Sex Differences in Hepatic Heme Oxygenase Expression and Activity Following Trauma and Hemorrhagic Shock

Balazs Toth, MD; Yukihiro Yokoyama, MD, PhD; Joachim F. Kuebler, MD; Martin G. Schwacha, PhD; Loring W. Rue III, MD; Kirby I. Bland, MD; Irshad H. Chaudry, PhD

Hypothesis: Sex differentially influences heme oxygenase (HO) expression following trauma and hemorrhagic shock.

Design: Prospective controlled animal study.

Setting: A university laboratory.

Interventions: Female Sprague-Dawley rats in the proestrus stage of their estrus cycle and male rats underwent a 5-cm midline laparotomy (ie, induction of soft tissue trauma) and were bled to a mean arterial blood pressure of 35 mm Hg for approximately 90 minutes, after which they were resuscitated with Ringer lactate solution (4 × the shed blood volume). In another group of animals, tin protoporphyrin IX was used to block HO activity.

Main Outcome Measures: Liver samples were collected for analysis of HO expression and activity, plasma samples were collected, and alanine transaminase levels were determined 5 hours after resuscitation. Portal pressure and bile production were measured in vivo 5 hours after resuscitation.

Results: Trauma and hemorrhage induced a 2-fold increase in hepatic HO1 expression (the inducible form of HO) in proestrus females compared with males. Hepatic expression of HO2 (a constitutive isoform of HO) was unaffected by sex or trauma and hemorrhage. Blockade of HO in vivo with tin protoporphyrin IX abolished the sex differences caused by diverse HO1 expression. Treatment with tin protoporphyrin IX also elevated the portal pressure, decreased bile production, and increased alanine transaminase to similar levels in proestrus females and males following trauma and hemorrhage.

Conclusions: Sex influences the hepatic expression of HO1 following trauma and hemorrhage. The enhanced induction of HO1 expression and activity in females after trauma and hemorrhage may attenuate hepatocellular dysfunction and injury by maintaining microcirculation via the increased production of carbon monoxide.

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TRAUMA WITH accompanying blood loss is the leading cause of death among young adults.1-3 Hemorrhagic shock decreases organ perfusion, resulting in organ and immune dysfunction. Several studies have suggested that carbon monoxide (CO) may participate in the maintenance of organ perfusion and thus produce beneficial effects following trauma and hemorrhage. The endogenous source of CO (which acts as a vasodilator agent via the activation of guanylate cyclase) is the heme oxygenase (HO) enzyme, which catalyzes the degradation of heme to biliverdin. Three isoforms of HO have been identified: HO1, the inducible isoform, and HO2 and HO3, the constitutive isoforms. The heme oxygenase inducible isoform is considered a heat shock protein (HSP) and is also identified as HSP-32. The HSPs protect the cellular machinery of many organs from a wide variety of insults and stresses, such as ischemia and oxidative stress. Studies have shown that the maintenance of hepatic microcirculation is essential following low-flow conditions to maintain liver function. Data indicate that the HO pathway, and specifically HO1, may contribute to the attenuation of hemorrhagic shock and the subsequently impaired circulation of the liver via the generation of CO. However, in the male animal model, HO1 fails to fully eliminate the deleterious effects of trauma and hemorrhage.

Clinical and laboratory studies have shown that sex differences exist in liver function following adverse circulatory conditions. Studies indicate that male sex steroids have deleterious effects and female sex steroids produce beneficial effects on hepatic function following circulatory stress. Testosterone receptor blockade after trauma and hemorrhage has been shown to improve hepatic function in
male animals. Alternatively, administration of 17β-estradiol improved cardiac and hepatic functions in male animals after trauma and hemorrhagic shock. In addition, Jarrar et al have shown that proestrus female rats have normal organ function 24 hours after trauma and hemorrhage, whereas males show marked depression in cardiovascular and hepatocellular functions. Estradiol levels are highest during the proestrus stage of the estrus cycle, a stage in which females are protected against the deleterious effects of trauma and hemorrhage. Furthermore, it has been shown that estrogens are protective during other adverse circulatory conditions such as ischemia or inflammation. We hypothesized that the elevated estrogen levels in proestrus females compared with males increase HO activity following trauma and hemorrhage and thus produce salutary effects on organ blood flow. The aim of our study, therefore, was to determine whether there is any difference in HO expression and activity in males vs proestrus females following soft tissue trauma and hemorrhagic shock.

