

EFFECT OF SELENIUM AND /OR VITAMIN E ON BOVINE HERPES TYPE 1 INFECTION IN VITRO CULTURED CELLS

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ABSTRACT

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Bovine herpes virus type 1 (BHV-1) still the causes of great economic losses in the livestock industry and trade because there are no available drug that proved to be fully effective against it. In this study, the antioxidant of selenium and/ or the vitamin E were evaluated as a natural anti-viral drugs. The antiviral activities against Bovine Herpesvirus type 1 (BHV-1) were evaluated by the reduction of the viral cytopathic effect in Madin-Darby Bovine Kidney cell line (MDBK) with, GSH (glutathione reduced) and MAD (malondialdehyde) assays. Selenium in concentrations 0.01 and 0.1 μM and / or Vit. E in concentrations 50 and 100 μM with two concentrations were added at different stages of the viral infection (pre and post treatment). This study concluded that the higher concentration of Selenium and Vit. E (0.1 μM +100 μM respectively) reduced the Cytopathic Effect (CPE) of BHV-1 in 50% and increased GSH level as well as decreased MDA level, in pre infection treatment assay.

Key words: Selenium, Vitamin E, Bovine Herpes, In Vitro

INTRODUCTION

Bovine herpesvirus-1 (BHV-1), also called infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis virus, is a member of the subfamily Alphaherpesvirinae and is one of the most common viral pathogens found in bovine semen (Afshar and Eaglesome., 1990). Reproductive disorders caused by BHV-1 include infectious pustular vulvovaginitis, endometritis, salpingitis, shortened estrus cycles and abortions in susceptible female cattle and balanoposthitis in susceptible bulls (Biswas *et al.*, 2013). In BHV-1 infection of the genital tract of the bull, the virus replicates in the mucosae of the prepuce, penis and distal part of the urethra, and semen is most likely to be contaminated during ejaculation by virus shedding from infected mucosae (Chowdhury, 2010). BHV-1 causes economically important genital, respiratory and neurological diseases in cattle populations world-wide. Infected animals may be immunosuppressed and thus be more susceptible to secondary bacterial infections. BHV-1 infection of the male genital tract can result in mild or severe clinical signs of balanoposthitis or can be clinically unapparent (Vander, 1995). Bulls play an important role in the dissemination of the disease because the virus is excreted in semen both during the

acute phase of infection and also following the establishment of latent infection (Smits *et al.*, 2000).

Oxidative stress has been implicated as a possible mechanism leading to selection of more virulent viral genotypes. Selenium and vitamin E deficiency increase viral disease progression with *Coxsackie virus* in mice (Beck *et al.*, 1994 and Beck *et al.*, 1995). A similar finding was observed when an attenuated strain of influenza A rapidly gained virulence upon passage through selenium deficient mice (Nelson *et al.*, 2001). These results suggest dietary effects on oxidative stress may influence viral disease progression. Selenium status in grazing crops is dependent on incorporation from soil. The Se status of Egyptian soils and plants is generally low and its location in the international field is definitely among low Se countries (Mikko and Hakan, 1992). Vitamin E is synthesized by plants but not by animals. Grass normally contains adequate levels of vitamin E, Cereals contain moderate levels of vitamin E, but the maturity and storage of feed materials affect their vitamin E content (Bradley *et al.*, 1986). However, it is known that animal requirements depend on age, physiological stage and species (Villar *et al.*, 2002). Adult cows with a selenium/vitamin E deficiency may be more susceptible to diseases (Kommisrud

et al., 2005) and (Allison and Laven, 2000). It has been reported that white cell function from cows with higher blood selenium concentrations have a better killing ability and therefore may be better able to resist diseases (Cebra *et al.*, 2003). Also Taylor *et al.* (1997) strongly suspect that various herpes viruses will prove responsive to selenium therapy that selenium is playing a role in cell signaling and attachment. Furthermore, Se may have not only preventive, but also therapeutic in the treatment of HIV infections (Taylor *et al.*, 1994). One function of Se is as a cofactor for the antioxidant enzyme, glutathione peroxidase. A deficiency in Se leads to decreased glutathione peroxidase activity. Malondialdehyde (MDA) released from oxidative damage (Rayman, 2000). The increased pathology and altered viral genome found in the Coxsackie virus infected Se-deficient mice may be due to a reduction in glutathione peroxidase activity (Beck *et al.*, 1995) reduced glutathione (GSH) functions as an antioxidant in several ways a substrate for glutathione peroxidase (GSH-P_x) (Moron *et al.*, 1979). Despite evidence supporting a beneficial effect on cow health with additional selenium/vitamin E supplementation (LeBlanc *et al.*, 2002; ADAS, 2004; Sivertsen *et al.*, 2005), but the effect of those antioxidants on BHV-1 is not studied yet. Therefore there is a need to continue research to control BHV-1. The present work aimed to study the antiviral effect of selenium (as sodium selenite) or / and Vitamin E on BHV-1 infection in vitro cultured cells in pre and post-infection.

