Susceptibility to HSV-1 infection and exercise stress in female mice: role of estrogen

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Abstract

Exhaustive exercise has been associated with an increased risk for URTI in mice and humans. We have previously shown that female mice are better protected from the lethal effects of HSV-1 infection, both at rest and following exercise stress, but little is known about possible mechanisms. This study tested the effects of estrogen on HSV-1 infection and macrophage antiviral resistance following repeated exhaustive exercise. Female mice were assigned to either exercise (Ex) or control (C): intact female (I-C or I-Ex), ovariectomized female (O-C or O-Ex), or ovariectomized estrogen-supplemented female (E-C or E-Ex). Exercise consisted of treadmill running to volitional fatigue (~125 min) for 3 consecutive days. Intact female mice had a later time to death than O and E (p < 0.05) and fewer deaths than both O and E (p < 0.05). Exercise stress was associated with increased time to sickness (p < 0.05) and symptom severity at days 6 and 12-21 post-infection (p < 0.05) and decreased macrophage antiviral resistance (p < 0.001) in all groups. E had increased symptom severity at days 6 and 13-21 post-infection (p < 0.05). Results indicate that intact female mice are better protected from the lethal effects of HSV-1 infection and that exercise stress had a similar negative impact in all groups. This protective effect was lost in ovariectomized mice, but was not reinstated by 17β-estradiol replacement. This indicates that other ovarian factors, alone or in combination with estrogen, are responsible for the protective effects in females.

Key words: virus; ovariectomy; gender; mortality; morbidity
Introduction

Sexual dimorphism is evident in respiratory diseases, with males being more susceptible to viral and some bacterial infections, and sepsis (7; 17; 34; 42), whereas females are more susceptible to many autoimmune diseases and have greater immunoreactivity to particular pathogens (7; 18; 19). We have previously shown that female mice appear to be less susceptible to intranasal herpes simplex virus type-1 (HSV-1) infection (4) and recent data suggests that greater macrophage antiviral function may account for this difference (5). Studies of potential mechanisms of the gender differences in infection often focus on estrogen supplementation, but research is conflicting due to differing species, dosages of estrogen, and experimental conditions. In animal models, estrogen treatment has been shown to both increase and decrease risk of infection (15; 30; 31; 38), inflammatory cytokine responses (22), and macrophage function (10). Estrogen has also been shown to affect macrophage phagocytic activity (33), enzymatic activity (2), and intracellular killing (8). Estrogen appears to alter inflammatory cytokine responses in a biphasic manner, physiologic dosages stimulate while supra-physiologic/pharmacologic dosages inhibit the responses (29) and this impact on cytokines (via estrogen’s influence on the production/release of cytokines from leukocytes (22; 31; 33; 38)) may heavily influence the host’s resistance to various infections (15; 31). It is also possible that greater immune responses in females versus males (i.e. macrophage antiviral function and plasma inflammatory responses to HSV-1) may produce greater sickness symptoms, but overall better protection from the lethal effects of HSV-1 infection and estrogen may partially account for these observed gender differences.
Estrogen, infection, and exercise

In humans, intense exercise is associated with increased risks for upper respiratory tract infections (URTI) and suppressed immune function (28). In our animal model of exercise and HSV-1 infection, we find that single or repeated bouts of exhaustive exercise increases susceptibility to infection (12; 13; 23) and decreases macrophage antiviral resistance (5; 12; 13), and we now have data to show that female mice are better protected from this effect than males (4), however, preliminary data suggest they also may have greater severity of symptoms. In addition, female mice have elevated macrophage antiviral resistance as compared to males (5). It is possible that estrogen exposure may partially account for the lower risk of infection while increasing symptoms of infection/sickness as part of a generalized inflammatory response. The primary purpose of this study was to test the effects of estrogen on susceptibility to in vivo HSV-1 infection and in vitro macrophage antiviral function to HSV-1 following repeated exercise stress. It was hypothesized that estrogen would be associated with lower mortality and greater macrophage antiviral function in females. We also hypothesized that exercise would negate the beneficial effects of estrogen.

