

**THE EFFECTS OF ESTROGEN ON THEILER'S VIRUS  
INFECTION OF ENDOTHELIAL CELLS**

A Senior Scholars Thesis

by

STEVEN M. MAHER

Submitted to the Office of Undergraduate Research  
Texas A&M University  
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Biomedical Science

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Approved by:

Research Advisor:  
Associate Dean for Undergraduate Research:

Jane Welsh  
Robert C. Webb

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## ABSTRACT

The Effects of Estrogen on Theiler's Virus Infection of Endothelial Cells. (April 2009)

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Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system. The pathogenesis of this disease is not fully understood, but it is thought to be triggered by viral infection during early adulthood and is followed by autoimmune-mediated demyelination. Sex hormones are involved in the pathogenesis of MS since during pregnancy, women with MS undergo remission of the disease. Our laboratory uses Theiler's virus-induced demyelination (TVID) as the animal model for studying MS. Theiler's virus is a naturally occurring pathogen of mice, which causes an autoimmune-mediated demyelination of the central nervous system resulting in inflammatory lesions with similarities to human MS. In the current study we examined the effects of estrogen on Theiler's virus infection of cells that form the blood-brain barrier (BBB): cerebral vascular endothelial (CVE) cells *in vitro*. The hypotheses to be tested were that (1) estrogen would have a protective effect on the CVE cells and that (2) estrogen treatment would alter the expression of microRNAs. Cloned CVE cells from BALB/c and C57Bl/6 mice were used in these experiments, which remain diploid and

retain their differentiation markers for at least 20 passages in culture. In order to determine the effect of estrogen on modulating microRNAs within CVE, RNA was isolated from the cells and incorporated into miRNA chip analysis. We found miRNAs that are highly expressed in CVE: Let-7a, mmu miR-21, mmu miR-25, mmu miR-341, mmu miR-423, mmu miR-690. Cells were pre-treated with either 17 $\beta$ -estradiol, or ER- $\alpha$  agonist propyl pyrazole triol (PPT) or ER- $\beta$  agonist diarylpropionitrile (DPN). CVE cells were infected with TMEV at MOI of 10.0 for 24, 48, and 72h. Subsequently, the supernatants were used to measure cytotoxicity by LDH release assay. The results demonstrate that estrogen has a protective effect on CVE cells infected with TMEV and may therefore aid in the maintenance of the BBB integrity during viral infection. These results suggest that estrogen may have beneficial effects in a virus model of MS and may also be relevant for MS therapy.

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## NOMENCLATURE

MS	Multiple Sclerosis
CNS	Central Nervous System
EAE	Experimental Autoimmune Encephalomyelitis
TVID	Theiler's Virus-Induced Demyelination
CVE	Cerebrovascular Endothelial
DPN	Diarylpropionitrile
PPT	Propy pyrazole triol
ER	Estrogen Receptor
MOI	Multiplicity of Infection
IMDM	Iscove's Modified Dulbecco's Medium
FBS	Fetal Bovine Serum
BSA	Bovine Serum Albumin
LDH	Lactate Dehydrogenase

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# CHAPTER I

## INTRODUCTION

### **Multiple Sclerosis (MS)**

Multiple Sclerosis is the most common, chronic inflammatory demyelinating disease of the central nervous system (CNS), occurring at a prevalence of approximately 350,000 in the United States and 2.5 million worldwide(Compston and Coles, 2002). A recent study estimated the total average cost to be \$47,215 per patient per year which included medical and non-medical costs(Kobelt et al., 2006). The pathogenesis of MS is not completely understood, but the disease produces a hallmark appearance as plaque formation in the white matter of the CNS. This plaque is thought to be a result from immune-mediated demyelination of axons(Noseworthy et al., 2000). MS is an extremely debilitating disease and has symptoms that reflect its pathological plaque formation which may include, but not limited to, gait ataxia, limb weakness, pain, depression, clumsiness and optic neuritis(Noseworthy et al., 2000). In most cases patients will need the use of a walking aid within 15 years of the initial onset of the disease(Weinshenker et al., 1989). The initial onset of MS usually occurs between the ages of 15 and 40, and typically follows the relapsing remitting course of pathogenesis, in which the patient has an acute relapse for approximately 24 hours and then remission

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This thesis follows the style of the Journal of Neuroimmunology.

which may last months or even years(Orhun and Brian, 2005). Most patients will eventually develop the secondary progressive form of the disease in which the clinical signs and symptoms progressively worsen. This transition is thought to be mediated by accumulative neurodegeneration and cerebral atrophy(Orhun and Brian, 2005).

Although MS is rarely a cause of direct death, a recent study has shown that increased suicide rates are associated with this disease, resulting in premature death(Goldman Consensus Group, 2005).

### *Viral etiology of MS*

Genetic background and environmental factors appear to attribute to the etiology of MS. Although the etiology of MS is not completely understood, epidemiological studies have implicated an infective agent as a probable initiating factor(Acheson, 1977). Another epidemiological study reported increased risk of developing MS was associated with measles, mumps, and Epstein-Barr viruses occurring at older ages in childhood(Hernan et al., 2001). Viruses also have known to cause demyelination in animals: mouse hepatitis virus, measles virus in rats, herpes simplex in rabbits and Theiler's virus in mice(Dal Canto, 1982). Theiler's virus infection in mice represents an excellent model for study of the pathogenesis of MS.

### **Theiler's virus as a model for MS**

There are two recognized animal models for studying MS: experimental autoimmune encephalomyelitis (EAE) and virus-induced demyelination. EAE is useful to study the

autoimmune aspects of MS by initiating auto reactivity to myelin components of the CNS. Theiler's virus-induced demyelination (TVID), is one of the most commonly researched viral models and provides a unique model for the study of MS. Theiler's virus is a cardiovirus belonging to the family *Picornaviridae*, which is a naturally occurring pathogen of mice(Lipton, 1975; Theiler, 1937). This virus causes autoimmune demyelination in the CNS of mice resulting in the inflammatory lesions which are similar to patients with MS(Lipton, 1975; Lucchinetti et al., 2000). The pathogenesis and mechanism of demyelination by TVID is not fully understood, but is partly mediated by: direct viral lysis of oligodendrocytes, autoimmunity, cytotoxic T cell reactivity, and bystander demyelination mediated by virus-specific DTH T cells(Borrow et al., 1998; Gerety et al., 1994; Rodriquez and Sriram, 1988; Roos and Wollmann, 1984).