MATERIALS and METHOD

- Cell culture: Continuous cell line of Madin Darby Bovine Kidney cells (MDBK) were supplied by Virology unit, Reproductive Diseases Department, MDBK cells were maintained at 37°C with 5% CO₂ for 24 h in culture flasks with MEM (Minimum Essential Media). Subcultures every 2-3 days after formation of confluent monolayer was done.

- Virus: BHV-1 was kindly obtained from Virology unit Reproductive Diseases Department. BHV-1 was grown in MDBK cells which were also used for measurement of viral infectivity (virus titration) by a dilution method using a 96-well micro titer plate. The infection titer was expressed as 50% Tissue Culture Infectious Dose (TCID₅₀) calculated by the formula of Reed and Muench., 1938. The infection titer of BHV-1 stock solution was 10⁷TCID₅₀.

Preinfection treatment assay: Confluent monolayer cultures of MDBK cells were grown in 24 well-plates, treated with different concentration of selenium and vitamin E, incubated for 24 h at 37°C and then infected with BHV-1. Results were examined after 3 days of inoculation. Percentages of Cytopathic Effect (CPE) were calculated as following:

$$\text{CPE\%} = \frac{\text{No of wellshaving CPE}}{\text{Total No. on infected wells}}$$

- Postinfection treatment assay: Confluent monolayer cultures of MDBK cells were grown in 24 well-plates, inoculated by BHV-1, incubated for 24 h at 37°C and then treated with different concentration of selenium and vitamin E. The plates were incubated for 3 days and % CPE was calculated as mentioned above.

- Preparation of antioxidant treatments:

Sodium selenite and vitamin E (di-alpha -tocopheryl acetate) were purchased from Sigma. Two concentrations of sodium Selenite (0.01 and 0.1 µM) diluted to the final concentration in assay medium (Minimum Essential Media; MEM). To be selenium adequate cells (Saguna, 2008) Two concentrations of vitamin E (50 and 100µM) were prepared first by dissolving in absolute ethanol then diluted to the final concentration in assay media to be vitamin E adequate cells (Norimasa *et al.*, 1999). Mixture of selenium and vitamin E was prepared. Six-well plates were prepared for each concentration of selenium and / or vitamin E. The controls consisted of untreated infected cells for virus control, confluent monolayer cells were infected with the virus at 10⁷ TCID₅₀/ ml of virus (virus control); and treated uninfected cells for Selenium and Vitamin E controls (cell control). MEM without selenium and vitamin E After 3 days of incubation, the CPE% was calculated. At the end of the incubation period, cell lysates were obtained by repeated cycles of freezing and thawing in liquid nitrogen. Samples of cells and supernatants were collected and centrifuged at 1500g for 10 min at 4 C. 0.5 ml of supernatant medium was collected from the different groups and stored frozen at -70 c until assayed for GSH and MDA concentration. Glutathione reduced and Malondialhyde were determined using kits according to Beutier *et al.* (1963) and Satoh (1978) respectively.

Statistical analysis:

Data from separate groups are expressed as means ± standard error. The statistical significance of observed differences between means was determined using ANOVA for comparing means of different samples and was defined as p > 0.05 according to Snedecor and Cochran (1987).

RESULTS

Cytotoxicity Effect of Antioxidant Agents, selenium and vitamin E, were applied with different concentrations and two infection protocols (pre, and post infection treatment assays) on the cell culture system. Firstly those antioxidants applied without inoculation of the virus to evaluate the toxicity effect of the agents. Both agents with different concentrations showed no toxicity on the cells (-ve cytotoxicity) for the whole 72 hours (Table 1).

