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

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Brown AS, Davis JM, Murphy EA, Carmichael MD, Carson JA, Ghaffar A, Mayer EP. Susceptibility to HSV-1 infection and exercise stress in female mice: role of estrogen. J Appl Physiol 103: 1592–1597, 2007. First published September 6, 2007; doi:10.1152/japplphysiol.00677.2007.—Exercise stress has been associated with an increased risk for upper respiratory tract infections in mice and humans. We have previously shown (Brown AS, Davis JM, Murphy AE, Carmichael MD, Ghaffer A, Mayer EP. Med Sci Sports Exerc 36: 1290–1295, 2004) that female mice are better protected from the lethal effects of herpes simplex virus type 1 (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 postinfection (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 postinfection (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 it 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.

Mice. Sexually mature female CD-1 mice, 7 wk of age (Harlan Sprague Dawley Laboratories), were acclimated to our facility for 1

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wk before 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 death.

**Ovariectomies.** Ovariectomies [ovariectomized (O) and ovariectomized plus estrogen (E) groups] and sham surgeries (I groups) were performed at least 10 days before 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 or removed. Mice were returned to home cages for 10 days (to allow for hormones to wash out in the O groups). Following death, 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 physiological dosage of ~1 μg 17β-estradiol (1 mg β-estriadiol and 24 mg cholesterol, Sigma Chemical) per day. This dosage was based on literature examining estrogen and infection (15, 30, 31) and on the work of Pung and Luster (32), who reported that 2–10 μg/animal in ovariectomized female mice resulted in normal estrogen serum levels (0.1–0.4 × 10^{-6} M), and because it was not likely to cause unwanted side effects, such as weight loss and carcinoma, and it would illicit constant physiological serum levels (30). A limitation to the study was the use of only one dose as well as using a consistent dose vs. a cycling dose which would mimic a normal cycling female across the estrus cycle. Serum levels were quantified with RIA procedures before experiments to ensure proper dosage. Placebo pellets contained inactive ingredients (25 mg cholesterol, Sigma Chemical).

**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 min/day for 3 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 ~70–80% maximal oxygen consumption (14, 36) until volitional fatigue. Fatigue was defined as the inability to keep up with the treadmill speed for 1 min with continual hand prodding. The exercise protocol was performed in the evening, 6 PM, for 3 consecutive days. Our model, which includes 3 consecutive days of prolonged treadmill running to fatigue, mimics a short period of severe exercise training similar to what may occur in athletes. Additionally, 3 days of exercise was chosen because 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 because 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 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 meningoencephalitis, 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% FBS and 2% penicillin, streptomycin and l-glutamine. 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 (LD40) 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, we 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 deprived of food and water during the exercise or control 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 8.2 × 10^5 plaque-forming units (PFU)/ml; therefore, each mouse received 3.2 × 10^5 PFU. A dose of 1.28 × 10^6 PFU of a similar preparation of this virus was found to yield an average of 1.55 × 10^5 PFU/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 laboratory, rates animals from 0 to 17 based on varying degree of symptoms 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, because 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 euthanized in a bell jar by halothane overdose. Death occurred within 1 min. 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, pH 7.2. Cells from two animals of the same group were pooled to obtain enough cells resulting in a final number of 6–7 cell pools per group. Cells in each pool were adjusted to a concentration of 2 × 10^6 cells/ml in cell culture media supplemented with 10% FBS and 2% penicillin, streptomycin and l-glutamine. Cells were seeded in 12-well plates at ~5 × 10^5 cells/well and infected with HSV-1 VR strain at an MOI of 0.005. Following infection, plates were incubated at 37°C for 2 h. Media were changed, and plates were returned to the incubator for 48 h. Culture media were collected from plates and frozen until analysis.

**Table 1. Symptom severity scores**

<table>
<thead>
<tr>
<th>Score of 1</th>
<th>Score of 2</th>
<th>Score of 3</th>
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<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.

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culture media. Viability was determined using trypan blue exclusion and was typically >94%. Subsequently, 200 μL 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 50 μL 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 250 μL), and the plates were incubated at 37°C and 5% CO₂ for 72 h. The HSV-1 virus 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 1-ml 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 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, Chicago, IL). Differences in morbidity and mortality between groups across a 21-day postinfection period were determined using a Lifetest Survival Analysis program in SAS (P < 0.05). Post hoc χ² 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 ANOVA (P < 0.05). Differences in macrophage antiviral resistance, plasma estrogen, body weights, and uteri-to-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). O mice weighed significantly more than I (25.95 ± 0.41 vs. 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 vs. 9.5 ± 1.6 mg; P < 0.001) and uterine-to-body weight ratio than O (10.2 ± 10⁻⁴ ± 5.7 ± 10⁻⁵ and 10.2 ± 10⁻⁴ ± 5.7 ± 10⁻⁵ vs. 3.7 ± 10⁻⁴ ± 6.4 ± 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-to-body weight ratio.

Morbidity. Figure 1 illustrates the time course in morbidity over the 21-day postinfection 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 vs. 7.6 ± 0.2 days C; P < 0.05). Based on these point estimates, post hoc χ² tests were performed on days 6–12 and 21 postinfection. Nonexercise groups recovered (recovery was defined as an animal having no visible signs on sickness) by day 16 (~85–90% of group appeared recovered), whereas 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 postinfection period. Intact females experienced a later time to death than ovariectomized and estrogen-supplemented females (Fig. 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 χ² tests were performed on days 9–12 and 21 postinfection 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 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 postinfection 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) postinfection. 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) postinfection. This increased severity persisted throughout day 21 (P < 0.05) of the postinfection 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 killed 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 compared with 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 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 were 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) compared with I and O (4.07 ± 4.45 and 2.57 ± 4.89 pg/ml respectively; $P < 0.001$). These results indicate that estrogen pellets in E groups maintained high circulating physiological 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. Our laboratory has previously shown that mortality is higher in males following intranasal HSV-1 infection than females in normal nonexercise controls and following exhaustive exercise stress (4). However, females tend to show greater symptoms of sickness and have greater peritoneal macrophage antiviral function against HSV-1 (5) The present study was done to determine whether 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 nonexercise control mice may involve ovarian function but not directly related to high physiological levels of estrogen. However, because only a high physiological consistent dose of estrogen was used in the present 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 are 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 physiological dosages increasing and decreasing host resistance (15, 30, 38), whereas pharmacological and supraphysiological dosages are associated with immunosuppression in animal models (30, 31). Our results indicate that stable high physiological levels of estrogen (~1 μg/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 laboratory’s previous study (4). Therefore, in this model, it appears that stable high physiological levels of estrogen is likely not the explanation for our laboratory’s previous observation that females were better protected from HSV-1 infection than males (4); however, the role of normal cycling physiological 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 compared with 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 indicates 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 (240 and ~40 μg/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 and/or 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). Because plasma cytokines were virtually nondetectable 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.

Our laboratory has 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 antiviral 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 physiological levels of estrogen, may be responsible for the protective effects in intact females, at least in this model.

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GRANTS

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