### METHODS

#### ANIMALS

Adult male (275 to 300-g) and female (250 to 275-g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) were used in this study. All experiments were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

#### EXPERIMENTAL PROCEDURES

A nonheparinized model of trauma and hemorrhage in the rat, as previously described, was used in this study. Age- and cycle-matched female and age-matched male Sprague-Dawley rats were fasted overnight before the experiment but were allowed water ad libitum. The female rats were in the proestrus state of the estrus cycle, as determined by daily examination of the vaginal smears. The proestrus stage was defined as the period when both leukocytes and nucleated epithelial cells were present in approximately equal numbers. Experiments were performed after at least 1 complete estrus cycle had been documented. To minimize the effects of circadian rhythms, vaginal smears were obtained between 7 AM and 9 AM, and the hemorrhage procedure began between 9 AM and 10 AM. The rats were anesthetized by isoflurane (Minrad Inc, Bethlehem, Pa) inhalation before the induction of soft tissue trauma (5-cm midline laparotomy). The abdomen was closed in layers, and catheters were placed in both femoral arteries and the right femoral vein (PE-50 polyethylene tubing; Becton, Dickinson and Company, Sparks, Md). The wounds were bathed with 1% lidocaine hydrochloride (Elkins-Sinn, Cherry Hill, NJ) throughout the surgical procedure to minimize postoperative pain. The rats were then allowed to awaken, after which they were bled to a mean arterial blood pressure of 35 mm Hg within 10 minutes. Rapid bleeding places the animals in a depressed state of sensibility, thereby minimizing stress. The point at which the animals could no longer maintain a mean arterial blood pressure of 35 mm Hg without the infusion of fluid was defined as the maximum bleed-out volume. The rats were maintained at this pressure until 40% of the shed blood was returned in the form of Ringer lactate solution. The animals were resuscitated with 4 × the volume of withdrawn blood using Ringer lactate solution for 60 minutes. Following resuscitation the catheters were removed, vessels were ligated, and skin incisions were closed with sutures.

Sham animals underwent the same groin dissection, which included ligation of the femoral artery and vein; however, neither trauma and hemorrhage nor resuscitation was carried out. All animals were returned to their cages and were allowed food and water ad libitum until death. The animals were killed 5 hours after resuscitation. The time point for death was selected on the basis of HO1 expression following trauma and hemorrhage, as described by Bauer at al.

#### DETERMINATION OF HO ACTIVITY

We determined HO activity in liver homogenates by measuring the amount of bilirubin formed in a 2-step enzymatic reaction, as described by Maines and Kappas and Wang et al. Liver tissue samples were homogenized in 10mM of phosphate-buffered saline (pH, 7.2) containing protease inhibitors (Complete; Roche, Basel, Switzerland). The homogenates were centrifuged at 10000g for 15 minutes at 4°C. The supernatant was further centrifuged at 105000g for 1 hour at 4°C. The resulting pellet was resuspended in phosphate-buffered saline with protease inhibitors. Protein content was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, Calif). The solution mixture was prepared containing 0.5 mL of microsomal protein (6-9 mg/mL), 200 µL of a 105 000g-centrifuged supernatant fraction of the rat liver (10 mg/mL of protein) as a source for biliverdin reductase, a nicotinamide adenine dinucleotide phosphate (NADPH)–generating system (0.8mM of B-NADPH, 0.8mM of glucose 6-phosphate, 1.3 U of glucose-6-phosphate dehydrogenase, and 2mM of magnesium chloride), and 20 µL of 2.5mM of heme solution. The final volume was adjusted to 1.5 mL with 0.1M of phosphate-buffered saline. Reagent blanks were prepared in a similar way but without the addition of the NADPH-generating system. Samples were prepared on ice and then incubated in the dark at 37°C with agitation for 60 minutes. Bilirubin content was measured spectrophotometrically at 460 nm and 530 nm; HO activity was expressed as nanometers of bilirubin per 60 minutes per milligram of protein.

#### WESTERN BLOT ANALYSIS

Liver samples were homogenized in 10 vol of cell lysis buffer (phosphate-buffered saline; pH, 7.2) containing 50mM of sodium fluoride, 100mM of tetrasodium pyrophosphate, 2.5mM of sodium orthovanadate, 50mM of phenylmethylsulphonyl fluoride, 1M of dithiothreitol, and protease inhibitors (Roche). The lysate was clarified by centrifugation at 105000g for 15 minutes at 4°C. The supernatant was resuspended in phosphate-buffered saline (pH, 7.2) containing protease inhibitors and centrifuged at 100000g for 1 hour at 4°C. The resulting pellet was resuspended in phosphate-buffered saline (pH, 7.2) containing protease inhibitors and centrifuged at 100000g for 1 hour at 4°C. The resulting pellet was resuspended in phosphate-buffered saline (pH, 7.2) containing protease inhibitors and centrifuged at 100000g for 1 hour at 4°C. The resulting pellet was resuspended in phosphate-buffered saline (pH, 7.2) containing protease inhibitors and centrifuged at 100000g for 1 hour at 4°C. The resulting pellet was resuspended in phosphate-buffered saline (pH, 7.2) containing protease inhibitors and centrifuged at 100000g for 1 hour at 4°C. The resulting pellet was resuspended in phosphate-buffered saline (pH, 7.2) containing protease inhibitors and centrifuged at 100000g for 1 hour at 4°C.
MEASUREMENT OF PORTAL PRESSURE AND BILE FLOW

