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*In vitro e*ffect of phototherapy with low intensity laser on HSV-1 and epithelial cells

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ABSTRACT

The effects of phototherapy on herpes lesions have been clinically demonstrated by either preventing the lesion formation or speeding their repair. The aim of this in vitro study was analyze the effect of phototherapy on epithelial cells and HSV-1 in culture. Cultures of HSV-1 and epithelial cells (Vero cell line) were used. The irradiations were done using a GaAlAs laser (660 e 780 nm, 4.0 mm^2). One, two and three irradiations with 6 h-intervals were done. The experimental groups were: Control: non-irradiated; 660 nm and 3 J/cm^2 (2.8 sec); 660 nm and 5 J/cm^2 (3.8 sec); 780 nm and 3 J/cm^2 (1.9 sec), and 780 nm and 5 J/cm^2 (2.5 sec). The HSV-1 cytopatic effect and the cell viability of irradiated cultures and controls were analyzed in four different conditions: irradiation of non-infected epithelial cells; epithelial cells irradiated prior infection; virus irradiated prior infection; irradiation of HSV infected cells. The mitochondrial activity and cytopathic effects were assessed. The number of irradiations influenced the cell growth positively and proportionally, except for the 660 nm/ 3 J/cm^2 group. Any variation in cytopathic effects was observed amongst the experimental groups. The viability of infected cells prior irradiation was significantly higher than that of non-irradiated cultures when 2 irradiations were done. Under the experimental conditions of this study we concluded that phototherapy is capable of enhancing epithelial cell growth and prolonging cell viability of HSV-1 infected cells. Positive benefits of phototherapy could be resultant from prolongation of infected cells viability, corroborating with host defenses.

KEYWORDS: cell culture, epithelial cells, phototherapy.

1. INTRODUCTION

Herpes labialis is a manifestation of the infection with herpes simplex virus type 1 (HSV-1). This is matter of concern once following the primary infection the virus persists in a latent state, most probably in the sensorial ganglia (Stevens, 1975). Reactivation of the virus by external stimuli such as ultraviolet light (Harbour et al., 1977) or trauma (Hill et al., 1978) may cause lesions expressed mostly as epithelial blisters in the skin, oral mucosa and/or perioral region.

Patients with frequent recurrences of herpes labialis can be treated by prophylactic administration of topic antiviral agents such as acyclovir (Spruance 1993). Others are given treatment at the onset of a recurrence episode to decrease the lesion duration and pain. The concerns with these treatments are the limited degree of efficacy and cost.

In the oral manifestations of some infectious diseases, such as recurrent herpes simplex virus infections, the phototherapy with low power lasers has shown effects that included, prevention of lesion formation, acceleration of the healing of lesions, and decreasing the frequency of recurrent lesions (Schindl et al., 1999). However, little is known about the mechanism underlying these supposed benefits of this therapy for HSV-1 infection. For this reason, the aim of this *in vitro* study was analyze the effect of phototherapy on epithelial cells and HSV-1 in culture.

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2. METHODOLOGY

2.1. Cell Culture

Vero cells (monkey kidney epithelial cell line - ATCC CRL 1587) were grown at 37 ºC in Minimum Eagle Medium (MEM, Cultilab, Campinas, SP, Brazil) supplemented by 10% fetal bovine serum (fbs) (Cultilab) and 100 U/ml of Penicillin and Streptomycin (nutritional regular culture medium). Cells were maintained in a humidified air-5 % CO_2 atmosphere.

2.2. Viruses Culture

The Herpes Simplex Virus, type I (HSV-1) was previously cultured in VERO cells and then used as standard for the standardization assay using the polymerase chain reaction (PCR).

2.3 Laser irradiation

Laser irradiation was delivered with GaAlAs/InGaAlP lasers (MM Optics LTD, São Carlos-SP-Brazil). Irradiations were done in contact using the punctual irradiation mode in a 3.6 mm^2 area. Two wavelengths were used 660 nm and 780 nm with powers of 40 mW and 70 mW (at the display) respectively. Two energy densities settings were used; for 660 nm: 3 J/cm^2 (2.8 s) and 5 J/cm^2 (3.8 s) and, for 780 nm: 3 J/cm^2 (1.9 s) and 5 J/cm^2 (2.5 s).

