Do Female Sex Steroids Adversely or Beneficially Affect the Depressed Immune Responses in Males After Trauma-Hemorrhage?

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Hypothesis: Administration of female sex steroids in males after trauma-hemorrhage has salutary effects on the depressed immune responses.

Design: Randomized laboratory experiment.

Interventions: Male C3H/HeN mice were subjected to midline laparotomy and hemorrhagic shock (35 ± 5 mm Hg for 90 minutes, then resuscitation) or sham operation and received subcutaneous 17β-estradiol (40 µg/kg body weight) or corn oil vehicle at the beginning of resuscitation.

Main Outcome Measures: At 24 hours after hemorrhage, the animals were killed and plasma 17β-estradiol and IL-6, splenocyte interleukin (IL) 2, IL-3, and IL-10 production as well as splenic and peritoneal macrophage IL-1β, IL-10, and IL-6 release were measured.

Results: Splenocyte IL-2 and IL-3 release were significantly depressed after hemorrhage in vehicle-treated mice (P<.05, analysis of variance). Treatment with 17β-estradiol after hemorrhage led to the restoration of splenocyte IL-2 and IL-3 release. The depressed proinflammatory cytokine (IL-1 and IL-6) release seen in splenic and peritoneal macrophages was restored in the 17β-estradiol–treated hemorrhage group. In contrast, the sustained release of the anti-inflammatory cytokine IL-10 by splenocytes and splenic and peritoneal macrophages in vehicle-treated mice after hemorrhage was decreased in 17β-estradiol–treated mice. The increase in circulating IL-6 levels after hemorrhage was significantly attenuated in 17β-estradiol–treated mice. Although administration of 17β-estradiol increased plasma 17β-estradiol levels by approximately 100% in sham as well as hemorrhage groups, improved immune responses were seen only in posthemorrhage 17β-estradiol–treated mice. There was no adverse effect of 17β-estradiol treatment in the sham or posthemorrhage groups.

Conclusion: Since administration of a single dose of 17β-estradiol in males after trauma-hemorrhage restores the immune functions and decreases circulating levels of IL-6, hormones with estrogenic properties should be considered as safe and novel therapeutic agents for restoring the immune responsiveness in male trauma victims.


Several studies indicate that immune functions are markedly depressed in male subjects after trauma-hemorrhage and that these changes persist for as long as 10 days after resuscitation.1,2 Furthermore, testosterone has been shown to play a significant role in producing immunodepression after trauma-hemorrhage. Support for this notion comes from studies that indicate that depletion of testosterone by castration of male mice before the insult prevents the depression of splenocyte immune functions.3,4 Furthermore, administration of a testosterone receptor antagonist, ie, flutamide, in healthy male animals after trauma-hemorrhage restored the depressed immune responses and increased the survival rate of animals subjected to subsequent sepsis.5,6

In contrast to male mice, female mice in the proestrus state of the estrus cycle demonstrate enhanced immune responses after trauma-hemorrhage.7 Thus, it appears that elevated levels of female sex hormones, ie, prolactin and estrogen, in the proestrus state contribute to the sexual dimorphism in the immune response after trauma-hemorrhage.8 Support for the notion that the female sex steroid 17β-estradiol might have protective effects on immune responses after trauma-hemorrhage comes from recent studies performed in castrated male mice.9 Although supplementation of testosterone for 2 weeks before the insult in the castrated male mice led to a depression of immune functions af-
MATERIALS AND METHODS

ANIMALS

Inbred male C3H/HeN mice (Charles River Laboratories, Wilmington, Mass), 7 weeks of age (weight, 24-27 g) were used. All procedures were performed in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health, Bethesda, Md. This project was approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital and Brown University, Providence.

EXPERIMENTAL GROUPS

Male mice were randomized into 4 groups (8-9 mice per group). The mice in groups 3 and 4 were subjected to trauma-hemorrhage, whereas the mice in groups 1 and 2 underwent the sham procedure. Groups 1 and 3 received a subcutaneous injection of 200 µL corn oil vehicle at the beginning of resuscitation, whereas groups 2 and 4 received a subcutaneous injection of 1 µg or 100 µg 17β-estradiol per mouse (40 or 4000 µg/kg body weight, respectively). In preliminary studies, administration of this dose of 17β-estradiol in male mice was found to increase plasma 17β-estradiol concentrations comparable to levels seen in female mice in the proestrus state (data not shown). In addition to these groups, sham and trauma-hemorrhage groups were treated with 100 µg 17β-estradiol per mouse to determine whether there are any deleterious or additional beneficial effects of a high dose of 17β-estradiol on immune responses.

