in presence of morphine virus production was completely inhibited (Fig. 2). The inhibitory effect of morphine was the highest till 4hr post infection and was reduced when added at later time.

Viral antigen in morphine treated cells

To determine presence of viral antigens synthesized in presence of morphine, infected cells 16 hrs post infection were fixed and stained with anti HSV fluorescent conjugate. As it is shown in Fig. 3, at first passage most of infected cells lacked viral antigens as compared with control infected cells untreated

with morphine. Only a small number of cells showed fluorescence in the first passage and in the third passage there was no HSV antigen in morphine treated cells (fig. 3).

The absence of viral proteins in morphine treated cells was confirmed by SDS-P AGE and western blotting.

Viral DNA synthesis

To determine presence of viral DNA in morphine treated infected cells, DNA was extracted from cells and PCR test was performed using HSV-1 specific primers. Although DNA was detected in morphine treated cells at the first passage (Fig. 4, lane 3) there was no detectable DNA after the third passage (Fig. 4, lane 4).

Effect of morphine blocking agents

To confirm the inhibitory effect of morphine on HSV growth in vitro, Naloxone (morphine inhibitor) was added to the morphine treated infected cells and the effect of morphine was detected by staining of cells with Acridine orange. It was found that HSV -1 virus could replicate in Naloxone treated cells in presence of morphine, which was shown by presence of inclusion bodies in infected cells (Fig. 5b). Whereas in the absence of Naloxone there was no apparent virus replication as shown by the lack of viral inclusions. (Fig. 5a).

Electron microscopy of morphine treated cells

Infected cells in presence of morphine lacked any detectable virus particles as compared with the control untreated cells indicating that there was no virus replication in the presence of morphine (Fig 6a, 6b).

Discussion

It has been reported that morphine could prevent pathogenesis of HSV in mice and reduces mortality following ocular infection with HSV-1. (16, 17, 18) We investigated the inhibitory effect of morphine on growth and replication of HSV in Vero cells. The first step was to determine the concentration of morphine which did not affect the viability of uninfected Vero cells. The viability of cell in presence of morphine was determined by the uptake of neutral red which is a vital dye and is

taken up by live cells. Using the nontoxic concentration of morphine, it was found that morphine inhibited replication of HSV in Vero cells. However, at first passage, there were some detectable virus particles infected cells which completely disappeared in third passage. The reason for complete inhibition of viral growth is not clear. It is possible that the inhibitory effect of morphine was dose dependent. In the second passage when virus titer was low the inhibition was complete. The absence of viral DNA as was determined by Pt.R and the lack of viral protein synthesis indicated the inhibition effect was not due to the prevention of viral assembly. Morphine inhibitors such as Naloxon blocked the inhibitory effects of the drug the effect of naloxone (Nallylnoroxymorphone), a narcotic antagonist, on the morphine blockade of ovulation was studied (21).

Although the mechanism of HSV growth inhibition of morphine is not clear it seems that the inhibition is due to prevention of viral macromolecular synthesis. Morphine inhibited the growth of both HSV -1 and HSV -2. The inhibitory effect was very similar for both viruses. We did not test the growth inhibition of other Herpes viruses. It would be interesting to investigate the effect of morphine on the replication of other viruses such as varicella and cytomegalovirus.

Acknowledgements

The authors would like to thank " The Iran University of Medical Sciences" for financial support of this project.

References

1. Roizman B, Furlong D. The replication of herpes viruses. In: Fraenkel-Conrat H, Wagner RR, eds. *Comprehensive virology*. New York: Plenum, 1974;2(29):395-403.
2. Lee FK, Nahmias AJ, Nahmias DG, McDougal JS. Demonstration of virus particles within immune complexes by electron microscopy. *J Viral Methods*.1983;7:167-181.
3. Cone RW, Hobson AC, Palmer J. Extended duration of herpes simplex virus DNA in genital

lesions detected by the polymerase chain reaction. *J Infect Dis.* 1991;164:757-760.

4. Leator GM, Klapper P. Herpes simplex, in; principal and practice of clinical virology. *Clinical Virology.* 2000;4:23-45.

5. Roizman A, Knipe GM. Herpes simplex viruses and their Replication, In: *Fields virology. Clinical Virology.* 2001;3:295-302.

6. Chatis PA, Crompacker CS. Resistance of HSV to Antiviral drug. *Antimicrobial Agentchewother.* 1992;36:1589-1595

7. Crumpacker C: Antiviral therapy In: DM Knipe, PM Hawley (eds): *Fields' Virology 4th Ed,* Philadelphia:Lippincott Williams and Wilkins.2001: 393-395.

8. Schinazi RF, Peters J, Williams CC. Effect of combinations of acyclovir with vidarabine or its 5-monophosphate on herpes simplex viruses in cell culture and in mice. *Antimicrobial agents Chemother.* 1982;22 499-507.

9. Neyts J, Declercq E. Mycophenolate mofetil strongly potentiates the anti-herpes virus activity of acyclovir. *Antiviral Res.* 1998;40:53-56.

10. Eberle R, Courtney RJ. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. *J Viral.* 1980;35:902-917.

11. Jacobson G. Protein blotting using semi-dry electrophoresis transfer equipment. In: BS Dunbar (Ed): *protein blotting, a practical approach 1 st Ed,* IRL press Oxford University Press. 1994;53-70.

12. Robards AW, Wilson AJ. Basic biological preparation techniques for TEM. In: *Procedures in electron microscopy 1 st Ed,* Letch worth John Wiley and Sons Ltd. 1993;5:9-14.

13. White DO, Feuner F. *Med Viral 4th Ed,* Glasgow. Academic Press. 1994;3:321-329.

14. Johnson FB, Black low NR, Hoggan MD. Immunological reactivity of antisera prepared Against the sodium dodecyl sulfate-treated structural polypeptides of adenovirus associated virus. *J Viral.* 1972;9:1017-1026.

15. Moattari A, Kabiri, M, Shahrabadi MS, Akbari S. Sodium valproate-induced potentiation of ant herpetic effect of acyclovir. *Iran J Med Sci.* 2002;27: 180-7.

16. Weeks Bs, Alston NI, Cadet P, Zhu W, Riaslas C, Stefano GB. Morphine reduce herpes simplex virus-I pathogenesis in the murine flank. *Int J Mol Med.* 2001;8(3):303-307.

17. Alonzo NC, Carr DJ. Morphine reduces mortality in mice following ocular infection with HSV-1. *Immunopharmacology.* 1999;41(3):187-97.

18. Liroy DT, Sheridan PA, Hurley SD. Acute morphine exposure potentiates the development of HSV-1 induced encephalitis. *J Neuroimmunol.* 2006;172(12):9-17.

19. Alonzo NC. Infection due to acyclovir resistant herpes simplex virus in patients undergoing allogeneic hematopoietic stem cell transplantation.: *Pathol Biol (Paris).* 2001;49(7):553-558.

20. Laboratory of Virology of the Hospices Civils de Lyon, Herpes simplex virus resistance to antiviral drugs. *J Clin Viral.* 2003;26(1):29-37.

21. Packman PM, Rothchild JA. Morphine inhibition of ovulation: reversal by naloxone, *Endocrinology.* Endocrine Society. 1997;99:7 -10.