We set up the experiments to standardize the procedures as previously described (Pereira et al,., 2002; Marques et al., 2004). Briefly, knowing that the distance between the laser source and the surface of application is critical, the laser application was done through the bottom of the optically clear 96 well-microtitration plates. Therefore, the laser beam did not transpose the culture medium being applied straight on the cell instead. Additionally, the distance between the laser beam and the cell monolayer was held constant at 1 mm. Finally, the laser irradiation was carried out in a partial darkness without other light influence than laser. The LaserCheck power meter (Coherent, Inc., Santa Clara, CA) verified the output power of the laser equipment. The control groups were treated under identical conditions except that the laser equipment was kept off.

2.4 Cell mitochondrial activity analysis

Cell mitochondrial activity was analyzed using the MTT-based cytotoxicity assay. The MTT assay involves the conversion of the water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan. The formazan is then solubilized, and the concentration determined by optical density at ≅ 570 nm. A MTT reduction analysis kit (Vybrant MTT, Molecular Probes, Eugene, OR, USA) was used. Immediately after the end of the assay procedures the absorbance was read in a micro plate reader (Biotrak II, Biochrom Ltd, Eugendorf, Austria) using a 562 nm filter. The absorbance data was used to plot the cell growth curves.

2.5 Statistical Analysis

The optical density data, corresponding to the cell viability, obtained in quadruplicate for the serum concentration determination experiment and in triplicate for the irradiation experiments, are presented as mean ± the standard error of mean. The data were compared by the Kolgomorov-Smirnof (Lilliefors) test followed by ANOVA test. The level of significance was 5% ($P < 0.05$).

2.6 Experimental groups

Control: no laser irradiation
Group 1: 660 nm, 3 J/cm^2
Group 2: 660 nm, 5 J/cm^2
Group 3: 780 nm, 3 J/cm^2
Group 4: 780 nm, 5 J/cm^2

2.7 Experiments

The experiments were done in four steps:
Step 1: Laser irradiation on non-infected VERO cells:
Step 2: Laser irradiation on non-infected VERO cells prior HSV-1 inoculation
Step 3: Laser irradiation on HSV-1 cultures prior inoculation on VERO cells;

Step 4: Effect of laser irradiation on HSV-1 infected VERO cells.
The laser irradiation effect was analyzed in the cell growth at the Step 1, and in the cell survival and cytophatic effects at the other 3 steps.

2.7.1 Step 1: Laser irradiation on non-infected VERO cells: effect of laser irradiation on cell growth

For cell growth analysis, control (non-irradiated) and treated cultures (irradiated) were plated on 96 well-microtitration plates. The cultures were incubated in humidified air-5 % CO_2 atmosphere for 6 h before the irradiation. Before irradiation, the culture medium was replaced by the nutritional deficient culture medium supplemented with 2 % fbs. Then, one, two or three irradiations were applied. The second and the third irradiations were successively done with 6 h intervals. Samples from each group were taken for mitochondrial activity analysis 20, 24, 48 and 72 h after the first irradiation. The cell mitochondrial activity analysis was also used to infer the cell viability and to plot the cell growth curves. All the experiments were done in triplicate.

2.7.2 Step 2: Laser irradiation on non-infected VERO cells prior HSV-1 inoculation: effect on the cell survival and the HSV-1 cytophatic effect

VERO cells (1 x 10^4 cells/well) were plated on 96 well-microtitration plates. The irradiations were performed as described at the Step 1. Then, the a HSV-1 suspension was inoculated in the irradiated cultures that were maintained in an incubator (37 ◦C) for 24 h. After this incubation time the cytophatic effects of the HSV-1 in the VERO cells was recorded at first ant then, this was repeated each 24 h for the next 3 days, using the Reed-Muench method. At the end of the experimental time (96 h) these VERO cells were submitted to the MTT test for checking the cell survival, as described above.