TRAUMA-HEMORRHAGE PROCEDURE

Mice in the trauma-hemorrhage groups were lightly anesthetized with methoxyflurane (Metofane; Pitman Moore, Mundelein, Ill) and restrained in a supine position, and a 2.5-cm midline laparotomy (ie, soft tissue trauma–induced) was performed, which was then closed aseptically in 2 layers using 6-0 sutures (Ethilon; Ethicon, Inc, Somerville, NJ). Both femoral arteries then were aseptically cannulated with polyethylene-10 tubing (Clay-Adams, Parsippany, NJ) using a minor dissection technique, and the animals were allowed to awaken. Blood pressure was monitored constantly by attaching one of the catheters to a blood pressure analyzer (Micro-Med, Inc, Louisville, Ky). Lidocaine hydrochloride was applied to the incision sites to provide analgesia during the study period. On awakening, the animals were bled rapidly through the other catheter to a mean (± SEM) arterial blood pressure of 35 ± 5 mm Hg (prehemorrhage mean [± SEM] arterial blood pressure, 95 ± 5 mm Hg), which was maintained for 90 minutes. At the end of that period, the animals were resuscitated with 4 times the shed blood volume in the form of lactated Ringer solution. The catheters were then removed, the vessels were ligated, and the groin incisions were closed. Sham groups underwent the same surgical procedure, which included ligation of both femoral arteries, but neither hemorrhage nor fluid resuscitation. There was no mortality observed in this model of trauma-hemorrhage.

BLOOD, TISSUE, AND CELL HARVESTING PROCEDURE

The animals were killed by methoxyflurane overdose 24 hours after the completion of the experiment to obtain the spleen, peritoneal macrophages, and whole blood samples. The mice were killed at the same time of day to avoid fluctuations due to circadian rhythm.

PLASMA COLLECTION AND STORAGE

Whole blood was obtained using cardiac puncture and placed in microcentrifuge tubes (Microtainer; Becton Dickinson and Co, Rutherford, NJ). The tubes then were centrifuged at 16 000g for 15 minutes at 4°C. Plasma was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen, and stored (−80°C) until assayed.

DETERMINATION OF PLASMA 17B-ESTRADIOL CONCENTRATION

Concentration of 17β-estradiol was determined using a commercially available radioimmunoassay (RIA) (ICN Biomedical, Costa Mesa, Calif) as described by the manufacturer.

PREPARATION OF SPLLENOCYTE CULTURE

Twenty-four hours after trauma-hemorrhage, the spleens were removed aseptically and placed in separate Petri dishes containing ice-cold phosphate-buffered saline (PBS) solution. Splenocytes were isolated as previously described,9 and the ability of the splenocyte cultures to produce lymphokines in response to a mitogenic challenge was assessed by incubation for 48 hours (at 37°C, 5% carbon dioxide [CO2], and 90% humidity) in the presence of 2.5 µg/mL concanavalin A (Con A; Pharmacia/LKB Biotech Inc, Piscataway, NJ). After incubation, the cell suspension was centrifuged at 300g for 15 minutes, and the supernatants were harvested and stored at −80°C until assayed for interleukin (IL) 2, IL-3, IL-10, and interferon γ (IFN-γ).

CELL-LINE MAINTENANCE

The IL-2-dependent CTLL-2 cells and the IFN-γ-dependent RAW 264.7 cells were obtained from the American
Type Culture Collection and maintained according to their directions. The IL-3–dependent FDC-P1 cells were maintained as previously described. The IL-6–sensitive murine B-cell hybridoma (7TD1) was maintained as previously described.

**ASSESSMENT OF LYMPHOKINE RELEASE**

The capacity of the mixed splenocyte culture to produce IL-2 or IL-3 was assessed by determining the amount of IL-2 or IL-3 in the collected culture supernatant. Serial dilutions of the supernatants were added to CTLL-2 cells (1 x 10^3 cells/mL) or to FDC-P1 cells (2.5 x 10^5 cells/mL) and incubated for 48 or 24 hours, respectively, at 37°C and 5% CO_2. At the end of this period, 0.037 MBq of tritiated thymidine (specific activity, 24.79 x 10^10 Bq/mmol; New England Nuclear, Wilmington, Del) was added to each well, and the cultures were incubated for an additional 16 hours. The cells were then harvested onto glass-fiber mats, and the beta decay was detected using liquid scintillation radiography as previously described.

**PREPARATION OF PERITONEAL AND SPLENIC MACROPHAGE CULTURE**

Resident peritoneal macrophages were obtained from mice, and monolayers were established as previously described. The spleens were removed aseptically and placed in separate Petri dishes containing cold (4°C) PBS solution. The spleens were dissociated by grinding and then suspended and used to establish a macrophage culture as previously described. The macrophage monolayers were stimulated with lipopolysaccharide (LPS), 10 µg/mL Click medium containing 10% fetal calf serum for 48 hours (at 37°C, 5% CO_2, and 90% humidity) to assess the cells' ability to release IL-1β, IL-6, and IL-10. At the end of the incubation period, the culture supernatants were removed, divided into aliquots, and stored at −80°C until assayed.

