

# Administration of Progesterone After Trauma and Hemorrhagic Shock Prevents Hepatocellular Injury

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**Hypothesis:** Administration of a single dose of progesterone following trauma and hemorrhage in progesterone-deficient rats would ameliorate the inflammatory response and hepatocellular damage.

**Setting:** A university laboratory.

**Interventions:** Ovariectomized female Sprague-Dawley rats (250-350 g; Charles River Laboratories, Wilmington, Mass) underwent a 5-cm midline laparotomy (ie, induction of soft tissue trauma), were bled to a mean arterial blood pressure of 35 mm Hg for about 90 minutes, and then were resuscitated using Ringer lactate solution. Progesterone (25 mg/kg of body weight) or vehicle was administered subcutaneously at the end of resuscitation. In additional animals, Kupffer cells were isolated following trauma, hemorrhage, and resuscitation and treated in vitro with progesterone, lipopolysaccharide, or both.

**Main Outcome Measures:** Six hours following resuscitation, plasma tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 6 (IL-6) levels and liver myeloperoxidase activity were determined. Hepatocellular function (maximum velocity of indocyanine green clearance [ $V_{max}$ ] and

the efficiency of the active transport or Michaelis-Menten constant [ $K_m$ ]) and plasma levels of transaminases were measured 20 hours after resuscitation. Kupffer cell IL-6 and TNF- $\alpha$  production were assessed.

**Results:** Plasma levels of TNF- $\alpha$ , IL-6, aspartate aminotransferase, and alanine aminotransferase, as well as hepatic myeloperoxidase activity were increased, whereas indocyanine green clearance was depressed in vehicle-treated rats following trauma-hemorrhage. Animals treated with progesterone showed significantly reduced levels of the TNF- $\alpha$ , IL-6, and transaminases as well as reduced myeloperoxidase activity in the liver. Progesterone-treated animals showed increased  $V_{max}$  and  $K_{max}$  values for indocyanine green. In vitro treatment of Kupffer cells with progesterone decreased TNF- $\alpha$  production but did not affect the production of IL-6.

**Conclusion:** Progesterone administration following trauma-hemorrhage ameliorates the proinflammatory response and, subsequently, the hepatocellular injury via direct action on immunocompetent cells.

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**C**LINICAL STUDIES have shown that the incidence of infectious complications and the outcome in patients with traumatic injuries are sex dependent.<sup>1,2</sup> These clinical observations agree with the findings from our laboratory and others that indicate that the susceptibility of rodents to trauma-hemorrhage and sepsis is sex dependent.<sup>3,4</sup> Female rodents especially in the proestrus stage of their menstrual cycle do not show evidence of depressed cell and organ functions following soft tissue trauma and hemorrhagic shock, whereas male rodents have significantly decreased cardiac output, hepatocellular function, and depressed immune functions.<sup>3,5,6</sup> The steroid progesterone is a female sex hormone that peaks during the

proestrus stage of the rodent menstrual cycle.<sup>7</sup> Recent studies have shown that progesterone receptors are expressed in a variety of nonreproductive tissues and the function of progesterone does not seem to be limited to its role within the female reproductive cycle and pregnancy.<sup>8</sup> Several studies have demonstrated that this female sex steroid influences important cellular and systemic stress response mechanisms, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) production,<sup>9</sup> regulation of inducible nitric oxide synthase expression,<sup>10</sup> and the activation of heat shock proteins.<sup>11</sup> However, it remains unknown whether progesterone can modulate the stress response under in vivo conditions. We hypothesized that administration of a single dose of progesterone following trauma-hemorrhage in progesterone-

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deficient (ie, ovariectomized) female rats ameliorates the inflammatory response, improves liver function, and reduces hepatocellular damage.

## METHODS

### EXPERIMENTAL PROTOCOL

Animals were divided into the following 3 groups: sham animals and animals that were subjected to trauma-hemorrhage and treated with either vehicle (sesame oil) or progesterone (25 mg/kg of body weight) subcutaneously. Additional animals were used to determine the proinflammatory cytokines and hepatic myeloperoxidase (MPO) activity 6 hours after trauma-hemorrhage and resuscitation and to measure active hepatocellular function and damage 20 hours after resuscitation. In a different set of rats, Kupffer cells were isolated after completion of resuscitation and treated *in vitro* with different doses of progesterone. We chose the earlier time points to observe potential effects of progesterone administration on the inflammatory response to trauma-hemorrhage and the 20-hour time point as a subsequent outcome measurement.

### OVARECTOMY

Rats were ovariectomized 14 days prior to the experiment. Briefly, after initiation of general anesthesia using methoxyflurane (Mallinckrodt Veterinary Inc, Mundelein, Ill), a small incision was made in the skin on the back of the animal midway between the last rib and the hip. A second incision was made through the muscle layer about 1 cm lateral to the spinal muscle into the peritoneal cavity. The ovaries were separated and tied off with a silk ligature (3-0 surgical silk; Deknatel Inc, Fall River, Mass). The ovaries were then removed and the incisions closed with 4-0 nonabsorbable nylon monofilament sutures (Ethilon; Ethicon, Sommerville, NJ).