2.7.3 Step 3: Laser irradiation on HSV-1 cultures prior inoculation on VERO cells: effect on the cell survival and the HSV-1 cytophatic effect.

The HSV-1 suspension in an Eppendorf tube was irradiated as described for the VERO cells. Then, this HSV-1 irradiated viral suspension was inoculated in VERO cells plated on 96 well-microtitration plates. The analyses of cytophatic effect and cells survival were carried out as described at the Step 2.

2.7.4 Step 4: Effect of laser irradiation on HSV-1 infected VERO cells: effect on the cell survival and the HSV-1 cytophatic effect.

Vero cells plated on 96 well-microtitration plates were inoculated with HSV-1 suspension. Then, the infected cultures were submitted to the same irradiation protocol described for the other Steps of this study. The analyses of cytophatic effect and cells survival were carried out as described at the Steps 2 and 3.

3. RESULTS

3.1. Step 1: Laser irradiation on non-infected VERO cells: effect of laser irradiation on cell growth

The cell growth curves of the VERO cells submitted to one, two and three irradiations are plotted in Figures 1m, 2 and 3 respectively. There was a progressive cell growth during the experimental time independently of both the experimental group and the number of irradiations. Comparing the curves of each figure it is possible to observe a difference in the curves of cells submitted to three irradiations where the irradiated cultures presented cell growth significantly higher than control non-irradiated cultures.

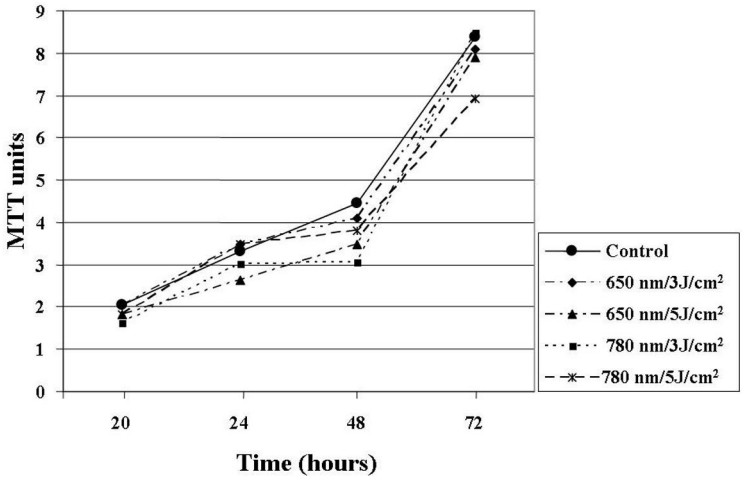

Figure 1- Growth curves of Vero cells irradiated once.

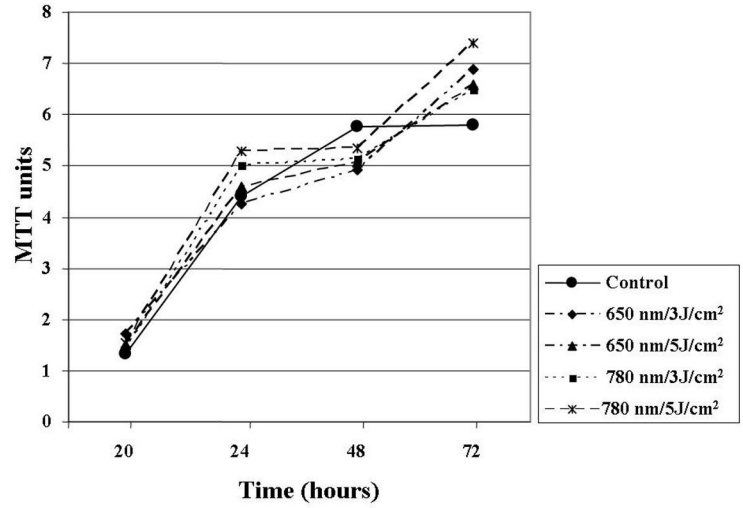

Figure 2- Growth curves of Vero cells irradiated twice.