### **Estrogen hormones**

During the development of select neuronal groups, estrogen has shown to promote growth and differentiation as well as stimulate the expression of growth factors, cytoskeletal proteins and enzymes. There are two forms of estrogen receptors (ER) that have been identified, the classical estrogen receptor (Green et al., 1986), now called ER- $\alpha$ , and the newly cloned ER- $\beta$ (Mosselman et al., 1996). Activated ER interacts with a number of other target proteins, which initiate chromatin remodeling and recruit RNA polymerases. Estrogen has been shown to reduce neurotoxicity, improve cerebral blood flow and reduce infarct volume in a variety of experimental models(Rusa et al., 1999). Although the hormonal mechanism is not understood, it is likely that estrogen acts on

both neuronal and vascular components. A recent study showed that estrogen can affect the endothelium, by increasing responsiveness to endothelial-vasodilators seen after ischemia(Watanabe et al., 2001). There are three commonly used estrogens; 17 $\beta$ -estradiol, the ER- $\alpha$  ligand agonist propyl pyrazole triol (PPT) and the ER- $\beta$  ligand agonist diarylpropionitrile (DPN). 17 $\beta$ -estradiol can bind to both estrogen receptors, therefore potentially producing a more profound effect.

### **Gender and sex hormones in MS and EAE**

Multiple sclerosis is similar to other autoimmune diseases, in that there is a marked gender biased where women are twice as likely to develop MS as men(Confavreux et al., 1980). Although females seem to be more susceptible to the disease, their condition is less severe than males with MS who tend to deteriorate faster(Kremenchtzky et al., 1999). Although females have a higher risk of MS, female hormones appear to help improve symptoms of established MS. Therapy with estrogen has shown to alter T-cell activation and cytokine expression, as well as have strong neuroprotective effects by promoting axon and myelin survival(Tomassini and Pozzilli, 2006). In pregnant women, the relapse rate was reduced in the third trimester when estrogen levels are the highest, but worsened and then returned to normal after giving birth(Confavreux et al., 1998). Interestingly MS symptoms worsen during pre-menstruation and menopause when estrogen levels are decreased, but these symptoms are reduced by hormonal replacement(Smith and Studd, 1992). These studies suggest a neuroprotective effect of estrogen on MS. The effects of estrogen have also been investigated in the autoimmune

model EAE, which demonstrated that ER- $\alpha$  ligand treatment abrogated the disease at onset and ER- $\beta$  ligand treatment promoted recovery during the chronic stage. Also, ER- $\alpha$  ligand treatment was anti-inflammatory in the systemic immune system as well as the CNS, whereas ER- $\beta$  ligand treatment was not (Tiwari-Woodruff et al., 2007). 17 $\beta$ -estradiol has shown to reduce EAE in mice by inhibiting cell migration to the CNS, increasing T-cell regulatory effects, and through neuroprotective measures that promote myelin survival (Halina Offner, 2006).

### **Impact of sex hormones in Theiler's virus**

The effects of sex hormones on viral models of MS are not completely understood but do show some positive results. One study showed that male mice infected with TVID had higher viral loads in the early stage of the disease and worse demyelinating effects in the late stage compared to females (Alley et al., 2003). The result of this particular experiment concludes that estrogen could have a potential anti-viral effect. Another study showed that in-vitro estrogen treatment had inhibitory effects on viral replication in macrophages which correlates with earlier findings. Also, estrogen treatment inhibited VCAM-1 expression induced by tumor necrosis factor-alpha in cerebrovascular endothelial cells via inhibition of nuclear factor-kappa B (NFkB) (Fuller et al., 2005). Overall, estrogen treatment appears to show anti-inflammatory effects, decreased viral replication, and reduced infiltration of activated immune cells into the CNS. Although studies of estrogen have shown to have neuroprotective roles in the early stage of infection, the effects of estrogen have not been studied on the late demyelinating stage in

this model. The results of this research will aid in our understanding of the pathogenesis of multiple sclerosis and may lead to the development of therapeutic treatments.

## CHAPTER II

### METHODOLOGY

#### **Cerebrovascular endothelial cell isolation and purification**

For our studies it was imperative to produce CVE cells completely free from contamination of microglia, astrocytes and fibroblasts. The CVE cells were taken from 2-4 week old BALB/c and C57BL/6 mice and were isolated from the cerebrovascular microvessels by means of homogenization and filtration (Sapatino et al., 1993). These cells were then characterized with respect to Factor VIII-related antigen, angiotensin converting enzyme and uptake of acetylated low density lipoprotein. They retain their differentiation markers and remain diploid at least 20 passages. The cells were then frozen down using standard techniques and placed into a liquid nitrogen container for future use.

#### *Virus*

The BeAn 8683 strain of Theiler's virus (kindly provided by Dr. H. L. Lipton, Department of Microbiology-Immunology, University of Illinois at Chicago, IL) was initially propagated in lung tumor (L2) cells (Welsh et al., 1995). The virus was tittered using plaque assay method and found to have a concentration of  $8.3 \times 10^6$  pfu.



### **Experimental design for infection of BALB/c CVE cells**

A preliminary experiment was conducted using BALB/c CVE cells.

#### *Cell count and calculation of MOI for BALB/c CVE cells*

Cloned CVE cells were first grown in culture, a T25 flask, with Iscove's modified Dulbecco's medium (IMDM) containing 10% charcoal stripped fetal bovine serum (FBS), which has all estrogens removed, until a monolayer was established. Trypsin was used to release all cells adhering to the flask and the cells were re-suspended in 5ml of media. The cell suspension was mixed thoroughly and 20 $\mu$ l was removed and placed into a 1ml tube along with 20 $\mu$ L of trypan blue. This mixture was then placed into a hemacytometer. The hemacytometer was viewed under microscope and the average number of cells was determined by counting the cells in five different boxes. This number was then used to determine the amount of virus needed in order to make a MOI of 1.0 and 0.1.