Methods

Mice

Sexually mature female CD-1 mice, seven weeks of age (Harlan Sprague Dawley Labs), were acclimated to our facility for one week prior to any experimentation. Mice were purchased as pathogen free stock, and periodic screening of sentinel mice yielded negative results for common murine viral or bacterial pathogens. Mice were housed four per cage and cared for in the animal facility at the University of South Carolina Medical School. Mice were maintained on a 12:12-h light-dark cycle in a low stress environment (22°C, 50% humidity, low noise) and
given food (Purina Chow) and water *ad libitum*. Body weights were taken before and after the exercise or control period. Dry uterine weights were taken following sacrifice.

**Ovariectomies**

Ovariectomies (ovariectomized (O) and ovariectomized plus estrogen (E) groups) and sham surgeries (I groups) were performed at least 10 days prior to exercise treatment and standardized procedures were used. Briefly, a midline dorsal incision was made in the lumbar region to reveal the dorsal fat pads containing the ovaries and the ovaries were tied off and removed. Sham surgeries were performed in the same manner but ovaries were not tied off nor removed. Mice were returned to home cages for 10 days (to allow for hormones to wash out in the O groups). Following sacrifice, dry uteri were weighed to confirm that the ovariectomy and estrogen replacement procedures were successful.

**Estrogen treatment**

Estrogen (E groups) or placebo (I and O groups) pellets were surgically implanted subcutaneously in the dorsal region at the time of ovariectomy or sham surgery. The estrogen pellets released a high-physiologic dosage of approximately 1 µg 17-β estradiol (1 mg β-estradiol and 24 mg cholesterol, Sigma Chemicals Co.) per day. This dosage was based literature examining estrogen and infection (15; 30; 31) and on the work of Pung and Luster (1986) (32) who reported that 2-10µg/animal in ovariectomized female mice resulted in normal estrogen serum levels (0.1-0.4 X 10⁻⁶M) and because it was not likely to cause unwanted side effects, such as weight loss and carcinoma, and it would illicit constant physiologic serum levels (30). A limitation to the study was the use of only one dose as well as using a consistent dose versus a
cycling dose which would mimic a normal cycling female across the estrus cycle. Serum levels were quantified with RIA procedures prior to experiments to ensure proper dosage. Placebo pellets contained inactive ingredients (25 mg cholesterol, Sigma Chemicals Co.).

*Treadmill acclimation and exercise protocol*

The University’s Institutional Animal Care and Use Committee approved the protocol described. Exercise mice (I-Ex, O-Ex, and E-Ex) were acclimated (at least 10 days following surgeries) to the treadmill for a period of 20 minutes a day for three consecutive days. This acclimation consisted of treadmill running at 18 m/min at 5% grade. Following acclimation, mice ran on the treadmill (3 per lane) at a speed of 36 m/min and a grade of 8% grade, which elicits approximately 70–80% $\text{VO}_2\text{max}$ (14; 36) until volitional fatigue. Fatigue was defined as the inability to keep up with the treadmill speed for one minute with continual hand prodding. The exercise protocol was performed in the evening, 6 p.m., for three consecutive days. Our model, which includes three consecutive days of prolonged treadmill running to fatigue, mimics a short period of severe exercise training similar to what may occur in athletes. Additionally, three days of exercise was chosen as it is our experience that effects of a single session of such exercise on macrophage function and susceptibility to HSV-1 infection are less robust and often require a larger sample size. We interpret the exercise stress effects in this protocol to be a cumulative effect of repeated daily sessions of exercise. Electric shock was never used in these experiments as mice readily respond to a gentle tap of the tail or hindquarters encouraging them to maintain pace with the treadmill. Mice in the control groups (I-C, O-C, and E-C) remained in their cages in the treadmill room throughout the exercise bouts. These mice were exposed to similar
handling and noise in an attempt to control for extraneous stresses that may be associated with treadmill running. All mice were deprived of food and water during the exercise sessions.