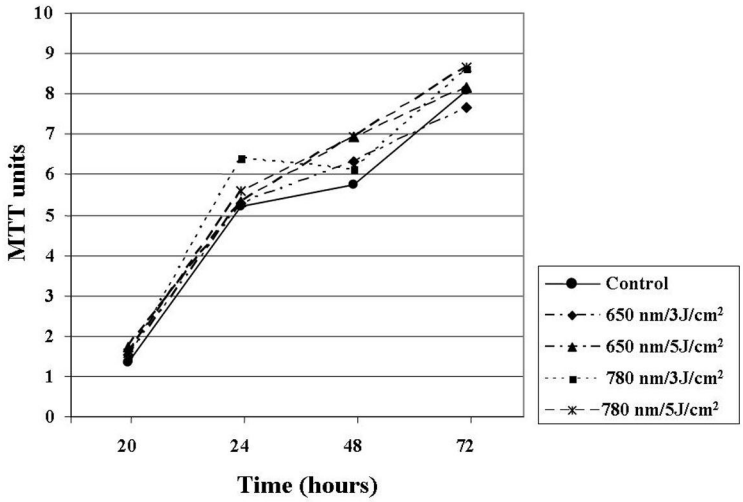

Figure 3- Growth curves of Vero cells irradiated three times.

3.2 Step 2: Laser irradiation on non-infected VERO cells prior HSV-1 inoculation: effect on the cell survival and the HSV-1 cytophatic effect

Figure 4 illustrates the results of cell survival 96 h after the last irradiation. When two irradiations were done the group treated with the parameter 780 nm/3J/cm^2 presented viable cell number significantly higher than non-irradiated cultures and cultures irradiated just once. The parameter 780nm/5J/cm^2 irradiated for three times also presented significantly higher viable cell number than the same group irradiated once.

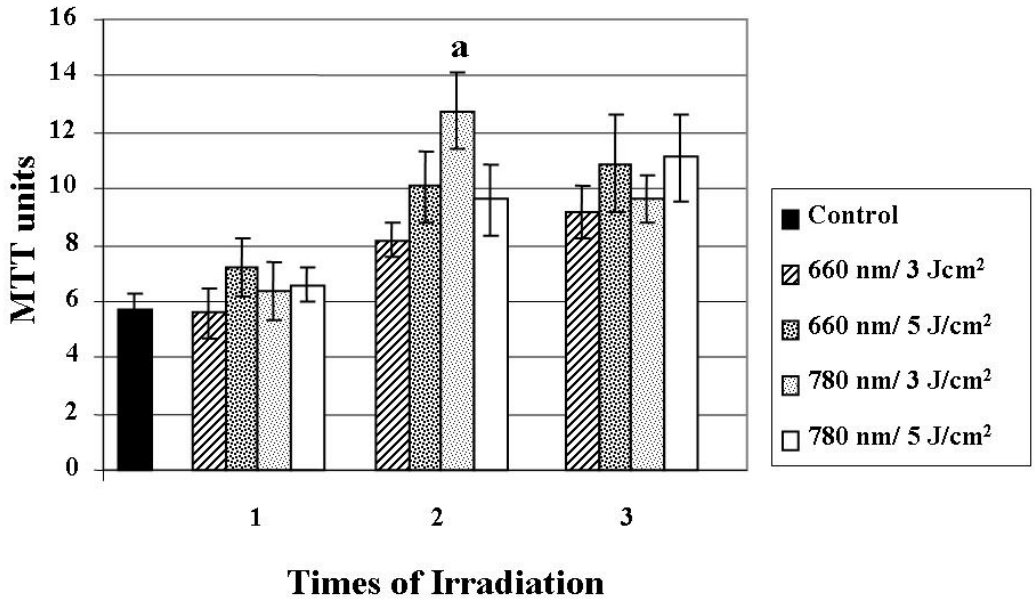

Figure 4- Graphic representation of the cell survival when the epithelial cells were firstly irradiated and then inoculated with the HSV-1 suspension. a: significantly different from the control and the same groups irradiated once.