**ASSESSMENT OF IL-1β AND IL-10 RELEASE**

Levels of IL-1β and IL-10 in the macrophage supernatants were determined using Sandwich enzyme-linked immunosorbent assay described by Mosmann et al. In brief, 96-well plates were coated overnight with 2 µg monoclonal hamster anti-mouse IL-1β capture antibody (Genzyme Diagnostics, Cambridge, Mass) per milliliter of 0.1-mol/L carbonate (pH 9.5) or 4 µg rat anti-mouse IL-10 capture antibody (clone JES-5; Pharmingen, San Diego, Calif) per milliliter of 0.1-mol/L sodium bicarbonate (pH 8.2). The plates were washed 3 times with PBS solution containing 0.05% polyoxyethylene-sorbitan 20 (Tween 20; Sigma-Aldrich Corporation, St Louis, Mo) and blocked with PBS solution containing 20% fetal calf serum for 2 hours. After the plates were washed, 100 µL of the samples and standard (1000 pg/mL murine IL-1β [Genzyme Diagnostics] or 1000 pg/mL murine IL-10 [Pharmingen]) were added to the plates in duplicate, and they were incubated overnight (4°C). After repeated washings, the plates were incubated for 1 hour with 100 µL of biotinylated polyclonal rabbit anti–mouse IL-1β (Genzyme Diagnostics) at a concentration of 0.8 µg/mL at 37°C or biotinylated monoclonal rat anti–mouse IL-10 (clone SXC-1; Pharmingen) at a concentration of 2 µg/mL at room temperature. For IL-1β detection, the washed plates were incubated with horseradish peroxidase-conjugated streptavidin (Genzyme Diagnostics) for 15 minutes at 37°C. After multiple washings, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich Corporation) was added for 10 minutes at room temperature. After the addition of 100 µL of stop solution (0.5 mol/L sulfuric acid), the optical density of each well was determined at 450 nm on a plate reader (EL-311; Bio-Tek Instruments Inc, Winooski, Vt). For detection of IL-10, the plates were washed and incubated at room temperature for 30 minutes with avidin-peroxidase (diluted 1:400; Sigma-Aldrich Corporation). After washing, 100 µL of 2,2'-azino-bis(3-ethylbenzthiazoline-sulfonic acid)–hydrogen peroxide substrate buffer was added to each well to initiate color development. The optical density at 405 nm for each well was then determined on a microplate reader. The concentration of IL-1β and IL-10 present in the samples was determined by interpolation using a standard curve produced with murine IL-1β and IL-10, respectively.

**ASSESSMENT OF IL-6 RELEASE**

Activity of IL-6 in culture supernatant was determined by the degree of proliferation of the murine B-cell hybridoma cell line 7TD1, which only grows in the presence of IL-6. Serial dilutions of macrophage supernatants were added to 4 x 10^5 7TD1 cells/mL, and the cells were incubated for 72 hours at 37°C in 3% CO_2. For the last 4 hours of incubation, 20 µL of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide solution (5 µg/mL in RPMI-1640 [Sigma-Aldrich Corporation]) was added to each well (only viable cells incorporate the solution). The assay was stopped by aspiration of 150 µL of supernatant from each well, with subsequent replacement by 100 µL of 10% sodium dodecyl sulfate solution in PBS (lauryl sulfate; Sigma-Aldrich Corporation) to dissolve the dark blue formazan crystals. Using an automated microplate reader (EL-311), the light absorbance was measured at 570 nm.

**STATISTICAL ANALYSIS**

Results are presented as mean ± SEM. One-way analysis of variance followed by the Newman–Keuls test as a post hoc test for multiple comparisons was used to determine the significance of the differences between experimental means. A P value of less than .05 was considered statistically significant.
significantly higher plasma estradiol levels than the vehicle-treated animals (sham group, 11 377.0 ± 1950.2 pmol/L [3100.2 ± 531.4 pg/mL]; trauma-hemorrhage group, 16 492.6 ± 1744.0 pmol/L [4493.9 ± 475.2 pg/mL]).