#### *Infection of BALB/c with BeAn*

The CVE cells were either treated with 2nM 17 $\beta$ -estradiol dissolved in water four hours prior to infection or in the case of the control, media alone. The stock solution of 17 $\beta$ -estradiol was dissolved in water to obtain the correct concentration. After treatment with 17 $\beta$ -estradiol, the media was removed and the CVE cells were infected with Theiler's virus at a MOI of 0.1 and 1.0 in BSA (Sigma, IL), with or without 17 $\beta$ -estradiol. The virus was left on the CVE cells for 45 minutes and then removed. The cultures were

then prepared with IMDM containing 2% charcoal stripped FBS, with or without 17 $\beta$ -estradiol and left for 24 hours at which point the cells were observed microscopically. The supernatant was collected and spun down at 250 x g for 10 min, to remove all of the cells. The supernatant was used for further experiments and the cells were collected for miRNA analysis. This experiment was conducted for preliminary data. Table 1 shows the set-up.

Table 1  
Set-up for infection with or without 17 $\beta$ -estradiol

Culture Number	Amount of Virus (MOI)	Amount of 17 $\beta$ -estradiol
1	0.0	0.0
2	0.0	2nM
3	0.1	0.0
4	0.1	2nM
5	1.0	0.0
6	1.0	2nM

Note: Each culture was grown in a T25 flask

#### *Detection for cell death of BALB/c cells by means of LDH assay*

Lactate Dehydrogenase (LDH) is a stable enzymatic enzyme that is present in all cells. LDH is rapidly release into the culture supernatant when the cell membrane is damaged, which makes it an ideal marker for determining cell death. This assay provides a simple yet precise colorimetric method to measure LDH activity. Supernatant was collected in separate labeled 10ml tubes to determine the cell-mediated cytotoxicity in each culture. The supernatant was spun down at 250 x g for 10 min to remove all dead cells and any other contaminants. A 96-well flat-bottom plate was then prepared by adding 100 $\mu$ l of IMDM with 2% FBS to each well except for the first column. After centrifuging the

tubes, 200ul of supernatant was removed from each tube and transferred to the corresponding well in the first column. Each well was diluted using the doubling dilutions method and 100ul of reaction mixture was added to each well then incubated for 30 min protected from light. The plates were then measured with a multi-well plate reader at an absorbance of 520nm with a reference wavelength no greater than 600nm. This experiment was conducted as a preliminary run. Table 2 shows the set-up.

Table 2  
Set-up for the 96-well flat-bottom plate

Dilution	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Low Control	200ul of cell free supernatant	100ul media + 100ul from column 1	100ul media + 100ul from column 2	100ul media + 100ul from column 3
High Control	↓	↓	↓	↓
MOI 1.0				
MOI 0.1				
Estrogen + MOI 1.0				
Estrogen + MOI 0.1	↓	↓	↓	↓

Note: Remove 100ul from column 5 when finished, final amount should be 100ul in each well

Note: Add 100ul of reaction mixture to each well when finished with set-up

### *Calculating the percent cytotoxicity*

In order to determine the effects of estrogen on the CVE cells, a calculation was derived to obtain the percent cytotoxicity. The calculation is given in Fig. 1.

$$\text{Cytotoxicity (\%)} = \frac{\text{Test Well LDH release} - \text{Min LDH release}}{\text{Max LDH release} - \text{Min LDH release}} \times 100$$

Fig. 1. Equation used to calculate percent cytotoxicity for BALB/c CVE cells.

### *Obtaining the maximum and minimum LDH release*

In order to compare the percent cytotoxicity between the test wells, a maximum and minimum control must be obtained. To determine the maximum amount of LDH released by cell death, cells were treated with Triton X-100 at a final concentration of 1% in solution. This detergent will cause 100% cell death at this concentration without interfering with LDH activity. To obtain the minimum LDH release, cells were cultured without virus and estrogen, which corresponds to spontaneous cell death.

### *MicroRNA analysis*

RNA was extracted from CVE cells in collaboration with Dr. Steelman and Dr. Farida Sohrabji. The sample was then divided into two aliquots and labeled with Cy-3 and Cy-5 fluorescent dyes and hybridized competitively to a miRNA microarray.

### **Experimental design for infection of C57Bl/6 CVE cells**

This experiment was conducted after the conclusion of the preliminary experiment.

### *Cell count and calculation of MOI for C57Bl/6 CVE cells*

Cloned C57Bl/6 CVE cells were first grown in three 24-well plates with IMDM containing 10% charcoal stripped FBS, until a monolayer was established. The cells

were then trypsinized to release all cells from adhering to the well and re-suspended in 2ml of media. The cell suspension was mixed thoroughly and 20 $\mu$ l was removed and placed into a 1ml tube along with 20 $\mu$ L of Trypan blue. This mixture was then placed into a hemacytometer. The hemacytometer was viewed under microscope and the average number of cells was determined by counting the cells in five different boxes. This number was then used to determine the amount of virus needed in order to make a MOI of 10.0.

#### *Infection of C57Bl/6 with BeAn*

The CVE cells were either treated with 2nM 17 $\beta$ -estradiol dissolved in water, or 10nM DPN dissolved in ethanol, or 2nM PPT dissolved in ethanol four hours prior to infection or in the case of the control, media with or without ethanol. The stock solutions of the estrogens were diluted in their corresponding solution to obtain correct concentrations. After treatment with 17 $\beta$ -estradiol, DPN, or PPT the media was removed and the CVE cells were infected with Theiler's virus at a MOI of 10.0 in BSA (Sigma, IL), with or without 17 $\beta$ -estradiol, DPN or PPT. The virus was left on the CVE cells for 45 minutes and then removed. The cultures were then prepared with IMDM containing 2% charcoal stripped FBS, with or without 17 $\beta$ -estradiol, DPN or PPT and left for 24, 48 or 72 hours, at which point the cells were observed microscopically. The supernatant was collected in 1.5ml tubes at each time point and spun down at 250 x g for 10 min, to remove all cells. The collected supernatant was then placed in a -80°C freezer until needed for future experiments. Table 3 shows the set-up.