3.3. Step 3: Laser irradiation on HSV-1 cultures prior inoculation on VERO cells: effect on the cell survival and the HSV-1 cytophatic effect.

Figure 5 illustrates the results of cell survival 96 h after the inoculation of irradiated HSV-1 suspensions. There were no differences in the viable cell numbers when the comparisons were done among groups and number of irradiations.

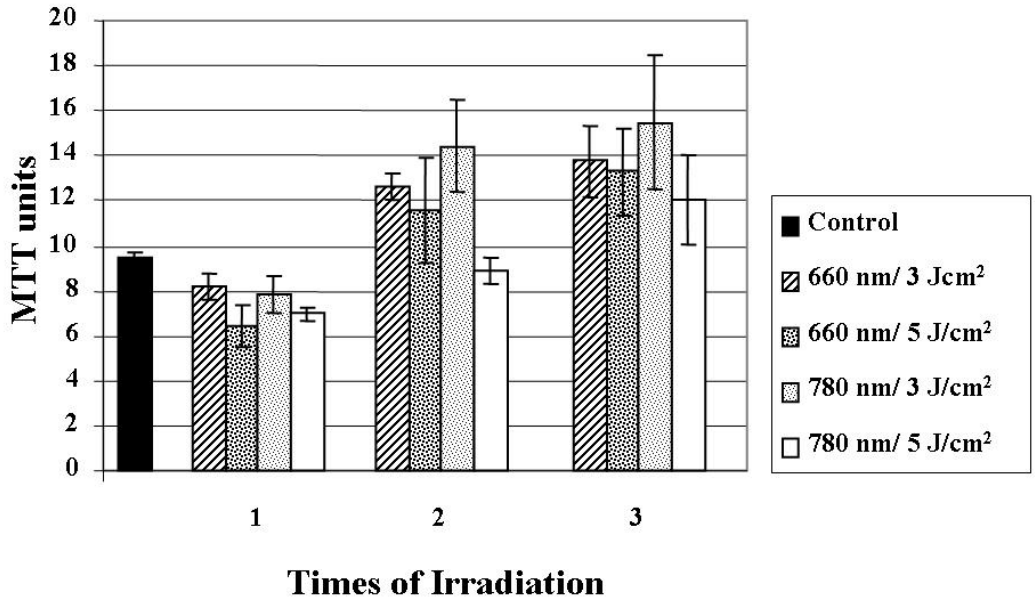

Figure 5: Graphic representation of the cell survival when the HSV-1 suspension were firstly irradiated and then inoculated into the epithelial cells. There are no differences amongst the groups.

3.4 Step 4: Effect of laser irradiation on HSV-1 infected VERO cells: effect on the cell survival and the HSV-1 cytophatic effect.

Figure 6 presents the results of the HSV-1 infected cells survival after the irradiation protocols. The numbers of viable cells were significantly higher than those of all groups irradiated once and non-irradiated, when two irradiations were done independently of the parameters used.