**EFFECT OF 17β-ESTRADIOL ADMINISTRATION ON PLASMA IL-6 LEVELS**

There was no significant difference in circulating levels of IL-6 between the vehicle-treated sham group (0.03 ± 0.00 U/mL) and the sham group treated with 17β-estradiol (0.03 ± 0.02 U/mL) (Figure 1, bottom). Plasma IL-6 levels significantly increased in vehicle-treated animals that underwent the trauma-hemorrhage procedure (0.38 ± 0.16 U/mL). Treatment with 17β-estradiol after trauma-hemorrhage significantly attenuated the increased circulating levels of IL-6 (0.10 ± 0.06 U/mL).

**EFFECT OF 17β-ESTRADIOL ADMINISTRATION ON SPLENOCYTE FUNCTION AFTER TRAUMA-HEMORRHAGE**

There was no significant difference in IL-2 release capacity by splenocytes from the sham group receiving vehicle or 1 µg 17β-estradiol (Figure 2, top). Trauma-hemorrhage produced a significant depression of splenocyte IL-2 release in vehicle-treated animals (−46% compared with the vehicle-treated sham group). Administration of 17β-estradiol after trauma-hemorrhage significantly increased the release of IL-2 to levels comparable to those of the sham groups (97.8% compared with the vehicle-treated trauma-hemorrhage group). Comparable to IL-2, IL-3 release capacity was significantly depressed in animals receiving vehicle after trauma-hemorrhage (−56.6% compared with the vehicle-treated sham group) (Figure 2, bottom). Treatment with 17β-estradiol after trauma-hemorrhage led to a restoration of splenocyte IL-3 release (173.9% compared with the vehicle-treated trauma-hemorrhage group). In addition, the release of IFN-γ was significantly depressed in animals subjected to trauma-hemorrhage and treated with vehicle (−28.9% compared with the vehicle-treated sham group) (Figure 3, top). In contrast, no depression of IFN-γ release capacity was observed in animals that received 1 µg of 17β-estradiol at the beginning of resuscitation. The release of the anti-inflammatory cytokine IL-10 was maintained in vehicle-treated mice subjected to trauma-hemorrhage (Figure 3, bottom). In contrast, the release of IL-10 decreased in mice treated with 17β-estradiol compared with the corresponding sham group (−52.4%). However, these changes did not reach statistical significance.

**EFFECT OF 17β-ESTRADIOL ADMINISTRATION ON MONOKINE RELEASE BY SPLENIC AND PERITONEAL MACROPHAGES**

No significant difference in peritoneal macrophage IL-1β release was evident between the sham groups receiving ve-
hicle or 1 µg of 17β-estradiol (Figure 4, top). However, the trauma-hemorrhage group receiving vehicle had significantly decreased IL-1β release (−35.9% compared with the vehicle-treated sham group). In contrast, peritoneal macrophage IL-1β release was restored in mice that received 1 µg of 17β-estradiol after trauma-hemorrhage (61.2% compared with the vehicle-treated trauma-hemorrhage group). Peritoneal macrophage IL-6 release capacity was comparable in both sham groups. Vehicle-treated mice subjected to trauma-hemorrhage had significantly decreased peritoneal macrophage IL-6 release (−57.6% compared with the vehicle-treated sham group). Administration of 17β-estradiol increased but did not restore the depressed IL-6 release after trauma-hemorrhage (56.8% compared with the vehicle-treated trauma-hemorrhage group). At 24 hours after trauma-hemorrhage, peritoneal macrophage IL-10 release was maintained in vehicle-treated animals (Figure 5). However, the release of the anti-inflammatory cytokine IL-10 significantly decreased in animals that received 1 µg of 17β-estradiol after trauma-hemorrhage (−45.6% compared with the estradiol-treated sham group).

Splenocytic IL-1β release (Figure 6, top) was comparable in both sham groups. Animals subjected to trauma-hemorrhage and vehicle had significantly depressed splenic macrophage IL-1β release (−42.6% compared with the vehicle-treated sham group). Splenic macrophage IL-1β release in the trauma-hemorrhage group treated with 17β-estradiol, however, showed IL-1β release that was comparable to IL-1β release by macrophages from the sham groups (60.8% compared with the vehicle-treated trauma-hemorrhage group). Comparable findings were obtained for splenic macrophage IL-6 release (Figure 6, bottom). After trauma-hemorrhage, IL-6 release was significantly depressed in vehicle-treated animals compared with the vehicle-treated sham group (−64.4%). Animals treated with 1 µg of 17β-estradiol at the beginning of resuscitation had restored IL-6 release capac-

Figure 3. Splenocytes were harvested at 24 hours after trauma-hemorrhage or sham operation from male C3H/HeN mice treated with corn oil vehicle or 1 µg of 17β-estradiol. The release of interferon (IFN) γ by splenocytes stimulated with 2.5 µg/mL concanavalin A for 48 hours was determined using a specific bioassay (RAW 264.7) (top); interleukin-10 (IL-10) release was measured using enzyme-linked immunosorbent assay (bottom). Values are given as mean ± SEM of 7 to 8 animals in each group; comparisons were made using analysis of variance. Asterisk indicates P < .05 vs vehicle-treated sham group; dagger, P < .05 vs vehicle-treated trauma-hemorrhage group.