The experiments were performed using (250- to 350-g) female Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass), according to the guidelines of the Animal Welfare Act and The Guide for Care and Use of Laboratory Animals from the National Institutes of Health. The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved this project.

### TRAUMA-HEMORRHAGE AND RESUSCITATION

Our previously described nonheparinized model of trauma-hemorrhage in the rat was used with minor modifications.<sup>12</sup> Briefly, rats were fasted overnight before the experiment but allowed water *ad libitum*. The rats were anesthetized using methoxyflurane inhalation prior to the induction of trauma (ie, 5-cm midline laparotomy). The abdomen was then closed in layers, and catheters were placed in both femoral arteries and the right femoral vein (polyethylene [PE-50] tubing; Becton Dickinson and Co, Sparks, Md). The wounds were bathed with 1% lidocaine hydrochloride (Elkins-Sinn Inc, Cherry Hill, NJ) throughout the surgical procedure to reduce postoperative pain. The rats were then allowed to awaken and bled to and maintained at a mean arterial blood pressure of 35 mm Hg. This level of hypotension was continued until the animals could not maintain a mean arterial blood pressure of 35 mm Hg unless extra fluid in the form of Ringer lactate solution was given. This time was defined as maximum bleed out, and the amount of withdrawn blood was noted. Following this, the rats were maintained at a mean arterial blood pressure of 35 mm Hg until 40% of the maximum bleed-out volume was returned in the form of Ringer lactate solution. The animals were then resuscitated with 4 times

the volume of the withdrawn blood over 60 minutes (about 40 mL/rat) with Ringer lactate solution. The shed blood was not used for resuscitation. At the end of the resuscitation period, the rats received progesterone, 25 mg/kg of body weight (Sigma-Aldrich Corp, St Louis, Mo) subcutaneously or an equal volume (about 0.3 mL) of the vehicle (sesame oil). The catheters were then removed, the vessels were ligated, and the skin incisions closed with sutures. After returning the animals to their cages, they were allowed free access to food and water. Sham-operated on animals underwent the same groin dissection, which included the ligation of the femoral artery and the right femoral vein; however, neither laparotomy nor hemorrhage and resuscitation was carried out.

### MEASUREMENT OF HEPATOCELLULAR FUNCTION

Hepatocellular function was measured 20 hours after the end of resuscitation using the *in vivo* indocyanine green (ICG) clearance technique described in detail previously.<sup>13</sup> Briefly, ICG was administered by bolus injection (50  $\mu$ L) of 1, 2, and 5 mg/mL of ICG in aqueous solvent. The arterial concentration of ICG was recorded each second for 5 minutes using a computer-assisted data acquisition program. Following this, the initial velocity of ICG clearance for each dose was calculated after performing a nonlinear regression of the ICG clearance curves according to an e-raised second-order polynomial function. The initial velocities of ICG clearance were then plotted against the ICG doses according to the method of Lineweaver-Burk.<sup>13</sup> This results in a straight line, allowing the determination of a maximum velocity of ICG clearance ( $V_{max}$ ) and the Michaelis-Menten constant ( $K_m$ ). The  $V_{max}$  represents the functional hepatocyte ICG receptors while  $K_m$  represents the efficiency of the transport process in this active hepatocellular membrane transport system.

### MEASUREMENT OF PLASMA LEVELS OF TRANSAMINASES

At the end of the measurements (ie, 20 hours after the end of trauma-hemorrhage and resuscitation), whole blood was obtained and placed in aliquot microcentrifuge tubes with heparin sodium (American Pharmaceutical Partners Inc, Los Angeles, Calif). The tubes were then centrifuged at 16000g for 15 minutes at 4°C. Plasma was separated, placed in pyrogen-free microcentrifuge tubes, and stored ( $-70^{\circ}\text{C}$ ) until assayed. Hepatocellular injury was determined by measuring the enzymatic activity of the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma samples using a colorimetric reaction kit according to the manufacturer's instructions (Sigma-Aldrich Corp).

### MEASUREMENT OF PLASMA LEVELS OF CYTOKINES

The levels of TNF- $\alpha$  and IL-6 were determined in the plasma 6 hours after the end of resuscitation, using enzyme-linked immunosorbent assays (PharMingen, San Diego, Calif) according to the manufacturer's instructions.

### MEASUREMENT OF CYTOKINE PRODUCTION IN ISOLATED KUPFFER CELLS

Kupffer cells were isolated from rats as previously described with some modifications.<sup>14,15</sup> Briefly, the portal vein was cannulated following a 5-cm midline incision, the aorta ligated, and the abdominal vena cava was severed. The liver was immediately perfused *in situ* with about 60 mL of Hanks balanced salt solution (without calcium [ $\text{Ca}^{2+}$ ] and magnesium [ $\text{Mg}^{2+}$ ]), at 37°C at a rate of 15 mL/min. This was followed by