In vivo titration of HSV-1

Intranasal inoculation of herpes simplex virus type one (HSV-1) VR strain in the mouse is an established experimental model of respiratory infection. Although HSV-1 is not a common respiratory virus in humans, it can cause various pathological conditions in humans such as meningo-encephalitis, hepatitis, esophagitis, tracheobronchitis, and pneumonia as well as being associated with cases of adult respiratory distress syndrome (12; 27; 39). The intranasal route was chosen to mimic the typical route of entry for viral infection. HSV-1 was propagated in Vero cells and stored at -70°C in medium supplemented with 10% fetal bovine serum (FBS) and 2% penicillin, streptomycin and L-glutamine (PSG). The virus was titrated by administering 50 µl of various dilutions to mice in an initial experiment to determine the lethal dose. Morbidity and mortality were monitored for 21 days. A dose that caused 40% mortality was selected for subsequent experiments. This dose was selected based on previous work (4) demonstrating that animals of this age can recover from the infection. Therefore chose a dose that would allow us to observe possible group differences in time to death, time to sickness, and overall symptom severity scores.

Intranasal infection with HSV-1

On the day of the experiment, mice (n = 21-23 per group; I-C, O-C, E-C, I-Ex, O-Ex, and E-Ex) were exposed to either control or exercise treatment. Fifteen minutes following exercise or control, mice were lightly anesthetized with halothane and inoculated intranasally with 50 µl of HSV-1 VR strain. The LD40 preparation of this virus strain contained 8.2X10^6 plaque forming units.
units (PFU)/mL; therefore, each mouse received $3.2 \times 10^5$ PFU. A dose of $1.28 \times 10^6$ PFU’s of a similar preparation of this virus was found to yield an average of $1.55 \times 10^6$ PFU’s/lung in a small sample (n=5) of mice at 3 days following intranasal inoculation. The pathogenesis and symptomatology of infection following intranasal inoculation of HSV have been well characterized (39). Following inoculation, the mice were returned to their respective cages and housed in an isolated P2 facility. All animals were monitored and scored (blinded to experimental groups) daily for a period of 21 days for signs of mortality and morbidity (time to sickness and symptom severity). The symptom severity scale (Table 1), which was developed in our lab, rates animals from 0-17 based on varying degree of symptoms of sickness such as lesions, ruffled fur, sore eyes, hindlimb paralysis, hunched back, and unresponsiveness. Mice that did not display any of these symptoms were considered healthy. It is not uncommon for animals at this age to recover once they exhibit these symptoms. Therefore, it was important to document the possibility that the experimental treatment may have either delayed time to death once animals were sick and/or allowed for full recovery, as this would have important immunological implications.

**Peritoneal macrophage antiviral resistance to HSV-1**

On the day of the experiment, mice (n = 12-14 per group) were exposed to either the control or exercise treatment. Immediately following exercise or control, mice were euthanised in a bell jar by halothane overdose. Death occurred within one minute. Peritoneal macrophages were collected, prepared and infected with HSV-1 as previously described (12). Briefly, peritoneal macrophages were obtained by lavage of the peritoneal cavity with 5 ml of culture media. Peritoneal lavage cells were washed and red blood cells were lysed with tris (hydroxymethyl) aminomethane-ammonium chloride, pH 7.2. Cells from two animals of the
same group were pooled in order to obtain enough cells resulting in a final n of 6-7 cell pools per group. Cells in each pool were adjusted to a concentration of 2X10^6 cells/ml in cell culture media. Viability was determined using trypan blue exclusion and was typically >94%. Subsequently, 200ul of the cell preparation was added to the wells of a 96-well microtiter plate and allowed to adhere at 37°C and 5% CO2. After 12 h, each well was washed gently to remove nonadherent cells. The adherent macrophages were infected with HSV-1 KOS strain virus contained in 50ul of medium. The virus was allowed to absorb for 90 min. Prewarmed RPMI-1640 supplemented with 10% FBS was added to each well (to a final volume of 250ul), and the plates were incubated at 37°C and 5% CO2 for 72 h. The HSV-1 virus used had been propagated in Vero cells and titrated on macrophages. Three doses that resulted in a macrophage viability of ~60, 70, and 80% (5-15 PFU/cell) were chosen for this experiment. Aliquots of the virus were stored at -80°C. Seventy-two hours after infection with HSV-1, a cytopathic effect was observed in the macrophages and was quantified by a neutral red dye uptake assay as previously described (12).