Table 1: Cytotoxicity Effect of selenium and vitamin E on MDBK cells

Antioxidant	Concentration	24hr Cytotoxicity	48 hr cytotoxicity	72 hrcytotoxicity
Selenium	0.01 and 0.1µM	-ve	-ve	-ve
Vitamin E	50 and 100µM	-ve	-ve	-ve
Selenium+Vit.E	0.1 and 100µ Mrespectively	-ve	-ve	-ve

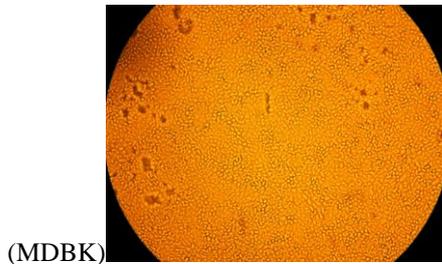


Fig. 1: Complete sheet of cell control culture

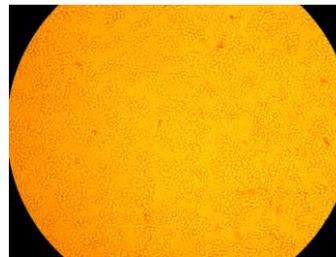


Fig. 2: 50% Cytopathic effect by the addition of higher con. of selenium +vitamin E in pre infection protocol.

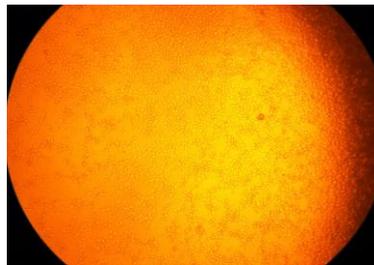


Fig. 3: 66% cytopathic effect by addition of higher conc. of selenium alone in pre infection protocol

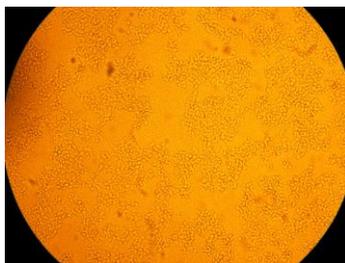


Fig. 4: 83% cytopathic effect by addition of higher con. of vitamin E alone in preinfection protocol.

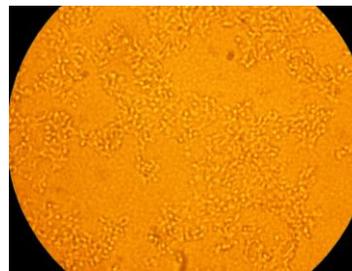


Fig. 5: Virus control with 100% cytopathic effect.

Table (2) shows effect of selenium or / and vitamin E treatments applied with different concentrations in pre-infection protocol on CPE%, GSH and MDA levels in MDBK cells by BHV-1. Effects of selenium on CPE% values were reached 83% and 66% in pretreatment assay, This reflected an inhibition percentage of BHV-1 by 17% and 34% in pretreatment assay at concentrations of selenium (0.01 and 0.1µM) respectively. GSH reached increased level in the treated control cells while the virus infected control cells (un-supplemented with antioxidants) showed the lowest level of GSH.

Selenium alone resulted in a concentration dependent significant ($p > 0.05$) increase in the levels of reduced glutathione at both the concentrations used a low (0.01µM) or high (0.1µM) (Table 2). Our results showed that the lipid- peroxidation in MDBK cells virus control cells (un-supplemented with antioxidant), expressed as the level of MDA (5.27 ± 0.15 nmol/L), was significantly higher ($p > 0.05$) in comparison with cell control (0.15 ± 0.03 nmol/L), as well as in comparison with the levels of MDA during selenium treatment alone. Selenium treatment alone showed significant lower concentration of MDA using the

higher concentration (0.1 μM) compared with the lower one.

Effect of vitamin E on CPE%, GSH, and MDA in MDBK cells by BHV-1 preinfection treatment, are shown in Table (2). CPE% values were 100% in pre-infection treatment when using concentration of 50 μM vitamin E assay and reached 83% in using concentration of 100 μM vitamin E. This reflected an inhibition percentage of BHV-1 by 0 and 17% in pretreatment assay at concentrations of vitamin E of about 50 and 100 μM respectively. Exposure of MDBK cells to 100 μM Vitamin E caused a significant increase in the GSH level than the lower conc. (50

μM). The higher concentration (100 μM) of Vitamin E alone showed lower level of MDA compared with the lower one.