Table 3  
Set-up of the 24-well plate for infection with or without estrogen

	1	2	3	4	5	6
A – 24hr	Control	High Control	Control + estrogen	Virus MOI 10.0	MOI 10.0 + estrogen	Blank
B – 48 hr	Control	High Control	Control + estrogen	Virus MOI 10.0	MOI 10.0 + estrogen	Blank
C – 72 hr	Control	High Control	Control + estrogen	Virus MOI 10.0	MOI 10.0 + estrogen	Blank
D	Blank	Blank	Blank	Blank	Blank	Cell count

Note: Each plate has either 17 $\beta$ -estradiol, DPN or PPT as the estrogen

*Detection for cell death of C57Bl/6 CVE cells by means of LDH assay*

Supernatant was collected in separate labeled 1.5ml tubes to determine the percent cytotoxicity in each culture. The supernatant was spun down at 250 x g for 10 min to remove all dead cells. A 96-well flat-bottom plate was then prepared by adding 100 $\mu$ l of IMDM with 2% charcoal stripped FBS to each well except for the 1<sup>st</sup>, 5<sup>th</sup> and 9<sup>th</sup> columns. After centrifuging the tubes, 200 $\mu$ l of supernatant was removed from each tube and transferred to the corresponding well. Each well was diluted using the doubling dilutions method and 100 $\mu$ l of reaction mixture was added to each well then incubated for 30 min protected from light. The plates were then measured with a multi-well plate reader (Fluostar Optima), at an absorbance of 520nm with a reference wavelength no greater than 600nm. This experiment was conducted in triplicates. Table 4 shows the set-up.

Table 4  
Set-up for the 96-well flat-bottom plate at 24, 48 and 72 hrs

Dilution	1	1/2	1/4	1/8
Media	200µl of cell free supernatant	100µl media + 100µl from column 1	100µl media + 100µl from column 2	100µl media + 100µl from column 3
Low Control	↓	↓	↓	↓
High Control				
Control + 17β-estradiol				
MOI 10.0 + 17β-estradiol				
MOI 10.0				

Note: Remove 100µl from column 4 when finished, final amount should be 100µl in each well

Note: Each 96-well flat-bottom plate has this set-up repeated three times

Note: Replace 17β-estradiol with DPN or PPT supernatant for other 96-well plates

### *Calculating the percent cytotoxicity*

In order to determine the effects of estrogen on the CVE cells, a calculation was derived to obtain the percent cytotoxicity. Fig. 2 shows the equation.

$$\text{Cytotoxicity (\%)} = \frac{\text{Average LDH release} - \text{Min LDH release}}{\text{Max LDH release} - \text{Min LDH release}} \times 100$$

Fig. 2. Equation used to calculate percent cytotoxicity for C57Bl/6 CVE cells

*Obtaining the maximum and minimum LDH release*

In order to compare the percent cytotoxicity between the test wells, a maximum must be obtained. To determine the maximum amount of LDH released by cell death, cells were treated with Triton X-100 at a final concentration of 1% in solution. This detergent will cause 100% cell death at this concentration without interfering with LDH activity. To obtain the minimum LDH release, cells were cultured without virus and estrogen, which corresponds to spontaneous cell death.



## CHAPTER III

### RESULTS

#### Experimental results for infection of BALB/c CVE cells

The preliminary study for this experiment showed signs that estrogen had neuroprotective effects on CVE cells infected with TVID.

#### *Cell count and calculation of MOI*

Cells were counted in order to determine the amount of virus needed to induce an infection of a certain magnitude. Fig. 3 shows the results of the calculation.

$$\begin{array}{r}
 \text{Cell Count: } 37 \\
 48 \\
 55 \\
 38 \\
 \hline
 47 \\
 225 / 5 = 45 \\
 \\
 45 \times 10^4 = 4.5 \times 10^5 / \text{ml} \cdot 10 = 4.5 \times 10^6 \text{ total cells}
 \end{array}$$

Dilution Factor

$$\frac{8.3 \times 10^6}{4.5 \times 10^6} = 1.844$$

MOI 1.0 = 1.0ml virus in 0.844ml media

MOI 0.1 = 0.1ml virus in 0.844ml media

Fig 3. Cell count and calculation of MOI of 1.0 and 0.1

*LDH cytotoxicity assay of BALB/c CVE cells*

Estrogen treated BALB/c cells that were infected with TVID, had decreased LDH activity, as shown in Fig. 4. The calculation given in the methods was used to determine the percent cytotoxicity of each culture. Cells that were infected at a MOI of 1.0 and treated with 17 $\beta$ -estradiol had an average percent cytotoxicity of 31.15% ( $p \leq .05$ ) less than non-treated cells. Also, cells infected at a MOI of 0.1 and treated with 17 $\beta$ -estradiol had an average percent cytotoxicity of 19.09% ( $p \leq .05$ ) less than non-treated cells.

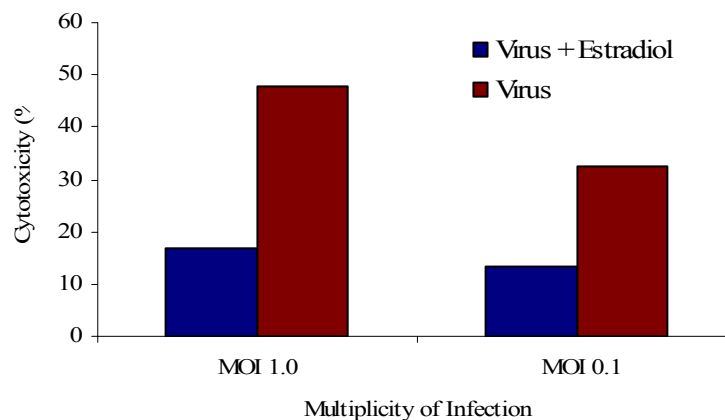


Fig. 4. The difference in cytotoxicity between infected cells. Cells were treated with 17 $\beta$ -estradiol and not treated with 17 $\beta$ -estradiol. At MOI 1.0 there was 31.15% ( $p \leq .05$ ) difference between cells treated with 17 $\beta$ -estradiol and not treated with it. At MOI 0.1 there was 19.09% ( $p \leq .05$ ) difference between cells treated with 17 $\beta$ -estradiol and not treated.