**Blood Collection**

Following halothane overdose, blood was collected by draining the inferior vena cava with a heparinized sterile 1mL syringe. Samples were then transferred to tubes, centrifuged, and plasma was stored at -70°C until assayed for estrogen.

**Plasma estrogen**

Plasma estrogen concentrations were determined in duplicate by a double antibody commercially available radioimmunoassay (RIA) kit (Diagnostics Products Systems, Los Angeles, CA) as per manufacturer’s instructions.
Statistical analysis

Statistical analyses were performed using commercially available statistical packages from SAS (SAS, Cary, NC) and Sigma Stat (SPSS Inc., Chicago, IL). Differences in morbidity and mortality between groups across a 21-day post-infection period were determined using a Lifetest Survival Analysis program in SAS (p < 0.05). Post-hoc \( \chi^2 \) analysis (p < 0.05) was used to determine the significance of differences between groups in percent morbidity and mortality at point estimates obtained from the Survival Analysis as well as several days surrounding the point estimates. Differences in run times to fatigue were compared using a One-Way Analysis of Variance (p < 0.05). Differences in macrophage antiviral resistance, plasma estrogen, body weights, and uteri/BW ratio were compared using a Two-Way Analysis of Variance with Student-Neuman-Keuls post-hoc comparisons (p < 0.05).

Results

Body and uterine weights

There was a significant group difference in final body weight (p < 0.001). Ovariectomized (O) mice weighed significantly more than I (25.95 ± 0.41 g versus 24.94 ± 0.41 g). There were no exercise effects on final body weight. There were no differences in body weight changes during the course of the study between any of the groups. As expected, I and E had greater uterine weights (25.6 ± 2.5 mg and 25.5 ± 3.2 mg versus 9.5 ± 1.6 mg; p < 0.001) and uterine/body weight ratio than O (10.2 \times 10^{-4} ± 5.7 \times 10^{-5} and 10.2 \times 10^{-4} ± 5.7 \times 10^{-5} versus 3.7 \times 10^{-4} ± 6.4 \times 10^{-5}, p < 0.001) suggesting complete ovariectomies in O groups with adequate estrogen.
supplementation to restore the uterine weight in E groups. There were no differences between E and I groups and exhaustive exercise did not have an effect on any group for uterine weight or uteri/body weight.

**Morbidity**

Figure 1 illustrates the time course in morbidity over the 21-day post-infection period. Survival analysis showed that exercise stress followed by intranasal inoculation of HSV-1 resulted in faster time to sickness than in resting controls (point estimates, 6.5 ± 0.1 days Ex versus 7.6 ± 0.2 days C, p < 0.05). Based on these point estimates, post-hoc \( \chi^2 \) tests were performed on days 6-12 and 21 post-infection. Non-exercise groups recovered (recovery was defined as an animal having no visible signs on sickness) by day 16 (~85-90% of group appeared recovered), while exercise groups remained sick throughout the 21 days (~65% of group appeared recovered) (p<0.05).