Figure 4. Peritoneal macrophages were harvested at 24 hours after trauma-hemorrhage or sham operation from male C3H/HeN mice treated with corn oil vehicle or 1 µg of 17β-estradiol and cultured in the presence of 10 µg/mL lipopolysaccharide for 48 hours. Peritoneal macrophage interleukin (IL) 1β release was measured using enzyme-linked immunosorbent assay (top); IL-6 release was determined using a specific bioassay (7TD1) (bottom). Values are given as mean ± SEM of 7 to 8 animals in each group; comparisons were made using analysis of variance. Asterisk indicates P < .05 vs vehicle-treated sham group.

Figure 5. Peritoneal macrophages were harvested at 24 hours after trauma-hemorrhage or sham operation from male C3H/HeN mice treated with corn oil vehicle or 1 µg of 17β-estradiol and cultured in the presence of 10 µg/mL lipopolysaccharide for 48 hours. Peritoneal macrophage interleukin (IL) 10 release was measured using enzyme-linked immunosorbent assay. Values are given as mean ± SEM of 7 to 8 animals in each group; comparisons were made using analysis of variance. Asterisk indicates P < .05 vs estradiol-treated sham group.
estradiol compared with vehicle-treated animals (Table). Significantly attenuated in animals receiving the high dose of 17β-estradiol after trauma-hemorrhage were significantly depressed in male subjects after trauma-hemorrhage (−44.0% compared with the vehicle-treated trauma-hemorrhage group). Similarly, IL-12 release was significantly depressed in male mice by castration 2 weeks before trauma-hemorrhage or sham operation from male C3H/HeN mice treated with corn oil vehicle or 1 µg of 17β-estradiol and cultured in the presence of 10 µg/mL lipopolysaccharide for 48 hours. Splenic macrophage interleukin (IL) 10 (top) and IL-12 release (bottom) were measured using enzyme-linked immunosorbent assay. Values are given as mean ± SEM of 7 to 8 animals in each group; comparisons were made using analysis of variance. Asterisk indicates \( P < .05 \) vs vehicle-treated sham group.

**EFFECT OF HIGH-DOSE 17β-ESTRADIOL ADMINISTRATION ON IMMUNE FUNCTIONS**

As shown in the Table, treatment of male mice with the high dose of 17β-estradiol (ie, 100 µg per mouse) after trauma-hemorrhage normalized splenocyte and splenic and peritoneal macrophage immune functions comparable to the pattern observed after treatment with the low dose of 17β-estradiol (ie, 1 µg per mouse). In addition, the increased plasma IL-6 levels after trauma-hemorrhage were significantly attenuated in animals receiving the high dose of 17β-estradiol compared with vehicle-treated animals (Table).

**COMMENT**

Several studies have shown that cell-mediated immunity is markedly depressed in male subjects after trauma-hemorrhage despite adequate fluid resuscitation.1 This depression in immune responses persists for up to 10 days after resuscitation and is associated with increased susceptibility to sepsis.2,9 Studies also have suggested that male sex steroids play a critical role in initiating the depression of immune responses.3,4 Moreover, administration of a testosterone receptor antagonist, eg, flutamide, in healthy male animals after trauma-hemorrhage restored the depressed immune responses after trauma-hemorrhage and improved survival rates after subsequent sepsis.5,6

In contrast to male mice, female mice in the proestrus state of the estrus cycle have enhanced immune functions after trauma-hemorrhage.7 Since the proestrus state of the cycle is characterized by increased plasma levels of female sex hormones such as estrogen and prolactin,8 it could be postulated that these hormones are responsible for producing immunoenhancing effects after trauma-hemorrhage. Indeed, administration of prolactin in males after trauma-hemorrhage has been shown to restore the depressed splenocyte and macrophage immune responses and to decrease mortality rates due to subsequent sepsis.13,14 However, little information is available concerning the effects of the female sex steroid 17β-estradiol on the immune responses after trauma-hemorrhage. Our aim, therefore, was to determine whether the female steroid hormone 17β-estradiol has...
any beneficial or deleterious effects on the depressed immune function after trauma-hemorrhage in males. Our results indicate that administration of 17β-estradiol at the beginning of resuscitation restores the depressed splenocyte as well as splenic and peritoneal macrophage function after trauma-hemorrhage. In this study, 17β-estradiol was administered subcutaneously at a dose of 1 µg per mouse, because preliminary studies from our laboratory have shown that this dose increased plasma 17β-estradiol concentrations in males comparable to levels observed in female mice in the proestrus state.8 In addition to this low dose of 17β-estradiol (ie, 1 µg per animal), we also investigated whether high-dose estradiol treatment (100 µg of 17β-estradiol per mouse) has any deleterious or additional salutary effects on immune responses. The results indicate that immune functions in animals treated with the high dose of 17β-estradiol were comparable to the findings observed after administration of the low dose. Thus, there appear to be no deleterious effects of administration of high doses of 17β-estradiol. Although administration of 17β-estradiol at a dose of 1 µg per animal after trauma-hemorrhage restored the immune responses, doses of 17β-estradiol even lower than 1 µg per animal may have salutary effects on the immune responses. This, however, remains to be determined.