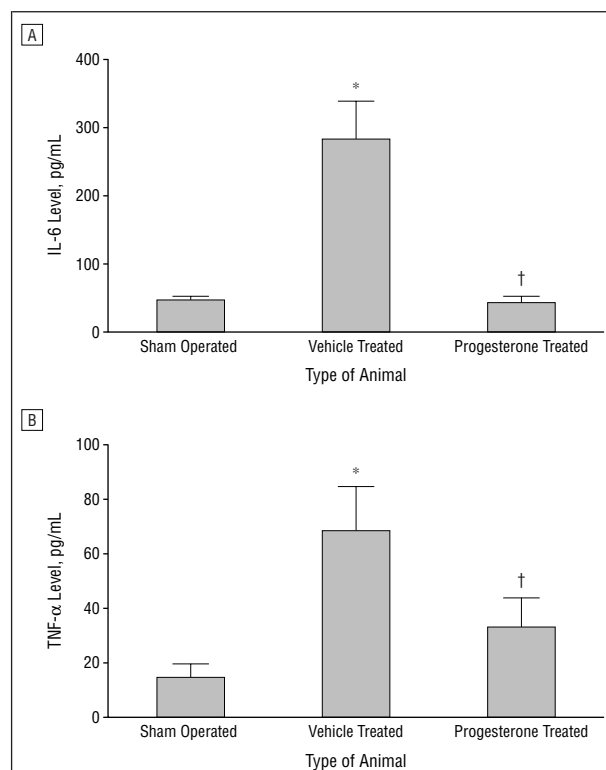
perfusion of 100 mL of Williams Medium E (Invitrogen Corp, Carlsbad, Calif) containing 0.02% collagenase (Worthington type II, 274 U/mg; Lorne Laboratories Ltd, Twyford, England), 0.02% trypsin inhibitor (Sigma-Aldrich Corp), and 0.5% 100mM calcium chloride solution at the same perfusion rate. The liver was then removed en bloc, minced in a Petri dish containing Williams Medium E with 0.02% collagenase, and incubated for 20 minutes at 37°C to further dissociate the cells. The cell suspension was then passed through a sterile 150-mesh stainless steel screen into cold Williams Medium E containing 10% heat-inactivated fetal bovine serum (final volume 125 mL) and centrifuged (700 revolutions per minute [rpm] for 2 minutes at 4°C) to sediment hepatocytes. The remaining cells in the supernatant were collected by centrifugation (1800 rpm for 15 minutes at 4°C). The supernatant was discarded and the cell pellets were gently laid over 4 mL of 16% metrizamide (Accurate Chemical & Scientific Co, Westbury, NY). The Kupffer cell layer was found at the interface of the metrizamide and the media following centrifugation (3200 rpm for 45 minutes at 4°C). The cells were further washed with 25 mL of Williams Medium E. Cell viability was determined by trypan blue exclusion, which was more than 95%. The isolated Kupffer cells from normal animals were cultured in Williams Medium E with 10% heat-inactivated fetal calf serum and 0.1% gentamycin sulfate at the concentration of  $10^6$  cells/mL. Kupffer cells were allowed to adhere on the bottom of the plastic culture dish for 3 hours, and unattached cells were removed by gentle washing. Kupffer cells were then cultured in Williams Medium E with progesterone (cyclodextrin-encapsulated progesterone; Sigma-Aldrich Corp) or vehicle with or without in vitro stimulation by 100 ng/mL of lipopolysaccharides for 24 hours. The supernatant was then collected and TNF- $\alpha$  and IL-6 levels were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions. Other stimulants such as endotoxin were not added to the culture system and every effort was made to minimize any contamination of endotoxin during Kupffer cell isolation and culture.

#### MPO ACTIVITY

Myeloperoxidase activity was determined in homogenates of hepatic tissues. All reagents were purchased commercially (Sigma-Aldrich Corp). Briefly, livers were excised, rinsed with an isotonic sodium chloride solution, and frozen in liquid nitrogen at -70°C until assayed. Frozen liver samples were thawed and homogenized in 10 volumes of 20mM potassium phosphate, pH 7.4, for 30 seconds. Samples were centrifuged at 14000 rpm for 30 minutes at 4°C. The pellets were resuspended in 10 volumes of 50mM potassium phosphate, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide. Samples were kept on ice and sonicated using a probe sonicator at two thirds maximum setting for about 40 seconds and centrifuged at 14000 rpm at 4°C for 10 minutes. Supernatants were then added to a 96-well plate at 5  $\mu$ L/well and 196  $\mu$ L of reaction buffer containing 530nM o-dianisidine and 150nM hydrogen peroxide in (added immediately before use) in 50mM potassium phosphate, pH 6.0. Light absorbances at 490 and 620 nm (reference wavelength) were read and compared with those obtained in wells containing a known activity of MPO standard purified from human leukocytes (Sigma-Aldrich Corp) (activity as declared on batch). Protein content in the samples was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, Calif).

#### STATISTICAL ANALYSES

Results are presented as mean (SEM). Data were analyzed using 1-way analysis of variance and the Student-Newman-Keul test, and differences were considered statistically significant at a  $P \leq .05$ .