**Mortality**

Group differences were found for mortality (time to death) over the 21-day post-infection period. Intact females experienced a later time to death than ovariectomized and estrogen-supplemented females (Figure 2, \( p = 0.001 \)). Point estimates were 11.3±0.2 days for I, 10.0±0.4 days for O, and 10.9±0.6 days for E. Based on these point estimates, post-hoc \( \chi^2 \) tests were performed on days 9-12 and 21 post-infection and showed differences between the groups in mortality rates. On days 9, 10, 11, and 12-21, ovariectomized (36.6% - 58.5%) and estrogen-supplemented females (51.1% - 61.7%) experienced significantly more deaths than intact females (11.9% - 33.3%) (p < 0.05). There were no differences between ovariectomized and
Estrogen-supplemented females. There was no significant effects of exercise in any of the groups.

**Symptom severity**

Severity of symptoms was also recorded in order to better address possible differences in morbidity. Morbidity data simply indicate the day on which the first symptom (see Table 1) was observed. Figure 3 shows the symptom severity scores of the six groups of mice across the 21-day post-infection period. Exercise significantly increased symptom severity at days 12 (1.8 ± 0.4 vs. 0.9 ± 0.4; p < 0.05) and 16-21 (1.1 ± 0.2 vs. 0.1 ± 0.2; p < 0.001) post-infection. Group differences were also observed with E having greater symptoms than the other groups (I and O) on days 6 (p < 0.05) and 12 (p < 0.05) post-infection. This increased severity persisted throughout day 21 (p < 0.05) of the post-infection period.

**Peritoneal macrophage antiviral resistance to HSV-1**

In this study, peritoneal macrophages were isolated from the six groups of mice (n = 6-7 cell pools) and their intrinsic antiviral resistance to HSV-1 was examined. Figure 4 compares the antiviral resistance (expressed as a viability index) of peritoneal macrophages from mice sacrificed immediately after the exercise or control period. Exhaustive exercise significantly decreased antiviral resistance by ~18% for 10 fold, ~20% for 30 fold, and ~13% for 90 fold dilution as compared to controls (p < 0.001). There was a trend toward I-C having greater macrophage viability than O-C and E-C (p = 0.1).
Exercise treatment

Run time to fatigue was significantly different between the exercise groups with I-Ex running longer than E-Ex (p < 0.05). Average run time to fatigue over the 3 exercise days was 129 ± 5 min for I-Ex, 120 ± 5 min for O-Ex, and 109 ± 5 min for E-Ex. Run times within each group was similar on days 1, 2 and 3, which indicates that the protocol was relatively well tolerated (run times did not decrease) and that there was no apparent training effect that occurred over this time period (run times did not increase).

Plasma estrogen

There were significant group differences in plasma estrogen concentrations. As expected, plasma estrogen was significantly greater in E (63.01 ± 4.48 pg/ml) as compared to I and O (4.07 ± 4.45 pg/ml and 2.57 ± 4.89 pg/ml respectively, p < 0.001). These results indicate that estrogen pellets in E groups maintained high circulating physiologic levels of estrogen (~63 pg/ml). Exhaustive exercise was not associated with altering plasma estrogen levels across any of the groups. Intact females were within normal basal serum levels for mice (1-5 pg/ml) (24).

Discussion

Gender differences have been reported in certain disease and infection models (7), but little information exists concerning the influence of estrogen on susceptibility to respiratory infection, or the immune mechanisms that might explain the difference. We have previously shown that mortality is higher in males following intranasal HSV-1 infection than females in normal non-exercise controls and following exhaustive exercise stress (4). However, females tend to show greater symptoms of sickness and have greater peritoneal macrophage antiviral resistance to HSV-1 (5) The present study was done to determine if estrogen may partially
Explain these gender differences in susceptibility to HSV-1 infection. We initially hypothesized that estrogen would account for the lower mortality and greater macrophage antiviral function previously observed in female mice; however, the results of this study suggest that some of the previously observed gender differences in non-exercise control mice may involve ovarian function but not directly related to high-physiologic levels of estrogen. However, because only a high-physiologic consistent dose of estrogen was used in the current study, a direct role of estrogen (at plasma levels seen in a normal cycling mouse) or a combined effect of estrogen and progesterone cannot be ruled out. The negative effects of exhaustive exercise was not affected by ovariectomy or estrogen.