Previous studies have demonstrated that normalization of the depressed macrophage and splenocyte functions in male mice treated with the immunomodulatory hormone prolactin after hemorrhagic shock was associated with decreased lethality owing to subsequent sepsis.13 Thus, it is possible that the restoration of immune responsiveness seen in 17β-estradiol–treated animals after trauma-hemorrhage might decrease the lethality rates due to a subsequent lethal septic challenge. However, as the animals in our study were not subjected to a subsequent septic challenge, further studies are necessary to address this issue.

Additional support for the findings that 17β-estradiol has immunoenhancing properties comes from the studies of Chao et al.15 These investigators demonstrated that 17β-estradiol at concentrations within the physiological range increases male rat peritoneal macrophage tumor necrosis factor-α release capacity in vitro.15 Further support for direct effects of 17β-estradiol on immune cells comes from studies by Fox et al,16 who demonstrated that 17β-estradiol markedly increased the activity of the IFN-γ promoter in lymphoid cells that express the appropriate hormone receptor. Support for the notion that the female sex steroid 17β-estradiol also has protective effects on immune responses after trauma-hemorrhage comes from recent studies from our laboratory.4 The results of those studies indicate that supplementation of testosterone in castrated male mice for 14 days before the insult led to depressed immune functions after trauma-hemorrhage comparable to the depression observed in healthy male mice under such conditions. However, when castrated mice received 17β-estradiol in addition to testosterone, immune functions after trauma-hemorrhage were restored to sham levels.4

In addition to restoration of splenic and peritoneal macrophage functional capacity, 17β-estradiol treatment significantly reduced circulating levels of IL-6 after trauma-hemorrhage. These findings are in line with the results of studies by Zuckerman et al,17 who demonstrated significantly decreased levels of plasma IL-6 in mice pretreated with 17α-ethynyl estradiol and subjected to endotoxemia. These findings suggest that under adverse conditions, estrogens might exert systemic effects by influencing the profile of circulating cytokines. In this regard, Kupffer cells have previously been shown to be the major source of circulating IL-6.18 Although the increasing IL-6 levels in circulation seem at first discordant with our observed restoration of macrophage IL-6 release capacity, a previous study from our laboratory has shown that the increased blood IL-6 concentrations are a product of Kupffer cell activation and not from the splenic or peritoneal macrophages.18 Kupffer cell IL-6 release was not measured in our study. Therefore, the finding that plasma IL-6 levels after trauma-hemorrhage are significantly lower in 17β-estradiol–

| Table 1: Effects of High-Dose 17β-Estradiol Administration on Plasma 17β-Estradiol Levels and Immune Functions 24 Hours After Sham Operation or Trauma-Hemorrhage |
|------------------------------------------|----------------|----------------|-----------------|----------------|
|                                       | Sham-Vehicle | Sham–17β-Estradiol | Hemorrhage-Vehicle | Hemorrhage–17β-Estradiol |
| Plasma 17β-estradiol, pmol/L (pg/mL)   | 69.4 ± 8.8   | 1137.0 ± 1900.2 | 58.4 ± 11.4      | 16492.6 ± 1744.0 |
| (18.9 ± 2.4)                         | (3100.2 ± 531.4)† | (15.9 ± 3.1)     | (4493.9 ± 475.2)‡ |                  |
| Plasma-IL-6, U/mL                   | 0.027 ± 0.011 | 0.018 ± 0.008  | 0.375 ± 0.162†  | 0.087 ± 0.027‡   |
| Splenocyte IL-2, U/mL              | 1.78 ± 0.07  | 1.74 ± 0.10    | 0.96 ± 0.22†    | 1.46 ± 0.21     |
| Splenocyte IL-3, U/mL              | 714.1 ± 59.9 | 797.9 ± 83.6   | 310.1 ± 64.8†   | 986.6 ± 90.3‡   |
| Splenocyte IFN-γ, U/mL             | 15177 ± 619  | 14531 ± 748    | 10789 ± 475§    | 13270 ± 798§    |
| Peritoneal macrophage IL-6, U/mL    | 2377 ± 248   | 2288 ± 468     | 1000 ± 112      | 1480 ± 532      |
| Peritoneal macrophage IL-10, pg/mL  | 373.9 ± 54.2 | 464 ± 43.5     | 303.9 ± 60.9    | 250 ± 24.4§     |
| Splenic macrophage IL-1β, pg/ml     | 28.6 ± 3.7   | 19.5 ± 2.5     | 16.4 ± 2.2†     | 26.9 ± 2.9‡     |
| Splenic macrophage IL-6, U/mL       | 16.3 ± 3.6   | 9.2 ± 1.3      | 5.8 ± 1.5†      | 11.4 ± 1.6      |
| Splenic macrophage IL-10, pg/ml     | 71.8 ± 15.0  | 71.7 ± 17.7    | 113.1 ± 25.2    | 49.3 ± 11.2     |