**Figure 1.** Effects of progesterone treatment on the plasma levels of the proinflammatory cytokines interleukin 6 (A) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (B) following trauma-hemorrhage. Comparison of sham-operated on animals and animals subjected to trauma-hemorrhage treated with either vehicle (sesame oil) or a single dose of progesterone. Data are presented as mean (SEM) and compared by 1-way analysis of variance and Student-Newman-Keuls test. There were 7 animals in each group. Asterisk indicates  $P < .05$  vs sham-operated on animals; dagger,  $P < .05$  vs vehicle-treated animals.

## RESULTS

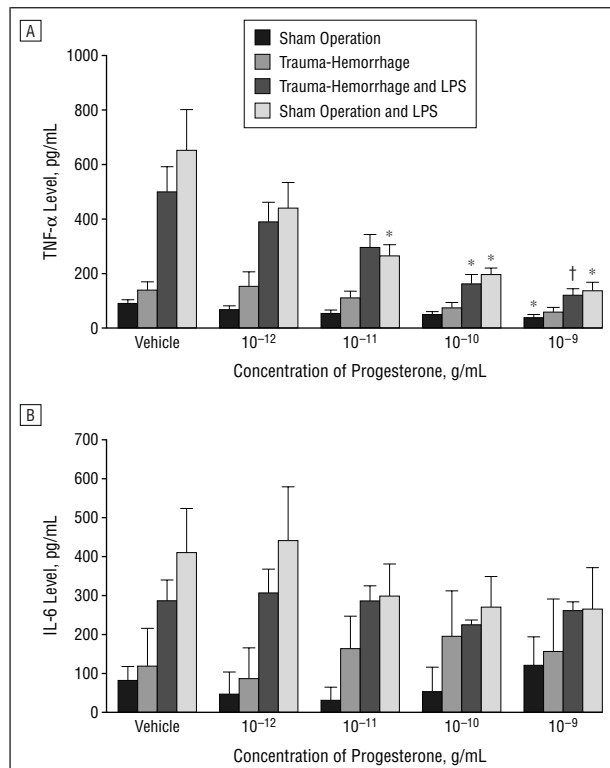
### EFFECTS OF PROGESTERONE ON THE CIRCULATING LEVELS OF CYTOKINES

As shown in **Figure 1 A**, the plasma levels of IL-6 were 17.6 (6.7) pg/mL in sham-operated on animals. Vehicle-treated animals had significantly increased IL-6 levels after trauma-hemorrhage 6 hours following the end of resuscitation (281.8 [56.3] pg/mL). Administration of progesterone at the end of resuscitation significantly decreased plasma IL-6 levels (40.6 [9.9] pg/mL).

Tumor necrosis factor  $\alpha$  levels in the plasma of sham-operated on animals were 14.2 (5.0) pg/mL (Figure 1B). Following soft tissue trauma and hemorrhagic shock, TNF- $\alpha$  levels increased significantly in vehicle-treated animals (68.2 [16.1] pg/mL). However, progesterone-treated animals showed no significant increase in the plasma levels of TNF- $\alpha$  and had significantly reduced TNF- $\alpha$  levels compared with vehicle-treated animals (32.4 [10.8] pg/mL).

### EFFECTS OF PROGESTERONE ON CYTOKINE PRODUCTION OF ISOLATED KUPFFER CELLS

**Figure 2A** shows the levels of TNF- $\alpha$  in the supernatant of isolated Kupffer cells in the presence of different concentrations of progesterone. With increasing con-



**Figure 2.** Effects of different concentrations of progesterone on the release of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (A) and interleukin 6 (IL-6) (B) from Kupffer cells in vitro. Cells were isolated from rats following sham operation or trauma-hemorrhage followed by resuscitation and treated with different doses of progesterone with or without stimulation by 100 ng/mL of lipopolysaccharide (LPS) in vitro. There were 7 animals in each group. Data are presented as mean (SEM) and compared by 1-way analysis of variance and Tukey test. Asterisk indicates  $P < .05$  vs vehicle-treated animals; dagger,  $P < .05$  vs vehicle-treated animals who also received a single dose of progesterone,  $10^{-12}$  g/mL.

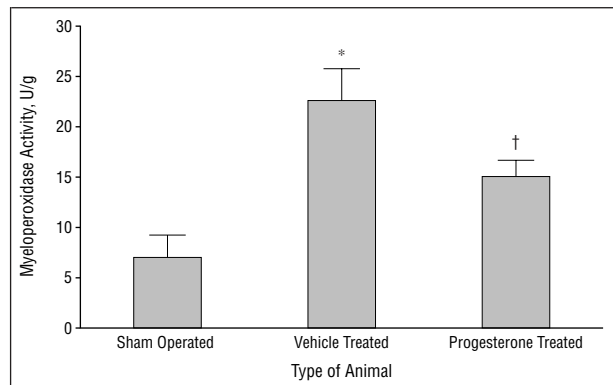
centrations of progesterone, the levels of TNF- $\alpha$  were reduced significantly. In contrast, IL-6 production of isolated Kupffer cells was not significantly affected by the presence of different doses of progesterone (Figure 2B).