Adding two different concentrations of selenium (0.01 and 0.1 μM) + Vitamin E (100 μM) showed 66% and 50% of CPE% in the two different concentration of selenium respectively. This means 34% and 50% inhibition values. Higher concentration of Selenium (0.1 μM) plus vitamin E (100 μM) treated cells showed significant (p>0.05) highest level of GSH in MDBK cells and significant lowest level of MDA compared with selenium or vitamin alone groups (Table 2).

Table 2: Effect of selenium or / and vitamin E treatments applied with different concentrations in pre-infection protocol on CPE%, GSH and MDA on MDBK cells of BHV-1.

Antioxidants in different conc.	CPE %	GSH (mg/dL)	MDA (nmol/ml)
Se (0.01 μM)	83%	0.053±0.011 ^a	2.37±0.68 ^a
Se (0.1 μM)	66%	1.93±0.35 ^b	0.99±0.024 ^d
Vitamin E (50 μM)	100%	0.024±0.018 ^a	4.4±1 ^b
Vitamin E (100 μM)	83%	0.059±0.029 ^c	2.89±0.17 ^a
Se+Vit E (0.01+100 μM)	66%	1.99±0.29 ^b	0.87±0.09 ^d
Se+Vit E (0.1+100 μM)	50%	2.86±0.25 ^e	0.57±0.012 ^e
Cell control	0	3.5 ±0.64 ^f	0.15±0.03 ^f
Virus control	100%	0.022±0.02 ^a	5.27±0.15 ^b

The values are means ± S.E., CPE% -cytopathic effect percentage, GSH- glutathione Reduced. MDA- malondialdehyde, different letters means significantly different at p>0.05 in the same Column.

Table (3) showed effects of selenium or / and vitamin E treatment supplied with different concentrations and post infection protocol treatment assay on CPE%, GSH and MDA in MDBK cells by bovine herpes virus -1. Results showed 100% cytopathic effect in the different groups after culture of virus. This reflected no inhibition

percentage of BHV-1 by the different treatments. Treated cell control showed higher level of GSH while there was non-significant difference between different groups. Virus control cell showed higher level of MDA while there was non-significant difference between the different treated groups.

Table 3: Effect of selenium or / and vitamin E treatments post infection on CPE%, GSH and MDA in MDBK cells by bovine herpes virus -1.

Antioxidants in different concentrations	CpE%	GSH (mg/dL)	MDA (nmol/ml)
Se (0.01µM)	100%	0.043±0.02 ^b	5.07±0.6 ^b
Se (0.1µM)	100%	0.033±0.03 ^b	4.37±0.1 ^b
Vitamin E (50µM)	100%	0.083±0.02 ^b	5±0.53 ^b
Vitamin E (100µM)	100%	0.093±0.03 ^b	3.8±0.6 ^b
Se+Vitamin E (0.01+100µM)	100%	0.043±0.02 ^b	4.9±0.52 ^b
Se+Vitamin E (0.1+100µM)	100%	0.043±0.02 ^b	4.8±0.65 ^b
Control cell	0	3.5 ±0.32 ^a	0.5±0.03 ^a
Virus cell	100%	0.053±0.04 ^b	5.27±0.8 ^b

The values are means ± S.E., CPE% - cytopathic effect percentage, GSH- glutathione Reduced. MDA- malondialdehyde, different letters means significantly different at $p > 0.05$ in the same Column.

DISCUSSION

BHV-1 is able to establish a latent infection in the trigeminal or sacral ganglia (Jones, 1998). Animals with a latent BHV-1 infection may serve as a source of infection for susceptible animals if and when the virus is reactivated (Engels, and Ackermann, 1996). Oxidative stress often accompanies viral infection, both in vivo (e.g. influenza (Oda *et al.*, 1989 and Clerici *et al.*, 1992) and in vitro (e.g. parainfluenza and herpes simplex type 1 (Rotilio *et al.*, 1994 and Palmara *et al.*, 1995). There is little understanding of how it influences virus replication. However the effect of the virus on the pro-/antioxidant balance in host cells, including virally induced inhibition of antioxidant enzymes such as glutathione peroxidase and virally induced increases in pro-oxidants such as nitric oxide; also effects of the redox state of the cell on the genetic composition of the virus as well as ROS-(reactive oxygenspecies) mediated release of host cell nuclear transcription factor-kappa-B, resulting in increased viral replication (Schwarz., 1996). In the search of a common redox event affecting virus replication, it has to be taken into account that the plasma membrane represents the earliest target of virus attack and reported that oxidative stress is able to modify the activity of cell membrane ion transport systems, such as Ca²⁺ movements, Na, K-pump, and sodium, potassium, and chlorine cotransport activity (Dawson., 1996 and Dröge *et al.*, 1994). These ion transport systems are essential for proper cellular function and viability, and their alterations may represent an early disturbance following oxidant exposure. Reduced glutathione (GSH) is the most abundant non-protein thiol in mammalian cells and the preferred substrate for several antioxidant enzymes