* Data are given as mean ± SEM. Sham-Vehicle indicates vehicle-treated sham group; Sham–17β-Estradiol, 17β-estradiol–treated sham group; Hemorrhage-Vehicle, vehicle-treated trauma-hemorrhage group; Hemorrhage–17β-Estradiol, 17β-estradiol–treated trauma-hemorrhage group; IL, interleukin; IFN, interferon; and ANOVA, analysis of variance. Groups are described in the “Experimental Groups” subsection of the “Materials and Methods” section.
†P < .05 vs Sham-Vehicle (ANOVA).
‡P < .05 vs Hemorrhage-Vehicle (ANOVA).
§P < .05 vs Sham–17β-Estradiol (ANOVA).
A number of studies have shown that various immune functions are markedly depressed in males after trauma-hemorrhage and that this dysfunction is associated with an increased susceptibility to subsequent sepsis. It also has become evident that sex steroids are involved in the regulation of immune responses under normal and pathophysiological conditions. Although the immunosuppressive role of testosterone has been well characterized and testosterone receptor blockade has been shown to be beneficial after trauma-hemorrhage, it remained unclear whether female sex steroids have any salutary or deleterious effects under such conditions. Our study indicates that administration of a single dose of the female sex steroid 17β-estradiol after trauma and hemorrhagic shock normalizes the depressed immune functions in male mice. In view of this, hormones with estrogenic properties should be considered novel therapeutic agents for the treatment of immune dysfunction encountered in male trauma victims.

Our results indicate that in contrast to proinflammatory cytokine release, IL-10 production by cells obtained from the trauma-hemorrhage groups was enhanced in splenic macrophages or unchanged in peritoneal macrophage or splenocytes. In this regard, it has been suggested that the increased IL-10 release in splenocytes from male mice after simple hemorrhage might contribute to the depression of immune functions.19 Thus, we suggest that the maintenance of anti-inflammatory IL-10 production in the face of diminishing cell-mediated proinflammatory cytokine productive capacity supports the role of IL-10 as one of several potential immunosuppressive agents present after trauma-hemorrhage. This is also supported by our observation that the release of IL-10 by splenic and peritoneal macrophages was decreased in 17β-estradiol–treated male mice after trauma-hemorrhage. Therefore, it could be speculated that the reduction of IL-10 production, or possibly concomitant suppression of other anti-inflammatory agents not assessed herein (eg, transforming growth factor-β, IL-4) in 17β-estradiol–treated animals subjected to trauma-hemorrhage might contribute to the maintenance of macrophage functions.

The underlying mechanisms by which 17β-estradiol mediates its beneficial effects on different immune cell populations after trauma-hemorrhage in males remain unclear. Our results showing that administration of 17β-estradiol did not enhance the immune responses in the sham groups suggest that 17β-estradiol is immunostimulatory only in an immunologically compromised host. Since 17β-estradiol plasma levels did not change significantly after trauma-hemorrhage, our findings further suggest that the number of 17β-estradiol receptors or the receptor affinity might be altered under such conditions. Therefore, provision of additional agonist, ie, 17β-estradiol, for the receptors might contribute to the improvement of the depressed immune responses after trauma-hemorrhage. The fact that estradiol receptors have been demonstrated on macrophages suggests that these cells may be susceptible to functional modulation by the additional estrogens administered after trauma-hemorrhage.20 Additional studies, however, are needed to determine whether the salutary effects of 17β-estradiol on immune responsiveness after trauma-hemorrhage are receptor mediated and which immune cell populations are primarily affected by this treatment. Alternatively, salutary effects of 17β-estradiol on immune functions after trauma-hemorrhage might be the indirect result of effects on other organ systems. For example, recent findings in cardiovascular disease provide evidence that estradiol acts via rapid, “nongenomic” as well as long-term, “genomic” mechanisms.21 In this regard, it is possible that the nongenomic effects of pharmacological doses of 17β-estradiol, such as vasodilation, are due to changes in ion-channel function22,23 as well as increased endothelial nitric oxide production.24,25 Such effects might contribute to the beneficial effects on immune functions by improving microcirculation after trauma-hemorrhage. However, the contribution of such a mechanism has not been assessed yet in our experimental setting.