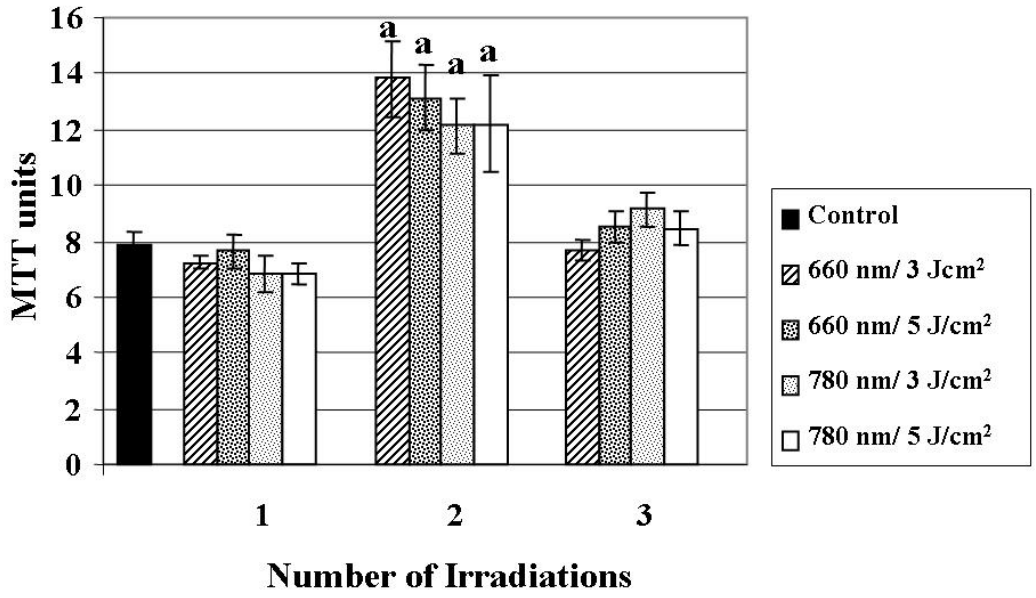

Figure 6: Graphic representation of the cell survival when the infected epithelial cells were irradiated. a: significantly different from the control and the same groups irradiated once.

4. DISCUSSION

Low intensity laser administration is capable of increasing non-infected epithelial cell growth rate for cells stressed by nutritionally deficient conditions. The improvement in cell growth rates is directly proportional to the number of irradiations. In the relationship VERO cells and HSV-1 cultures the phototherapy with low power laser showed to be innocuous to the HSV-1 cultures, once irradiated viruses suspensions were not able to modify the cytophatic effects of the HSV-1 in the VERO cells, neither changed the cell survival 4 days after the inoculation. The irradiation of VERO cells prior or after the HSV-1 inoculations were not able to change the cytophatic effects of the HSV-1, however, the cell survival was longer when two or more irradiations were done.

The positive effect of low intensity laser radiation on mesenchymal cells, especially fibroblasts, has been well established (Almeida-Lopes et al., 2001; Pereira et al., 2002; Marques et al., 2004; Azevedo et al., 2006). Exposure of cells to low intensity laser appears to be capable of stimulating the growth of these types of cells. This effect is particularly evident when the cell functions are compromised (Almeida-Lopes et al., 2001). *In vitro* cells can be compromised by growing them under nutritionally deficient conditions. These nutritional conditions must not abolish the cell growth completely but should only reduce the cell growth rate. Then, the effect on growth rates for cells grown under these conditions can be measured when cells are exposed to low intensity laser. *In vitro* stress can be achieved for each strain by varying fbs concentration. Some cell types are able to grow with half the fbs concentration of the nutritional regular culture medium that required for their characteristic normal growth rates (Almeida-Lopes et al., 2001; Marques et al., 2004; Azevedo et al, 2006; Fujihara et al., 2006) others require an accentuated decrease in this concentration to diminish the cell growth rate (Pereira et al., 2002). As there were no data about Vero cell growth in different fbs concentrations, three concentrations (10%, 5% and 2%) were tested for in order to find the appropriate fbs concentration for the subsequent irradiation experiments. We were looking for a fbs concentration that would lead to an accentuated

reduction of the Vero cell growth without totally preventing the cell growth. Using a culture medium supplemented with 2% fbs produced a condition where Vero cells viability was maintained but growth was significantly lower than that of the cells grown in nutritional regular culture medium supplemented with 10% fbs. Thus, this serum concentration was appropriate for the proposed experiments for exploring the effects of low intensity laser irradiation on cell growth.