in metabolism (glutathione peroxidase) and antioxidant defense (Meister, 1988). It plays an important role in many cellular processes, such as cell differentiation, proliferation and apoptosis (Lu, 1999). Malondialdehyde (MDA) is a three-carbon compound formed from peroxidized polyunsaturated fatty acids, mainly arachidonic acid. It is one of the end products of membrane lipid peroxidation. Since MDA levels are increased in various diseases with excess of oxygen free radicals, many relationships with free radical damage were observed (Ohkawa *et al.*, 1979 and Guichardant *et al.*, 1994).

In the current study, the viral CPE% of BHV-1 usually appeared within 3 days after inoculation. The CPE% of BHV-1 is characterized by cell rounding and ballooning, forming grape-like clusters of spherical cells gathered around a gap in the monolayer. Sometimes, giant cells with several nuclei may be observed (Figure- 1). The addition of antioxidant, selenium, with two different concentration was significantly reduced viral CPE% as showed in table (2). The CPE% values were decreased (66%) when host cells treated with the higher concentration of selenium (0.1µM) compared with the lower concentration of selenium (Fig -3).

In the current study, cells infected with the virus maintained in media un-supplemented with antioxidants (virus control) demonstrated a progressive decrease in glutathione reduced level and increased level of MDA. Similar results obtained by (Saguna 2008) who demonstrated that Se deficiency can be induced in Vero and SK-N-SH cells and infected with WNV (West Nile Virus). Infection of Se-deficient Vero cells leads to enhanced cell death

by apoptosis and increased CPE%. Exposure of tissue cells to selenium alone resulted in a significant dose dependent increase in the level of reduced glutathione compared to control virus infected cells. This can be attributed to the enzyme GSH Px which uses GSH to reduce cellular peroxides consuming reduced glutathione in this process. (Bucker *et al.*, 1998 and Shen *et al.*, 2001). On the other hand, MDA declined significantly in cells exposed to selenium at a concentration of 0.1 and 0.01 μM . This indicates that selenium acts as a pro-oxidant at these concentrations and contributes towards building up of an oxidative environment. The inhibitory activity of selenium on viral replication was evidenced by protecting the tissue culture cells against the damaging effects of lipid peroxides and free radicals produced during viral infection Fig (3). This is evident that the higher concentration of selenium (0.1 μM) is better than lower one (0.01) as antioxidant. Similar results with selenium has demonstrated that a deficiency in this trace element will lead to decreased glutathione concentration (Beck, 1998) and increased viral pathogenesis (Melinda., 2001 and Cai. *et al.*, 2003).

Moreover, it is believed that low intracellular GSH levels may facilitate the course of viral infections either by increasing viral replication (De Quay *et al.*, 1992) or activating transcription factors with consequent increased production of inflammatory cytokines, interleukin-1, and interleukin-6 (Sonia *et al.*, 2007 and Lopez *et al.*, 1996).

Vitamin E alone in higher concentration (100 μM) decreased the CPE% to 83% and increased glutathione reduced concentration and decreased malondialdehyde level while the lower concentration (50 μM) showed 100% cytopathic effect after culture of virus. This reflected no inhibition percentage of BHV-1 by lower concentration of vitamin E. The same results obtained by (Peterhans 1997 and Reddy *et al.*, 1985) with increased concentration of vitamin E inhibited replication of Infectious Bovine Rhinotracheitis Virus in tissue cultures. On the simplest level, vitamin E has protective effects on glutathione-dependent enzymes (van Haaften., 2003). Furthermore, vitamin E plays a role in resistance to viral infection, Vitamin E deficiency allows a normally benign virus to cause disease (Rachel *et al.*, 1992 and Beck *et al.*, 1994 and). In mice, enhanced virulence of a virus resulted in myocardial injury that was prevented with adequate supply of vitamin E (Beck, 1994). A selenium or vitamin E deficiency leads to a change in viral phenotype, such that a non-virulent strain of a virus becomes virulent and a virulent strain becomes more virulent (Beck, 1997, Li and Beck, 2007). Vitamin E protecting fats within the cell membrane from breaking down and prevent cellular tissue damage, in which peroxidation of lipids destroys structural integrity of the cell and causes metabolic derangements. Sung *et al.* (2000)

suggests that, in addition to its antioxidant activity, other mechanisms might be involved in vitamin E beneficial effect on lowering viral titer (Fig -4).