To calculate the average percent of overall cytotoxicity, the values of percent cytotoxicity for each dilution were combined. Fig. 5 shows the results of the individual dilution average percent cytotoxicity.

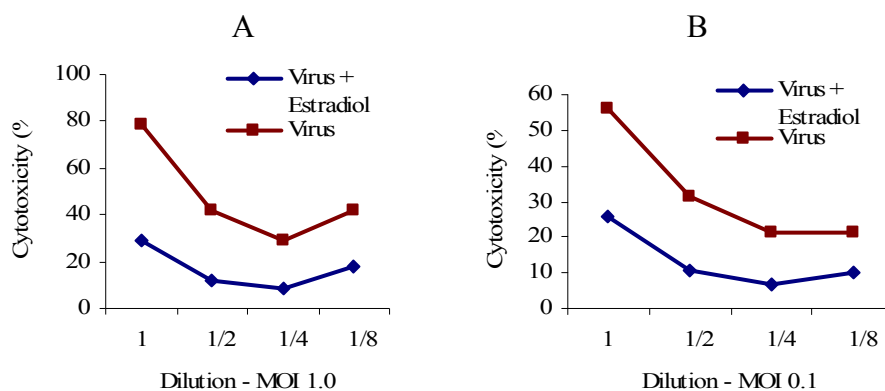


Fig. 5. Percent cytotoxicity for individual dilutions. (A) The average percent cytotoxicity was determined at a MOI of 1.0 at each dilution, and it showed that the levels maintained their differences. (B) The average percent cytotoxicity was determined at a MOI of 0.1 at each dilution, and it showed that the levels maintained their differences.

### *MicroRNA analysis*

The resulting “same on same” hybridization pattern revealed that highly expressed microRNAs in BALB/c endothelial cells are: Let-7a, mmu miR-21, mmu miR-25, mmu miR-341, mmu miR-423, mmu miR-690. These results indicate that these miRNA's are candidate markers for CVE cells, or at least CVE cells express them ubiquitously.

### **Experimental results for infection of C57Bl/6 CVE cells**

After obtaining data from the preliminary experiment it was decided to complete a full project using C57Bl/6 CVE cells.

### *Cell count and calculation of MOI*

Cells were counted in order to determine the amount of virus needed, to induce an infection of a certain magnitude. Fig. 6 shows the results of the calculation.

Cell Count: 3  
 5  
 2  
 4  
 3  
 $17/5 = 3.4$

$3.4 \times 10^4 / \text{ml} \times 4 = 1.36 \times 10^5$  total cells per well

Dilution Factor

$$\frac{8.3 \times 10^6}{0.136 \times 10^6} = 61.029$$

MOI 10.0 = 1.0ml virus in 6.0ml media

Fig. 6. Cell count and calculation of MOI of 10.0.

*LDH cytotoxicity assay of C57Bl/6 CVE cells – 24hrs*

After conclusion of the experiment the supernatant was used to determine the effects of estrogens by means of percent cytotoxicity. The supernatant was collected at 24, 48 and 72 hour marks. At 24hrs the cells were still confluent and maintained their monolayer. When the supernatant was collected and spun down, very few cells were seen at the bottom of the tube. The cell-free supernatant was tested for cell death LDH release. The LDH assay had similar results as the preliminary experiment. All of the estrogens decreased the percent cytotoxicity, but only 17 $\beta$ -estradiol was by a significant amount. 17 $\beta$ -estradiol decreased the percent cytotoxicity by 1.79% ( $p \leq .05$ ). The other two estrogens, DPN and PPT also decreased the percent cytotoxicity, which shows a strong trend. DPN decreased the average percent cytotoxicity by 1.83% ( $p \geq .05$ ) and PPT decreased the average percent cytotoxicity by 1.57% ( $p \geq .05$ ). Fig. 7 shows the results.

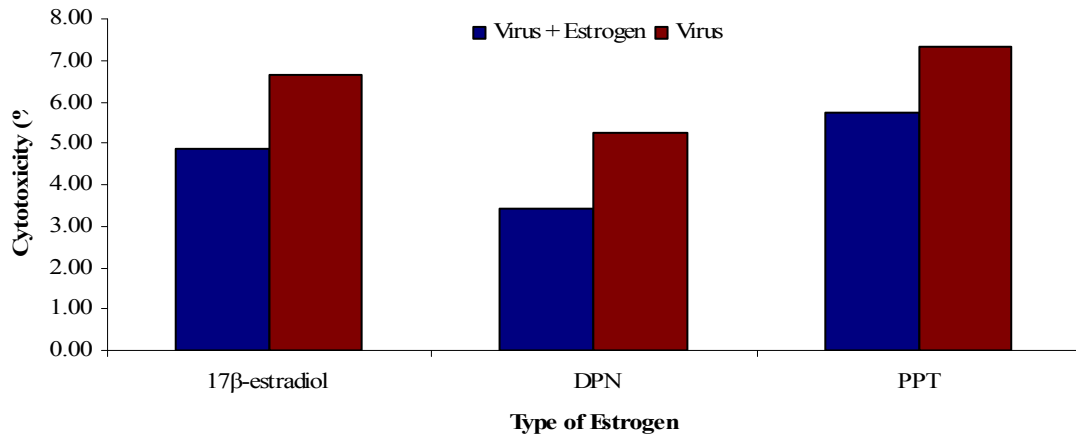


Fig. 7. Difference between estrogen treated and not treated cells at 24hrs. 17β-estradiol decreased the percent cytotoxicity by 1.79% ( $p \leq .05$ ). DPN decreased the average percent cytotoxicity by 1.83% ( $p \geq .05$ ) and PPT decreased the average percent cytotoxicity by 1.57% ( $p \geq .05$ ).

