

*Full Length Research Paper*

# Effects of morphine on replication of herpes simplex virus type 1 and 2

Seyed Hamid Reza Monavari\* and Mahmoud Shamsi Shahrabadi

Department of Virology, Iran University of Medical sciences, Tehran, Iran.

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Several drugs are being used in treatment of HSV (Herpesviridae) infection in human but still introducing an effective safe drug is desirable. We investigated the inhibitory effect of morphine on replication of HSV *in vitro*. The results indicated that a concentration of up to 200 µg/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 eliminated. The presence of viral antigens in infected cells in presence of morphine by immunofluorescent 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. 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.

**Key words:** Morphine, HSV1, 2.

## INTRODUCTION

Herpes simplex virus type 1 and 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 (Roizman et al., 1974; Cleator and Klapper, 2000; Roizman and Knipe, 2001). There are several antiviral drugs which are effective against HSV infections, and most of them inhibit viral DNA synthesis. Recently, physicians and researchers have been faced with the problem of elongated treatment with Acyclovir due to formation of drug resistant mutants and toxicity of the drug (Chatis and Crompacker, 1992; Schinazi et al., 1982; Venard et al., 2001; Morfin et al., 2003). 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 (Crumpacker et al., 2001; Neyts et al., 1998). 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 (Chibo et al., 2004; Stránská et al., 2004). It has been shown that Morphine has the potential in preventing HSV pathogenesis in certain laboratory animals. The main objective of this study was to determine the inhibitory effect of morphine on HSV replication and growth *in vitro* and to investigate its possible use for treatment of HSV infection.

## MATERIALS AND METHODS

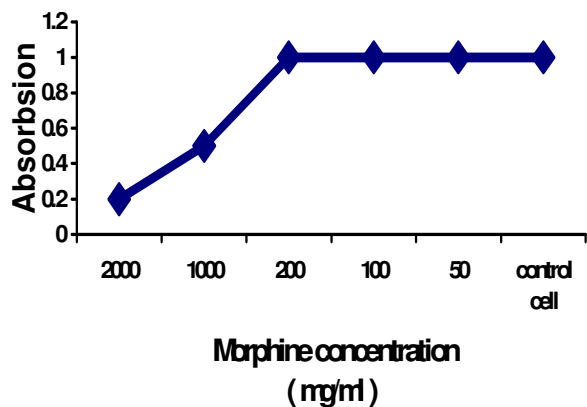
### Cell culture and virus

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 TCID<sub>50</sub> and plaque assay procedures. For TCID<sub>50</sub>, cells were grown in 24 wells tissue culture

\*Corresponding author. E-mail: [hrmonavari@yahoo.com](mailto:hrmonavari@yahoo.com).  
Tel./Fax: +98-021-66509183.



**Figure 1.** Effect of morphine on viability of Vero cells. The high absorbancy represents more uptake of neutral red and more cell viability.

dishes and the test was performed according to the Reid and 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 h. A second overlay containing 0.1% neutral red was used and plaques were counted after 12 h.

#### Determination of morphine cytotoxicity

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 µl of 120 µg/ml of neutral red was added to each cell monolayer and incubated for 3 h at 37°C. After that the cells were washed with 0.5% formaldehyde containing 1% CaCl<sub>2</sub>. The cells were extracted in citrate-ethanol buffer for 1 h at 4°C. The absorbency of extracted solution was measured at 550 nm.

#### Virus purification

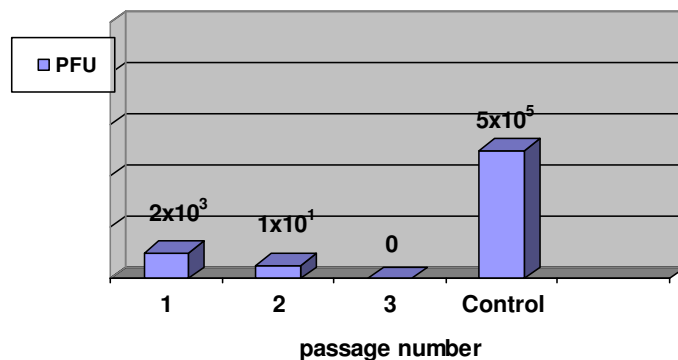
For animal immunization, purified virus was required. HSV was purified on sucrose cushion followed by sucrose gradient centrifugation. Discontinuous sucrose gradient was formed by successive layering of 50, 40, 30, 20% sucrose (made up in 0.01 M Tris buffer). These gradients were then centrifuged at 18000 rpm/min for 2 h 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 (Eberle et al., 1980; Johnson et al., 1972).