Vitamin E and selenium have complementary but independent roles as antioxidants in the protection of cells against the damaging effects of lipid peroxides and free radicals produced during normal metabolism (Villar *et al.*, 2002). The cells treated with selenium and vitamin E (0.1 μM +100 μM); promoted anti oxidative effect with the lower CPE% (50%) Fig-2 with a decrease in malondialdehyde and an increase in glutathione reduced levels. Saito *et al.* (2003) suggested the same results that selenium and vitamin E cooperate in the defense against oxidative stress upon cells by detoxifying and inhibiting the formation of lipid hydro peroxides.

When host cells were treated with (selenium and vitamin E.) Prior to infection, CPE% of BHV-1 infection was reduced (Figure 2), also MDA level reduced while GSH level increased compared with post treatment infection. Thus, pre-treatment of MDBK cells with different concentration inhibited BHV-1 infectivity (table-2). However, when the antioxidants were added after virus penetration (post-treatment infection), the viral CPE% of the BHV-1 was 100%. This reflects need of time for syntheses of antioxidant enzymes or may be for receptor rearrangement or translocation of preformed receptors from intracellular stores. This agrees with (Chirase *et al.*, 2004) who concluded that Pretreatment with selenium is more antioxidant than the post treatment one. This reflects selenium intake around 6 weeks prior to sampling, as GHPX is an antioxidant enzyme associated with red blood cells which are formed several weeks prior to release into circulation. In a previous study (Hayek *et al.*, 1997) showed that old mice whose diet was supplemented with vitamin E for 6 weeks had significantly lower lung viral titer compared with old mice fed vitamin E following influenza infection. Also Norimasa *et al.* (1999) demonstrated the importance of pretreatment effect of vitamin E in tissue culture polymorph nuclear leucocytes and explained that the cytoprotective effect of α -Tocopherol is attributed to its ability to act as a scavenger of highly reactive oxygen radicals, thus stabilizing membranes against lipid peroxidation and requires receptor rearrangement or translocation of preformed receptors from intracellular stores which require hours (Norimasa *et al.*, 1999).

CONCLUSION

The higher concentration of Selenium and Vit. E (0.1 μM +100 μM) reduced the Cytopathic Effect (CPE) of BHV-1 by (50%) and increased GSH level, decreased MDA level, in pre infection treatment assay.

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تأثير اضافة السيلينيوم و/ او فيتامين هـ على الخلايا المصابة بفيروس هربس الابقار- ١ في خلايا الزرع النسيجي

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يعتبر فيروس هربس الابقار - ١ من الفيروسات الخطيرة التي تسبب مشاكل في الجهاز التنفسي والجهاز التناسلي في كلا من الاناث والذكور مما يؤدي الي خسائر اقتصادية فادحة وحيث انه لا يوجد علاج يؤدي الي الشفاء التام من هذا المرض فقد اتجه التفكير حديثا الي استخدام مضادات الاكسدة مثل السيلينيوم وفيتامين هـ كدواء طبيعي مضاد للاكسدة وفي هذا البحث قد تم استخدام السيلينيوم بتركيزين هما (٠,١ ميكرومولار) و (٠,٠١ ميكرومولار) وفيتامين هـ بتركيزين هما (٥٠ ميكرومولار) و (١٠٠ ميكروملار) معا او كل علي حدى لدراسة التأثير على فيروس هربس الابقار-١ المحقون في خلايا الزرع النسيجي بطريقتين بعد المعالجة وقبل المعالجة وذلك من خلال قياس التأثير الخلوى الباثولوجى وقياس تركيز انزيم الجلوتاثيون وايضا قياس تركيز المالونداالدهد في الخلايا وقد وجد ان استخدام التركيز العالى للسيلينيوم وفيتامين هـ معا (٠,١ ميكرومولار (١٠٠ ميكروملار) ادى الى احسن النتائج وهي انخفاض نسبية التأثير الباثولوجى الخلوى الى ٥٠% وزيادة تركيز الجلوتاثيون المختزل وانخفاض تركيز المالونداالدهيد)