The averages for the above results were determined by combining the averages for each dilution. Fig. 8 shows the individual results for each type of estrogen with respect to the dilution principle.

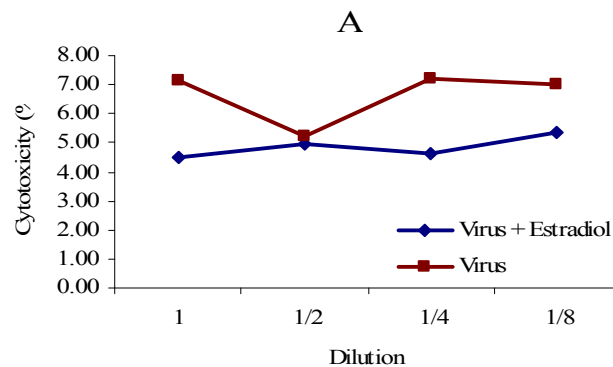


Fig. 8. Individual averages of percent cytotoxicity for each estrogen at 24hrs. (A) 17β-estradiol averages. (B) DPN averages. (C) PPT averages.

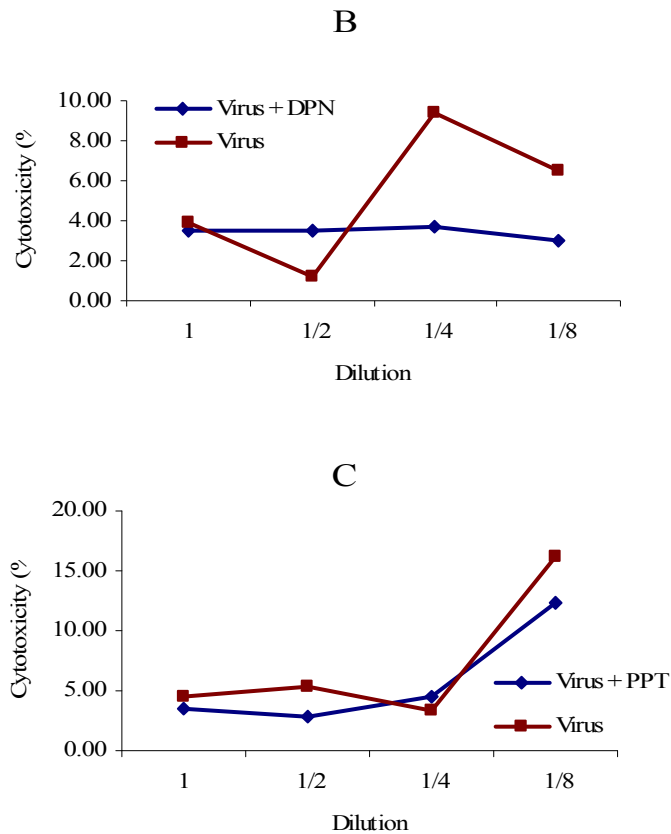


Fig. 8 Continued.

*LDH cytotoxicity assay of C57Bl/6 CVE cells – 48hrs*

The cell cultures were examined at 48hrs and the supernatant was removed. The cells treated with estrogen were approximately 90% confluent, compared to the cells not treated with estrogen which were approximately 75% confluent. The supernatant had few cells, but the supernatant that was treated with estrogen had less. The supernatant was measured using the LDH assay, and the results showed that estrogen was still decreasing the LDH activity. The results of the assay demonstrated that PPT had the

greatest decline in percent cytotoxicity on infected cells, 5.89% ( $p \leq .05$ ). PPT was the only estrogen that displayed statistically significant data, but  $17\beta$ -estradiol and DPN also decreased percent cytotoxicity of infected cells.  $17\beta$ -estradiol decreased the amount by 2.83% ( $p \geq .05$ ) and DPN decreased the percent by 2.36% ( $p \geq .05$ ). Although these two estrogens were not statistically significant they did display a strong trend in decreasing the percent cytotoxicity. Fig. 9 displays the results.

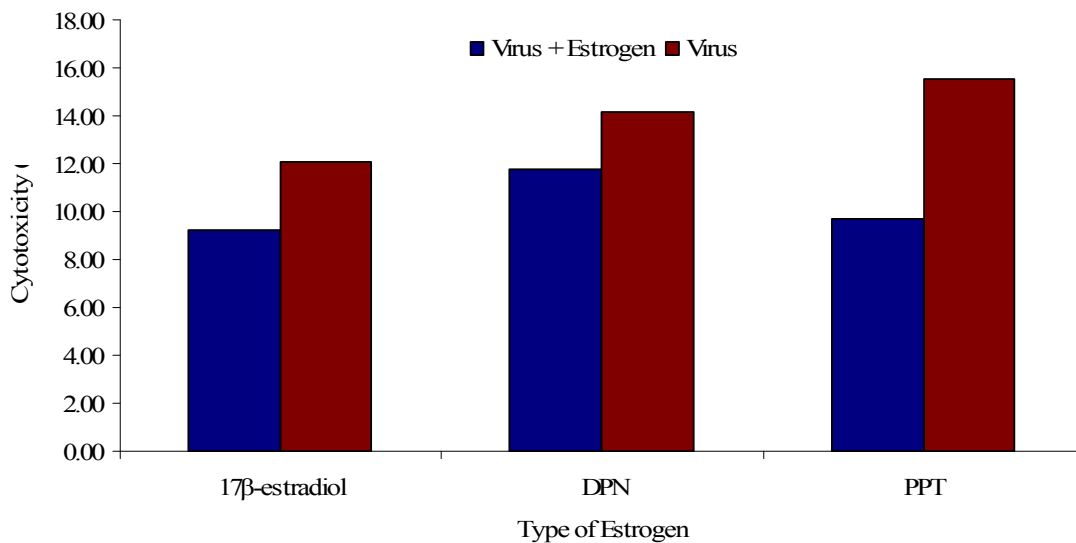


Fig. 9. Difference between estrogen treated and not treated cells at 48hrs.  $17\beta$ -estradiol decreased the percent cytotoxicity by 2.83% ( $p \geq .05$ ). DPN decreased the average percent cytotoxicity by 2.36% ( $p \geq .05$ ) and PPT decreased the average percent cytotoxicity by 5.89% ( $p \leq .05$ ).