There is a reported sexual dimorphism in susceptibility to many viral infections, and in general, females are less susceptible (6; 9; 21). One possible mechanism for these observed gender differences is the influence of various sex hormones, in particular, estrogen. We acknowledge that estrogen is not the only female ovarian reproductive hormone, but it is believed to be a predominant one in immune responses and it is generally the first candidate in studying gender differences. The effects of estrogen on immune function and susceptibility to infection is inconsistent with results showing increases and decreases in susceptibility to infection with differing dosages and pathogens, gender, and animal species (15; 30; 33; 37; 38). Estrogen affects host resistance in a biphasic manner with physiologic dosages increasing and decreasing host resistance (15; 30; 38), while pharmacologic and supra-physiologic dosages are associated with immunosuppression in animal models (30; 31). Our results indicate that stable high-physiologic levels of estrogen (~1µg/day) are not sufficient to account for the greater resistance to HSV-1 infection in intact females. This is due to the fact that estrogen did not reverse the increase in mortality or reduced macrophage antiviral function that occurred in
ovariectomized females. Additionally, the ovariectomized and estrogen-supplemented female groups displayed greater mortality rates compared with males from our previous study (4). Therefore, in this model, it appears that stable high-physiologic levels of estrogen is likely not the explanation for our previous observation that females were better protected from HSV-1 infection than males (4); however, the role of normal cycling physiologic estrogen as well as the combination of estrogen and progesterone cannot be ruled out. Further research is warranted to address the individual and combined roles of estrogen and progesterone on susceptibility to HSV-1 infection following intense and/or moderate exercise.

Macrophages are a vital part of the initial defense against pathogens functioning as phagocytic cells and cytokine producers. Our model analyzes antiviral resistance of peritoneal macrophages following in vitro infection with HSV-1, which we believe to be indicative of antiviral resistance of alveolar macrophages, which play an important role in resistance to HSV-1 induced respiratory infection (12; 26; 40; 41). Therefore, it is reasonable to assume that lower macrophage antiviral resistance may partially account for an increased risk of infection to HSV-1. The literature suggests that the effects of estrogen on macrophage functions are highly specific to the tissue analyzed and pathogen used. Peritoneal macrophages contain estrogen receptors-α and β (19; 25) and in vitro estrogen supplementation both suppress intracellular killing activity and increases phagocytic activity of macrophages (33). Gonadectomies in rats decrease superoxide catalase activities whereas estrogen restores these activities (2). We found a trend toward ovariectomy decreasing macrophage antiviral resistance as compared to intact females and in vivo estrogen supplementation did not restore macrophage antiviral function to that of intact females.

The symptom severity scale in this study was used to determine the severity of sickness in animals following infection, which is not reflected in the overall morbidity data which simply
indicating the day at which any symptom first appeared (incidence of symptoms). The scale was
developed due to previous observations (4) that symptoms of sickness seemed to differ between
mice even though time to sickness and overall incidence were similar. Gender differences in
symptomatology have also been reported by others following HSV-1 infection with female mice
showing greater sickness and hindlimb paralysis than males following pinna and intravenous
HSV-1 inoculation (3; 21). In contrast, males have shown greater sickness symptoms following
HSV-1 corneal inoculation (20). In the present study, the estrogen supplemented ovariectomized
group had a greater sickness score than both intact and ovariectomized females. These animals
appeared much worse and their symptoms persisted longer, with the majority of them never
recovering from their sickness. Our results differ from others who have shown that high dosages
(240μg/day and ~40μg/day, respectively) of estrogen administration result in smaller and fewer
lesions following intradermal inoculation with Leishmania in male hamsters (37) and decrease
disease scores following experimental autoimmune encephalomyelitis in female mice (35).
Differences in pathogens, inoculation pathways, species/strain variations, and the use of intact
animals could account for these differences. Our observed increases in symptom severity
following HSV-1 infection in estrogen replaced females may be related to increases in
inflammatory cytokines. Inflammatory cytokines (in particular IL-1β, IL-6, and TNF-α) have
been implicated as possible mechanisms involved in various sickness behaviors (i.e. fever,
myalgia, fatigue) (1; 11; 16). Since plasma cytokines were virtually non-detectable in our study
(data not shown), this is probably not a viable mechanism in our model. However, it is certainly
possible that plasma cytokines do not reflect tissue specific cytokine content especially in the
brain where sickness behavior is mediated. Further research is warranted to determine the
potential role of central nervous system cytokines on the increased symptom severity with
estrogen supplementation.
We have previously demonstrated in male mice that repeated exhaustive exercise increases susceptibility to HSV-1 infection (4; 12) that is due, at least in part, to decreased lung macrophage antiviral resistance to HSV-1 (5; 12). In this study with female mice, we also show an exercise-induced decrease in macrophage anti-viral resistance to HSV-1 but this was not associated with a significant increase in mortality following infection. However, the statistical power associated with the mortality data was very low (0.2) in this experiment. There was no association between estrogen and either macrophage function or mortality following exercise stress. Therefore, it seems reasonable to conclude that a decrease in macrophage antiviral resistance to HSV-1 is not sufficient to alter susceptibility to HSV-1 infection in female mice.