The beneficial effects of phototherapy with low intensity laser depend on the number, timing and frequency of irradiations and the type of laser used. In some studies the desired beneficial effect are more obvious when the irradiations are repeated (Pereira et al., 2002, Albertini et al., 2004), while in others excessive repetitions caused undesirable effects, such as pathological changes in the newly formed muscular structures during muscle regeneration. (Bibikova & Oron , 1994). Thus it was decided to perform single irradiation as well as repeated 2 and 3 exposures.

In vivo phototherapy protocols have utilized either daily or every other day irradiation schedules. Cells in culture have a much faster cell division rate than that generally observed in vivo, and for this reason we followed an in vitro protocol previously reported in the literature (Pereira et al., 2002) with 6 hours between the irradiations.

Independently of the laser parameter tested (wavelength and energy density), positive cell growth effects were observed primarily when the cells were irradiated for three times. It was noted that repeated irradiations increased the cell growth rate of cells grown under nutritional deficit conditions. This would seem to support the utilization of repeated irradiations with low intensity laser when attempting to impact clinical lesions and conditions.

It was interesting that the improvement in Vero cell growth following phototherapy was apparently independent of the wavelength used. However, there was a trend for a more pronounced increase in growth rate when the infrared (780 nm) was used. In fact, when three irradiations were administered, the cultures irradiated with the 780 nm laser presented significantly higher cell growth rates compared to non irradiated cells grown in nutritional deficit conditions.

When the effect of the phototherapy with low power laser on the HSV-1 infection *in vitro* was studied, we tried to understand in which point of the infection development this therapy was actually acting. For this reason we divided this study in steps to scan all possibilities of action. First of all it was tested the effect of the phototherapy in the epithelial cells in order to test if this therapy could improve the resistance of the irradiated cell to the infection. There are studies showing that phototherapy applied in between the herpes labialis manifestations can decrease the severity of the lesions, as well as increases the timing between the crises. Then, one could imagine that the epithelial cells could be modified by the laser irradiation becoming less prompt to the virus inoculation. However, neither the cytophatic effects of the HSV-1 in the VERO cells, nor the cell survival were observed consistently when the cells were irradiated prior to the HSV-1 inoculation. It means that the photoherapy is able to biostimulate the VERO cell growth; however does not change the susceptibility of these cells to the HSV-1 infection.

Other target for the phototherapy would be the HSV-1 itself. It is known that this virus entries the epithelial cells and after the cells death they reach the extra cellular environment and then they are exposed to the laser irradiation. One hypothesis for the phototherapy effect could reside in a modification of the virus potency or virulence. Then, suspensions of HSV-1 cultures were previously irradiated and then inoculated into the epithelial cells. Once again a negative result was observed. The infection course was not modified. Both, cytophatic effects and cell survival were similar amongst the groups. It means that the phototherapy was unable to change the virulence of the irradiated HSV-1 cultures.

The last situation to be tested would be the effect of phototherapy in epithelial cells already infected with the HSV-1 cultures. And in this step of pour study a very interesting result was obtained. Although the cytophatic effects were again not affected by the phototherapy, the cell survival presented a significant increase when two irradiations were done, independently of the parameters used. This result is very important, because if the infected cell survival is increased, the time for the virus to be drive out from the infected cells to reach the extra cellular environment and then be able to infect a great number of epithelial cells, resulting in a more severe lesion, would be longer. This has two implications that could explain the benefit of the phototherapy of herpes simplex infection. First of all, the severity of the lesions can be reduced; and more than that, there will be more time for the local immunity system organizes the local defense against the virus.

Those are only suppositions; however they open many questions related to the effect of phototherapy in the herpes simplex infection. In fact other authors also published their concerns the uncertainties about the mechanisms of phototherapy action on the herpes simplex infection (Rallis and Spruance, 2000) Thus new researches must be done in order to understand the mechanisms of laser biostimulation. Moreover, these studies must also determine the correct laser parameters for fully biostimulate the epithelial cells.

5.CONCLUSIONS

The laser radiation does not change either the susceptibility of the VERO cells to the HSV-1 infection or the HSV-1 virulence; however, prolongs the cell viability of HSV-1 infected cells

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