In summary, our study indicates that posttreatment with 17β-estradiol not only restores splenic and macrophage cytokine productive capacity but suppresses the release of a potential immunosuppressant, IL-10, and attenuates the increase in plasma IL-6 levels seen after trauma-hemorrhage. Although the exact mechanism for the immunomodulatory properties of 17β-estradiol remains unknown, these findings suggest salutary effects of this steroid hormone after adverse circulatory conditions. Therefore, administration of 17β-estradiol should be considered a novel and useful approach for the treatment of immune dysfunction in male trauma victims.

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**DISCUSSION**

Herbert B. Hechtman, MD, Boston, Mass: Dr Knöferl has continued the focus train of Dr Chaudry’s research regarding the ability of female steroid hormones to modulate immune function. These data are in concert with prior reports from his laboratory as well as the reports of several other groups regarding the ability of estrogen and other female steroids to alter production of a variety of cytokines. The net effect favors an anti-inflammatory action. The story is complex. Not only are there multitudes of pro- and anti-inflammatory cytokines released as a result of stress, but there are lymphoid and phagocytic cells of origin which may respond quite differently to similar stimuli. Thus an article by you of more than 10 years ago indicated that LPS will stimulate peritoneal macrophage production of IL-1, and this is in contrast to the effects of trauma and hemorrhage that are now reported to limit production of IL-1.

Indeed, one of the problems in interpretation is that the authors utilize a number of study stimuli. First is the application of laparotomy as a form of trauma. Second, there is hemorrhage and resuscitation. And third, there is in vitro stimulation of splenocytes with concanavalin A, but stimulation of peritoneal and splenic macrophages with LPS. The difficulty in simple interpretation is the multiplicity and lack of definition of stimuli. Thus, what are the independent roles of laparotomy, hemorrhage, and LPS of bacterial sepsis?

Another problem in interpretation is the report of depressed in vitro IL-6 production by splenic macrophages but elevated in situ plasma IL-6 concentrations after trauma. The conclusion that Kupffer cell synthesis of IL-6 is stimulated by trauma and depressed by estradiol is based on previous studies using gado-linium, which suggests the importance of Kupffer cell synthesis of IL-6. This remarkable heterogeneity, however, of macrophage function requires more proof of the role of each class of cells, such as changes in cytokine messenger RNA.

These comments should not detract from the novel results that demonstrate the clinical ability of female steroids to modulate immune function. How important this will prove to be remains open to question.

John A. Mannick, MD, Boston: I too enjoyed the paper and I have only one question, being a devotee of the motto in vivo veritas. Do you have any evidence that estradiol therapy in your model of trauma-hemorrhage with later challenge with cecal ligation and puncture will actually increase survival after an infectious challenge?

Dr Knöferl: In previous studies from our laboratory, the independent roles of trauma in the form of a midline laparotomy, as well as simple hemorrhagic shock alone, have been studied. In the present study, we used a combined model of trauma and hemorrhagic shock in order to have a severe model which is clinically relevant. Dr Hechtman mentioned the fact that splenocyte IL-6 production is decreased in response to LPS stimulation, whereas plasma IL-6 levels are increased. As you mentioned, in previous studies from our laboratory, it has been shown that the Kupffer cell population in the liver is a major cellular source for production of IL-6. Therefore, it appears that administration of 17β-estradiol indeed has influence on Kupffer cell proinflammatory cytokine production. This, however, was not studied in the present work.

He mentioned whether we have any clinical indication for the trend differences we observed in our animal models of trauma and hemorrhagic shock. Indeed, there is a wealth of information from epidemiological studies that indicate a predominance of the male gender for infection. In addition, a study recently published by a group in Denver indicated that in the surgical ICU male gender is a major risk factor for infection. Another study published from Germany indicates that even mortality from sepsis is increased in the male gender.

Dr Mannick asked whether we subjected our animals in the present study to cecal ligation and puncture in order to see the clinical relevance of our cytokine changes. This was not performed; however, the result that estradiol administration restores peritoneal macrophage cytokine release capacity as well as splenocyte cytokine release suggests that those animals would have a survival benefit, since the restoration of those immune functions in previous studies was associated with increased survival rates following a septic challenge.