#### EFFECT OF PROGESTERONE ON THE MPO ACTIVITY IN HEPATIC TISSUES

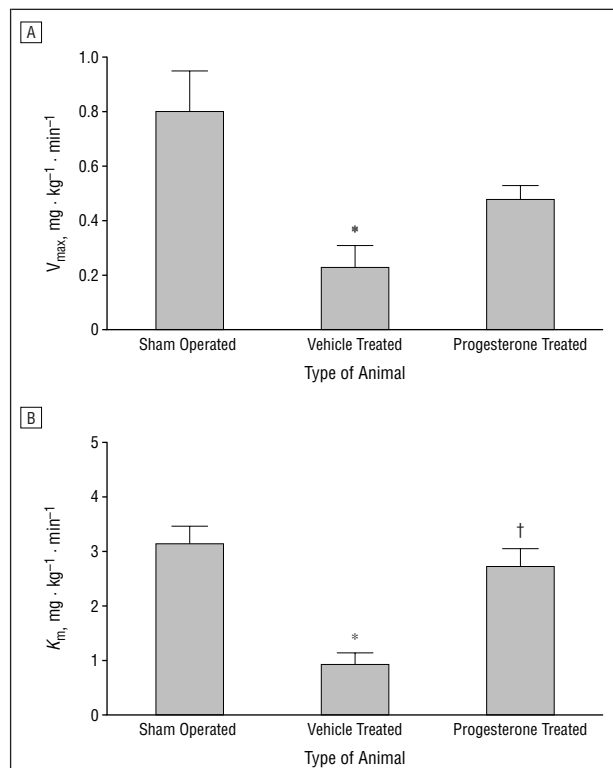
As shown in **Figure 3**, the MPO activity in hepatic tissues 6 hours after resuscitation was significantly increased in vehicle-treated animals following trauma and hemorrhagic shock (22.6 [3.2] U/g) compared with sham-operated on animals (7.0 [2.2] U/g). Treatment with a single dose of progesterone significantly reduced the MPO activity (15.1 [1.6] U/g); however, the values were still significantly higher than values of sham-operated on animals.

#### ALTERATIONS IN HEPATOCELLULAR FUNCTION

The mean value of  $V_{\max}$  was 0.80 [0.14]  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  in sham-operated on animals receiving vehicle (**Figure 4A**). In hemorrhage animals receiving vehicle,  $V_{\max}$  decreased significantly (0.23 [0.07]  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) 20 hours after trauma-hemorrhage and resuscitation. There was no significant difference in  $V_{\max}$  between progesterone-



**Figure 3.** Effects of progesterone treatment on the myeloperoxidase activity in hepatic tissues following trauma-hemorrhage. Comparison of sham-operated on animals, and animals subjected to trauma-hemorrhage and then treated with either vehicle (sesame oil) or a single dose of progesterone. Data are presented as mean (SEM) and compared by 1-way analysis of variance and Student-Newman-Keuls test. There were 7 animals in each group. Asterisk indicates  $P < .05$  vs sham-operated on animals; dagger,  $P < .05$  vs vehicle-treated animals.



**Figure 4.** Effects of progesterone administration on the active hepatocellular function 20 hours following sham operation or trauma-hemorrhage and resuscitation as measured by the indocyanine green (ICG) clearance technique. A,  $V_{\max}$  represents the maximum velocity of ICG clearance. B,  $K_m$  represents the overall efficiency of the active ICG transport. Comparison of sham-operated on animals treated with vehicle (sesame oil) and trauma-hemorrhage animals treated with vehicle, or trauma-hemorrhage animals treated with a single dose of progesterone. There were 7 animals in each group. Data are presented as mean (SEM) and compared by 1-way analysis of variance and Student-Newman-Keuls test. Asterisk indicates  $P < .05$  vs sham-operated on animals; dagger,  $P < .05$  vs vehicle-treated animals.

(0.46 [0.05]  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) or vehicle-treated animals following trauma-hemorrhage. As shown in Figure 4B,  $K_m$  was 3.15 [0.31]  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  in sham-operated on



animals and it decreased to 0.94 [0.21] mg·kg<sup>-1</sup>·min<sup>-1</sup> following trauma-hemorrhage and resuscitation in vehicle-treated rats. Progesterone treatment increased  $K_m$  at 24 hours after resuscitation compared with vehicle-treated animals and the values were similar to the sham-operated on animals (2.74 [0.33] mg·kg<sup>-1</sup>·min<sup>-1</sup>).