To our knowledge, this is the first study to address the effects of estrogen on susceptibility to HSV-1 infection and macrophage antiviral resistance to HSV-1 following repeated exhaustive treadmill exercise stress in female mice. Results indicate that normal ovarian function is important in resistance to HSV-1 respiratory function, but that this protective effect is most likely not due specifically to high levels of estrogen. Estrogen was, however, associated with greater sickness symptoms. Other ovarian factors, alone or in combination with normal physiologic levels of estrogen, may be responsible for the protective effects in intact females, at least in this model.

Acknowledgements

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**Figure Legends**

Figure 1. Time course of morbidity across 21-day post-infection period, * p < 0.05 for mean time to sickness for Ex (I-Ex, O-Ex, and E-Ex) versus C (I-C, O-C, and E-C) at days 16-21 post-infection.

Figure 2. Time course of mortality across 21-day post-infection period, * p = 0.001 for mean survival time for I (I-C and I-Ex) versus O (O-C and O-Ex), and E (E-C and E-Ex) at days 9-21 post-infection.
Figure 3. Daily symptom severity scores across 21-day post-infection period. Points represent the mean of the highest severity scores of those animals that were sick. Scores were determined based on the scale illustrated in Table 1. * p < 0.05 for Ex (I-Ex, O-Ex, and E-Ex) versus C (I-C, O-C, and E-C) at days 12 and 16-21 post-infection. # p < 0.05 for E (E-C and E-Ex) versus I (I-C and I-Ex), and O (O-C and O-Ex) at days 6 and 12-21 post-infection.

Figure 4. Peritoneal macrophage intrinsic antiviral resistance as measured by magnitude of cytopathic effect (neutral red dye uptake) after 72-h incubation with herpes simplex type 1 virus and expressed as % viability mean ± SEM. * p < 0.01 for Ex (I-Ex, O-Ex, and E-Ex) versus C (I-C, O-C, and E-C) at 10, 30, and 90 fold viral dilutions.
### Table 1. Symptom Severity Scores

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<thead>
<tr>
<th>Score of 1</th>
<th>Score of 2</th>
<th>Score of 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>sore eyes</td>
<td>hunched back</td>
<td>unresponsive</td>
</tr>
<tr>
<td>lesion on body</td>
<td>swollen head</td>
<td>hindlimb paralysis</td>
</tr>
<tr>
<td>lesion on ear</td>
<td></td>
<td>twitching</td>
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<tr>
<td>ruffled fur</td>
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Mice were scored blindly on a daily basis. Scores ranged from 0-17 based on the number of symptoms that the animal displayed. Symptoms and scores were previously determined from a prior group of age-matched male mice infected with the same dosage and viral stock.