The averages for the above results were determined by combining the averages for each dilution. Fig. 10 shows the individual results for each type of estrogen with respect to the dilution principle.

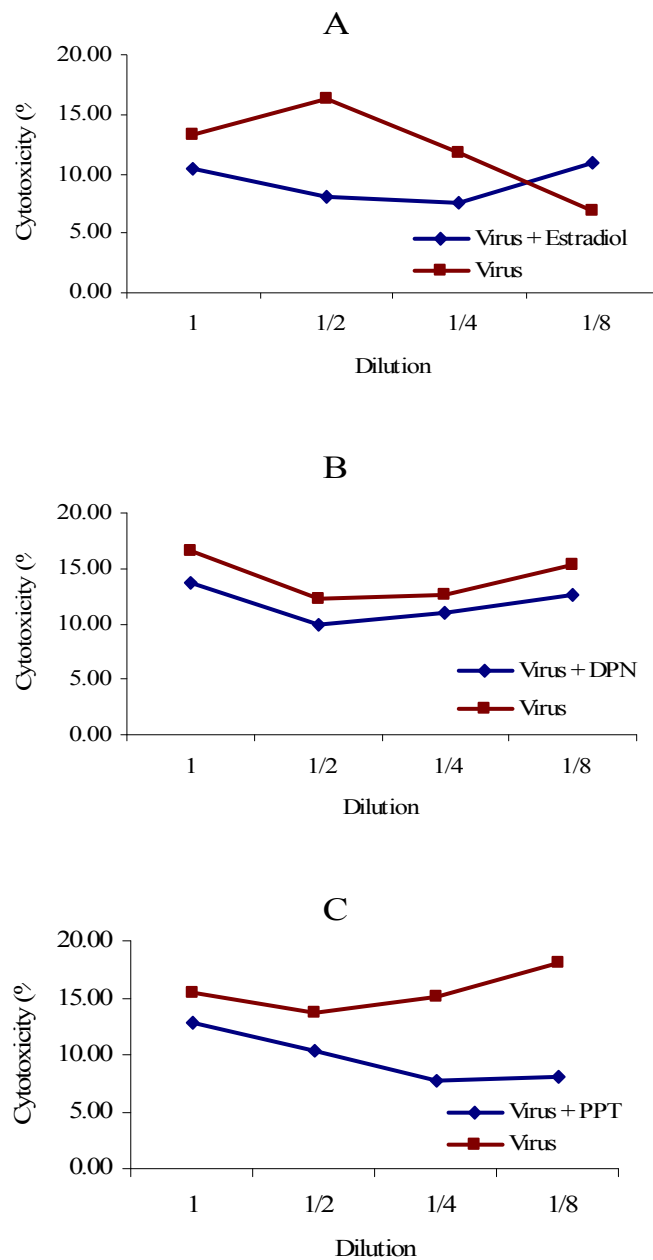


Fig. 10. Individual averages of percent cytotoxicity for each estrogen at 24hrs. (A) 17 $\beta$ -estradiol averages. (B) DPN averages. (C) PPT averages.



*LDH cytotoxicity assay of C57Bl/6 CVE cells – 72hrs*

The last extraction of supernatant was made at the 72hr mark and the cells were observed closely. The cultures that were infected and not treated with estrogen were approximately 60% confluent and there were a lot of dead cells floating. The cultures that were infected and treated with estrogen were approximately 75% confluent but there were less dead cells floating compared to the cultures not treated with estrogen. The supernatant was measured using the LDH assay, and the results showed that estrogen was still decreasing LDH activity. The results of the assay demonstrated that both 17 $\beta$ -estradiol and PPT decreased the percent cytotoxicity compared to the cultures that were not treated with estrogen. 17 $\beta$ -estradiol decreased the percent cytotoxicity by 8.12% ( $p \leq .05$ ) and PPT decreased the percent cytotoxicity by 2.46% ( $p \leq .05$ ). DPN on the other hand had no effect on the percent cytotoxicity compared to the culture not treated with estrogen. Fig. 11 displays the average results for the effects of estrogen on TVID infected CVE cells at the 72hr mark.

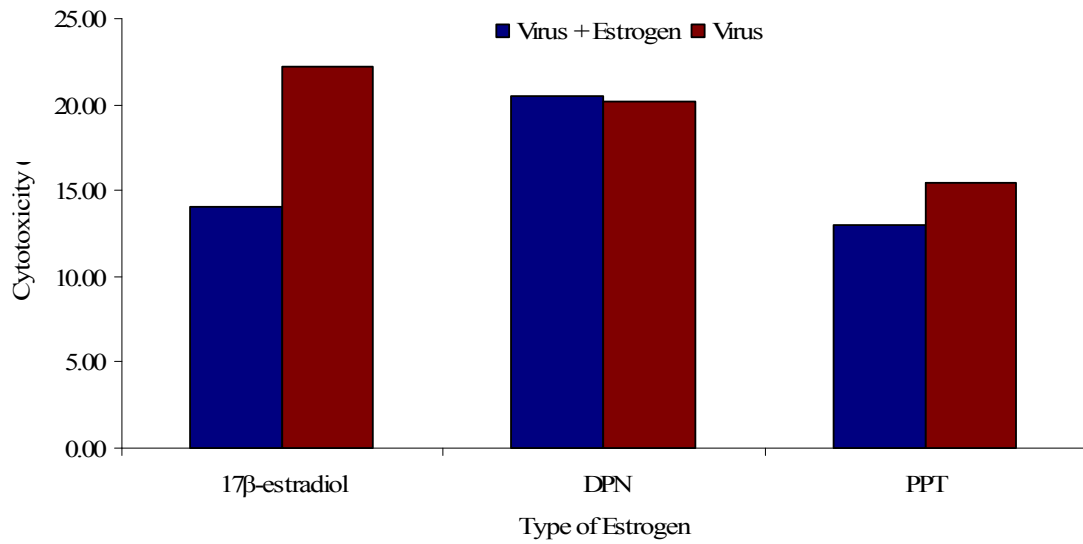


Fig 11. Difference between estrogen treated and not treated cells at 72hrs. 17β-estradiol decreased the percent cytotoxicity by 8.12% ( $p \leq .05$ ) and PPT decreased the percent cytotoxicity by 2.46% ( $p \leq .05$ ). DPN on the other hand had not effect on the percent cytotoxicity compared to the culture not treated with estrogen.