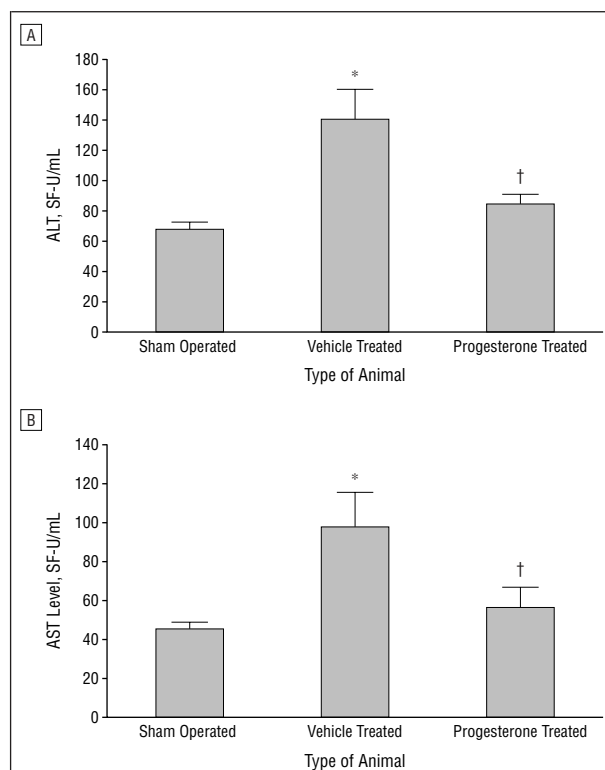
#### EFFECTS OF PROGESTERONE ON PLASMA ACTIVITY OF ALT AND AST LEVELS

In sham-operated on animals, the mean activities of ALT and AST were 67.5 (4.7) Sigma-Frankel units per milliliter (SF-U/mL) and 45.4 (3.4) SF-U/mL, respectively (Figure 4). In hemorrhaged and vehicle-treated rats the enzyme activities increased significantly (ALT, 140.1 [19.7] SF-U/mL; AST, 97.6 [17.7] SF-U/mL) 20 hours after resuscitation. Progesterone-treated, hemorrhaged animals had transaminase activities that were similar to sham-operated on animals (ALT, 84.0 [6.3] SF-U/mL; AST, 56.2 [10.3] SF-U/mL) (**Figure 5**).

#### COMMENT

The liver is one of the first organs affected by trauma and hemorrhagic shock.<sup>16</sup> Hepatocellular injury is a common feature in trauma patients<sup>17</sup> as well as in animal models of hemorrhagic shock.<sup>13</sup> Hepatic failure is second only to pulmonary failure as a manifestation of the multiple organ dysfunction syndrome.<sup>18</sup> Furthermore, impairment of hepatic functions, such as protein synthesis and detoxification, are thought to cause remote organ damage and, thus, to contribute to the progressive development of multiple organ dysfunction syndrome under such conditions.<sup>19</sup> The exact pathways of the development of the hepatocellular dysfunction seem multifactorial and are not yet totally identified. However, it is well known that the activation of the immune system following major injuries can contribute to organ damage following trauma-hemorrhage. The release of proinflammatory cytokines such as TNF- $\alpha$  and IL-6 into the circulatory system, activation of circulating neutrophils, and the production of free radicals have been shown to contribute to vascular and parenchymal damage.<sup>20</sup> Therefore, it appears that in addition to the rapid replacement of the lost fluid volume, further therapeutic interventions are needed to ameliorate the inflammatory response in the treatment of the severely injured.

Our studies have shown that trauma and hemorrhagic shock decreases several hemodynamic parameters such as cardiac index, stroke volume, mean arterial blood pressure, total peripheral resistance, hematocrit, maximum rate of left ventricle pressure increase, and maximum rate of left ventricle pressure decrease compared with sham-operated on animals.<sup>6</sup> The depression in the above parameters occurs early after trauma-hemorrhagic shock and remains depressed for a prolonged period.<sup>19</sup> Nevertheless, the depressed cardiovascular functions can be improved or restored by administration of flutamide<sup>21</sup> or estradiol<sup>6</sup> following trauma-hemorrhagic shock in males. Our recent studies have shown that administration of progesterone following trauma-hemorrhage in ovariectomized rats significantly improved cardiac output, maximum rate of left ventricle



**Figure 5.** Effects of progesterone administration on the enzymatic activity of alanine aminotransferase (ALT) (A) and aspartate aminotransferase (AST) (B) in the plasma of rats 20 hours following sham operation or trauma-hemorrhage and resuscitation. Comparison of sham-operated on animals treated with vehicle (sesame oil) (sham) and trauma-hemorrhage animals treated with vehicle, or trauma-hemorrhage animals treated with a single dose of progesterone. There were 7 animals in each group. Data are presented as mean (SEM) and compared by 1-way analysis of variance and Student-Newman-Keuls test. Asterisk indicates  $P < .05$  vs sham-operated on animal; dagger,  $P < .05$  vs vehicle.

pressure increase and maximum rate of left ventricle pressure decrease, and circulating blood volume but did not significantly improve mean arterial blood pressure.<sup>22</sup> Whether progesterone administration in males following trauma-hemorrhage produces cardiovascular and hemodynamic responses similar to those observed in ovariectomized females remains to be determined.