#### Immunofluorescence

Immunofluorescence was used in order to recognize viral antigens



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

in infected cells. For immunofluorescence, cells were grown in monolayer on glass cover slip in 24-wells tissue culture dish and infected with HSV. 16 h post infection, cover slips were removed, washed in PBS and fixed in acetone at 4°C. They were stained indirectly with fluorescing conjugated anti-rabbit IgG using specific viral antiserum.

#### Electron microscopy

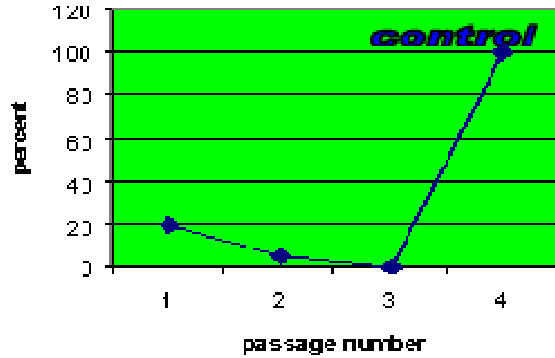
Cells in monolayer were infected with HSV and fixed in 3% glutaraldehyde at 4°C for 3 h, then post fixed in 1% osmium tetroxide. After dehydration in serial dilutions of ethanol, they were embedded in araldite. Sections were cut, stained with uranyl acetate followed by lead citrate. Negative staining of virus particles was performed using purified virus and 1% PTA (Lee et al., 1983).

## RESULTS

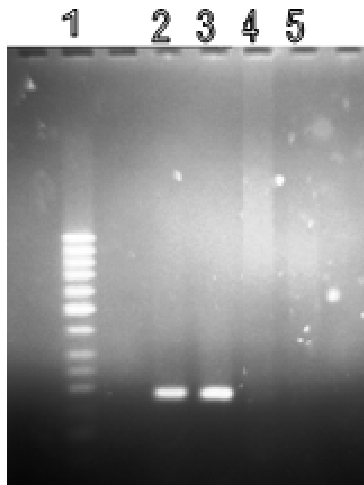
The optimum concentration of morphine which was non toxic for Vero cells was determined using neutral red uptake in presence of various amount of morphine (Figure 1). It was found that concentration of 200 µg/ml was non toxic to Vero cells. The concentration used for HSV infected cells did not exceed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

#### 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 h was determined by both TCID<sub>50</sub> and plaque assay methods. As it is shown in Figure 2, the titer of virus produced in presence of morphine was reduced to logs but the virus growth was not inhibited completely. After the third passage in presence of morphine virus production was completely inhibited (Figure 2). The inhibitory effect of morphine was the highest till 4 h post infection and was reduced when added at later time.



**Figure 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 immuno-flourescence and percentage was counted



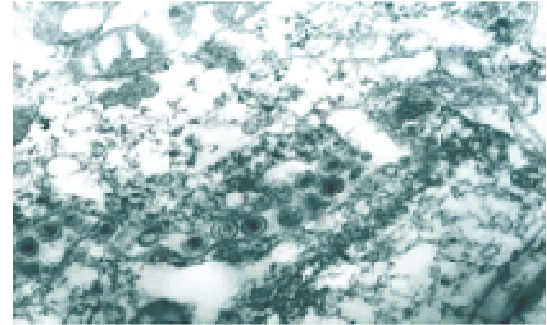
**Figure 4.** Effect of morphine on Viral DNA synthesis. Lane 1, marker; Lane 2, positive control; Lane 3, first passage; Lane 4, third passage; Lane 5, negative control.

**Viral antigen in morphine treated cells**

To determine presence of viral antigens synthesized in presence of morphine, infected cells 16 h post infection were fixed and stained with anti HSV fluorescent conjugate. As shown in Figure 3, at the 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 (Figure 3).

**Viral DNA synthesis**

To determine presence of viral DNA in morphine treated



**Figure 5a.** Electron micrograph of morphine treated infected cells in presence of naloxone showing HSV-2 virus particles.



**Figure 5b.** Electron micrograph of morphine treated infected cells showing no HSV-2 virus particles.

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 (Figure 4, lane 3) there was no detectable DNA after the third passage (Figure 4, lane 4).

**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 (Robards et al., 1993) (Figure 5a, b).

**DISCUSSION**

It has been reported that morphine could prevent pathogenesis of HSV in mice and reduces mortality following ocular infection with HSV-1 (Weeks et al., 2001; Alonzo and Carr, 1999; Liroy et al., 2005; Packman et al., 1976). 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 (Moattari et al., 2002). 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 in 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 PCR and the lack of viral protein synthesis indicated that the inhibition effect was not due to the prevention of viral assembly (Cone and Hobson, 1991).

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.

## ACKNOWLEDGMENT

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