The averages for the above results were determined by combining the averages for each dilution. Fig. 12 shows the individual results for each type of estrogen with respect to the dilution principle.

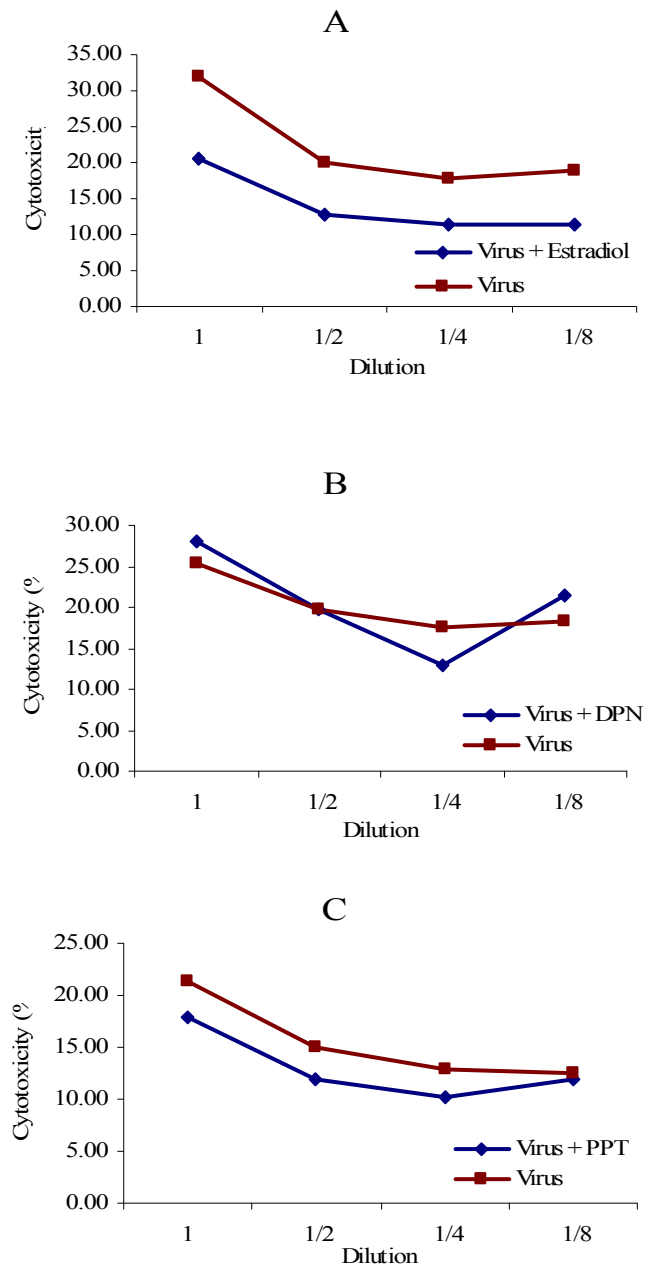


Fig. 12. Individual averages of percent cytotoxicity for each estrogen at 72hrs. (A)  $17\beta$ -estradiol averages. (B) DPN averages. (C) PPT averages.

## CHAPTER IV

### SUMMARY AND CONCLUSIONS

The present studies indicate that both BALB/c and C57Bl/6 CVE cells are susceptible to TVID infection *in vitro*. The studies also demonstrate that both strains of cells are directly affected by estrogen but the pattern and extent of neuroprotection are not the same. Estrogen reduced the LDH activity in both BALB/c and C57Bl/6 CVE cells, but the treatment decreased the cytotoxicity of TVID on BALB/c cells by a much greater margin. Also, 17 $\beta$ -estradiol and PPT showed to have a significant reduction in LDH activity on C57Bl/6 CVE cells, while DPN did not have a significant decrease.

#### **Discussion**

So what does all this mean? There are a couple of possibilities that can be drawn from this experiment. First of all, the data suggests that BALB/c CVE cells are more susceptible to estrogen response compared to C57Bl/6 CVE cells. This could mean that BALB/c CVE cells have more estrogen receptors than C57Bl/6 CVE cells. This conclusion can be drawn because of the greater significant difference in LDH activity between BALB/c CVE cells and C57Bl/6 CVE cells treated with estrogen. Further studies should be completed to compare the difference in CVE cells between strains to determine the difference in cytotoxicity. Another conclusion that can be drawn from this experiment is that 17 $\beta$ -estradiol and PPT have a more profound effect on CVE cells than compared to DPN. This could possibly be due to the fact that CVE cells have more ER- $\alpha$

ligand binding sites than ER- $\beta$  ligand binding sites. While estrogens have proven to be neuroprotective, some are considered useful during onset of disease while others show their action during the chronic stage of the disease. The experiment that was conducted shows the results of the onset of the disease because cells were only infected for 72hrs. This shows that the ER- $\alpha$  agonist PPT has a greater effect on CVE cells during the onset of TVID than DPN. These results coincide with a study completed on EAE, another model for Theiler's virus, which shows the ER- $\alpha$  agonist PPT abrogated the disease at onset and throughout the course of the disease, while DPN only showed neuroprotective effects during the chronic stage (Tiwari-Woodruff et al., 2007). This experiment allowed us to obtain crucial preliminary data for further studies with estrogen on Theiler's virus. The results in this experiment have set-up a basic understanding of the effects of estrogen on TVID. Future experiments should be conducted on the effects of estrogen at pregnancy concentrations, the effect of estrogens on CNS helper cells and the effects of estrogens on Theiler's virus *in vivo*. With further studies, a possible treatment could be obtained for patients with MS.

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