Recent studies have shown that the female sex steroid progesterone plays an important role in the adaptation of the immune system to pregnancy and regulates key mechanisms of inflammatory response.<sup>23</sup> In the present study, we investigated whether a single dose of progesterone administered after trauma-hemorrhage could ameliorate the inflammatory response and beneficially influence active liver function and prevent hepatocellular damage under such conditions. Since progesterone is released in pulsatile spikes, we used ovariectomized female rats to assure stable baseline levels of progesterone.<sup>24</sup>

Our results demonstrated that treatment with progesterone during resuscitation significantly decreased the circulating levels of the proinflammatory cytokines IL-6 and TNF- $\alpha$  and reduced the accumulation of polymorphonuclear granulocytes in the hepatic tissue as assessed by MPO assay. Previous studies from our laboratory have shown that levels of IL-6 and TNF- $\alpha$  significantly increase following trauma-hemorrhage and remain el-

evated for several hours.<sup>25</sup> Furthermore, studies using gadolinium chloride to deplete the Kupffer cell population indicated that this macrophage population plays a major role in the release of IL-6 and TNF- $\alpha$  and in the initiation of the inflammatory cascade under shock conditions.<sup>26,27</sup> Studies by Miller and Hunt<sup>9</sup> and Chao et al<sup>28</sup> have demonstrated that progesterone at physiological concentrations can inhibit TNF- $\alpha$  production and release in isolated macrophages. We observed in our *in vitro* experiments that progesterone directly inhibits the TNF- $\alpha$  production by Kupffer cell population. In this regard, inhibition of TNF- $\alpha$  effects by administration of anti-TNF- $\alpha$  antibodies has been shown to reduce the accumulation of polymorphonuclear leukocytes in the liver,<sup>29</sup> suggesting that the observed reduction in TNF- $\alpha$  production might contribute to the reduced polymorphonuclear leukocyte accumulation within the hepatic tissue. However, other mechanisms of the beneficial effects of progesterone administration in ovariectomized female rats following trauma-hemorrhage cannot be ruled out. Although we did not observe a direct modulation of IL-6 using different doses of progesterone, it is well known that progesterone, besides its direct effects on immune competent cells,<sup>30</sup> can modulate immune functions by stimulating lymphocytes to release progesterone-induced blocking factor, which is considered to play a major role in the alterations of the immune system observed during pregnancy.<sup>23</sup> Further studies are, therefore, needed to determine the role of progesterone-induced blocking factor in our model.

It has been previously shown that injury, such as trauma-hemorrhagic shock, can induce proinflammatory cytokine production, for example, IL-6 and TNF- $\alpha$ .<sup>3</sup> The transcriptional regulation of these cytokines is regulated by nuclear factor- $\kappa$  B activity in macrophages, B and T cells, monocytes, and other types of immune cells under such conditions.<sup>31</sup> *In vitro* studies have shown that a direct mutual repression exists between the hormone-activated progesterone receptor and the RelA(p65) subunit of nuclear factor- $\kappa$  B activity.<sup>32</sup> This transrepression is independent of progesterone receptor isoforms or cell types used. However, whether such an interaction also occurs *in vivo* has not been demonstrated thus far. Most target tissues for progesterone express receptors for cytokines, which can induce nuclear factor- $\kappa$  B activity. Thus, it could be postulated that there is a physiological significance of this antagonism.<sup>31</sup> To extrapolate these *in vitro* results to *in vivo* effects requires extensive additional work. Nevertheless, it is possible that the observed effects of progesterone on TNF- $\alpha$  and IL-6 in this study are, at least partly, due to this inhibitory effect. In additions, in human leiomyomas, the elevated TNF- $\alpha$  production was decreased in the secretory, progesterone-dominated phase of the menstrual cycle compared with the proliferative phase.<sup>33</sup> Furthermore, TNF- $\alpha$  expression was down-regulated in leiomyoma cells *in vitro*.<sup>33</sup> Our recent studies have shown that progesterone administration following trauma-hemorrhage up-regulates progesterone receptors.<sup>22</sup> Thus, it can be postulated that progesterone administration inhibits induction of nuclear factor- $\kappa$  B activity through the up-regulated progesterone receptors.

The reduction of proinflammatory mediators following trauma-hemorrhage in the plasma of progesterone-treated animals was correlated with a significant reduction in the accumulation of polymorphonuclear leukocytes in hepatic tissue 6 hours after resuscitation. This was associated with significantly diminished activities of the transaminases ALT and AST, parameters of hepatocellular damage, 20 hours after resuscitation in the plasma of progesterone-treated animals compared with vehicle-treated controls. Since it is well known that activated neutrophils can contribute to tissue damage following low-flow conditions such as ischemia and reperfusion or hemorrhagic shock, it is likely that the prevention of the accumulation of granulocytes in the hepatic tissue of progesterone-treated animals contribute to the reduced parenchymal liver damage observed in those animals following trauma-hemorrhage. Moreover, studies by Wang et al<sup>34</sup> have demonstrated that TNF- $\alpha$  itself can have deleterious effects on the liver, that is, infusion of TNF- $\alpha$  produces hepatocellular dysfunction. Thus, the reduction of TNF- $\alpha$  by progesterone could be a potential mechanism for the reduced liver damage in our model. Other possible mechanisms of the protective effect of progesterone include direct effects on the hepatocytes and changes in bile flow,<sup>35</sup> and potential indirect effects such as increased ventilation in the spontaneously breathing animal<sup>36</sup> cannot be excluded. In this study, the increase in the active hepatocellular function, that is, the combined increase in both  $V_{\max}$  and  $K_m$  in progesterone-treated animals did not reach significance ( $V_{\max}$ ,  $P < .01$ ;  $K_m$ ,  $P = .10$ ). Using the same ICG clearance method, Wang et al<sup>34</sup> were able to show that hepatocellular dysfunction following hemorrhagic shock is characterized by a reduction of both, the  $V_{\max}$ , the maximal clearance rate as well as the  $K_m$ , or Michaelis-Menten constant, the concentration of half maximal clearance of ICG in the circulation. Wang and colleagues<sup>13</sup> clearly demonstrated that hepatocellular dysfunction is a very early and sensitive parameter of liver injury. Our results showed only a trend toward improvement in this very sensitive parameter of impaired hepatocellular function after the severe insult of combined soft tissue trauma and decompensated hemorrhagic shock. However, the amount of cellular damage in the liver parenchyma was significantly reduced. Further studies are needed to investigate whether the promising effects observed with a single administration of progesterone could be improved with repeated treatment or a different dosage of progesterone, to achieve complete restoration. Moreover, although progesterone seems to be beneficial in our model of trauma-hemorrhagic shock, it remains unclear whether the direct modulation of the immune response by progesterone can beneficially influence the ability of the immune system to react to subsequent infectious insults. Studies using proestrus female rats and male rats suggest that rats with high levels of progesterone are protected in a model of trauma-hemorrhagic shock and induction of subsequent sepsis by cecal ligation and puncture.<sup>37</sup> Nevertheless, further studies are needed to determine whether high levels of progesterone or estradiol, low levels of testosterone, or the combination of those factors are responsible for the sex differences observed in that "double-hit" model of trauma-hemorrhagic shock and subsequent induced sepsis.

Several clinical studies suggest that sex affects humoral and cell-mediated immune responses. Remarkable female preponderance has been observed in a number of autoimmune diseases, that is, systemic lupus erythematosus, rheumatoid arthritis, Hashimoto thyroiditis, and primary biliary cirrhosis. Furthermore, higher levels of circulating antibody titers were observed in females compared with males. In addition to humoral immune responses, cell-mediated immune functions also seem to reveal similar sex dimorphism. Moreover, significantly higher intraindividual variability was observed in the number of colony-forming cells in peripheral blood from women compared with men. Gender and sex steroids seem to be responsible for these differences in the humoral immune response.

Numerous epidemiological studies indicate the importance of sex and age as risk factors of sepsis and multiple organ failure following trauma. These studies have shown that the victims of trauma are mostly young males. Nevertheless, not only the prevalence of trauma but also the mortality from trauma seem to be sex related. A multicenter study revealed that severely injured women younger than 50 years have a survival advantage compared with males of equal age and injury severity: young men have a 27% greater chance of dying than women after trauma. Recent retrospective analysis from our institution suggests that premenopausal adult women have a survival advantage compared with males following nonthermal blunt trauma; however, the opposite was noted following thermal injury. Furthermore, studies have shown female survival advantage in nonthermally injured patients younger than 50 years. Nevertheless, other studies could not find sex differences in mortality following blunt trauma. A potential reason for the controversy in the clinical findings may be related to the fact that the hormonal status of the females was not known or recorded in those studies. Since the experimental studies indicate that the advantage females have in tolerating the deleterious effects of trauma-hemorrhage and sepsis is only evident in the proestrus phase (with high estrogen, prolactin, and progesterone levels), it is imperative that a prospective study be performed in which the hormonal status of premenopausal women is correlated with complications or lack of those following trauma. Additional studies suggest that not only the outcome after severe injury shows sex dimorphism, but also the susceptibility to septic complications following trauma showed preponderance in males compared with females. These clinical findings are supported by experimental observations, which indicate significantly higher survival rates in proestrus females compared with males following trauma-hemorrhage and induction of subsequent sepsis. In this study, a single dose of progesterone at the end of resuscitation could prevent hepatocellular function in ovariectomized female rats following trauma and hemorrhagic shock. Whether progesterone administration as an adjunct following trauma in postmenopausal or ovariectomized females protects the liver from damage and dysfunction remains to be determined. Thus, studies are required to determine the potential clinical usefulness of progesterone in patients following trauma.

The role of the end organs following reperfusion injury is a clinically valid problem; however, our present study was focused only on the liver since this organ is affected very early following trauma-hemorrhage.<sup>13</sup> Although other organs such as the lungs should also have been examined in this study, our previous studies have shown that unless a second hit such as sepsis is inflicted following trauma-hemorrhage, the lungs are relatively unaffected in our model of trauma-hemorrhage.<sup>38</sup> Since sepsis following trauma-hemorrhage was not induced in the present study, we did not examine the lungs in this study. Nevertheless, studies using more sensitive techniques or methods to measure the various functions of the lung should be carried out in the future.

## CONCLUSIONS

Our results indicate that the pregnancy hormone progesterone ameliorates the inflammatory response of ovariectomized female rats following trauma-hemorrhage, and reduces polymorphonuclear leukocyte accumulation in the liver and the hepatocellular damage observed under those conditions. Further studies are needed to determine whether the protective effects of progesterone are sex specific and to better understand its mechanisms, as well as to identify potential adverse effects. Nevertheless, our data indicate that administration of progesterone might be a novel approach in the treatment of trauma and hemorrhagic shock related liver damage.

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