To block HO activity, a second set of animals was treated with tin protoporphyrin IX (SnPP) (50 µmol/kg of body weight, subcutaneously) at the beginning of resuscitation. The portal pressure measurements were performed according to Baveja et al26 with modifications. Five hours after resuscitation, the carotid artery was cannulated and the cannula was connected to a pressure transducer. A laparotomy was performed, after which the intestines were covered with wet gauze to minimize evaporative loss during the measurements. The portal vein was identified and exposed. The common bile duct was cannulated with polyethylene-10 tubing (Becton, Dickinson and Company), and the bile flow was measured in preweighted tubes for 10 minutes. A polyethylene-10 catheter filled with saline was inserted into the portal vein without compromising the flow. The catheter was connected to a low-pressure analyzer (Digi-Med, Louisville, Ky) to monitor the portal pressure. The pressure transducers were calibrated and zeroed against a column of fluid open to the atmosphere at the level of the cannula tips.

MEASUREMENT OF HEPATOCYTE DAMAGE

Five hours after resuscitation, blood samples were obtained and placed in microcentrifuge tubes. After centrifugation, plasma samples were separated, immediately frozen, and stored at –80°C until assayed. Hepatocyte injury was determined by measuring levels of alanine transaminase (ALT) using a commercially available colorimetric reaction kit according to the manufacturer’s instructions (Sigma, Ronkonkoma, NY).

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Statistical differences between groups were determined by analysis of variance followed by the Newman-Keuls post hoc test or t test, and differences were considered significant if \( P < .05 \).

RESULTS

HO ACTIVITY AND EXPRESSION

Trauma and hemorrhagic shock increased HO activity in the livers of both male and female animals as compared with sham animals (Figure 1). The trauma and hemorrhage–induced increase in HO activity was significantly greater \( (P < .05) \) in proestrus females than in males. To determine which isoform of the HO enzyme was responsible for the observed increase in activity, liver homogenates were analyzed for HO1 and HO2 protein using Western blot analysis. No significant differences were observed in HO1 expression in livers from male and female sham animals (Figure 2A and B). Trauma and hemorrhage induced significantly greater \( (P < .05) \) HO1 expression in proestrus females than in males. In contrast, HO2 expression and protein levels remained constant: neither trauma and hemorrhage nor sex influenced its expression (Figure 2C and D).

INHIBITION OF HO ACTIVITY

In normal conditions, there was no difference in portal pressure between males and females. Trauma and hemorrhage elevated portal pressure in vehicle-treated males; however, the differences were not significant when compared with sham animals. In contrast, vehicle-treated females exhibited significantly lower pressure compared with males following trauma and hemorrhage (Figure 3). Blockade of HO activity with SnPP did not affect portal pressure in sham animals; however, trauma and hemorrhage and SnPP increased the portal pressure significantly \( (P < .05) \) in both males and proestrus females.

Following sham operation, neither sex nor blockade of HO suppressed bile production. Trauma and hemorrhage significantly decreased bile flow in males, whereas bile flow was maintained in proestrus females (Figure 4). In addition, SnPP did not alter the suppressed bile production in males following trauma and hemorrhage; however, SnPP significantly \( (P < .05) \) decreased bile production in proestrus females following trauma and hemorrhage (Figure 4).

Mean arterial blood pressure was not significantly different at the beginning of the experiment or at the time point of euthanasia in sham animals. Furthermore, SnPP treatment did not significantly alter the pressure in either group. Although there was a significant decrease in mean arterial blood pressure 3 hours after trauma and hemorrhage and resuscitation, neither sex nor SnPP affected it (Table).

CHANGES IN PLASMA ALT LEVELS

In sham animals, neither sex nor blockade of HO altered ALT activity (Figure 5). Trauma and hemorrhage significantly increased ALT activity in males \( (P < .05) \) but not in proestrus females. However, SnPP treatment further increased ALT activity in males and also increased activity in proestrus females following trauma and hemorrhage (Figure 5).
Recent studies have suggested that CO generated via the HO may act as a vasodilator in the liver following trauma and hemorrhagic shock.6,27 Studies have also shown that hemorrhagic shock and resuscitation can induce the expression of HO1 (the inducible isoform) in the liver of male animals,6 whereas levels of HO2 (constitutive HO) are unchanged in such conditions.4 Our study shows a difference in magnitude of the increased expression pattern of HO1 in the liver of male and proestrus female rats following trauma and hemorrhage: HO activity (which reflects the combined activity of HO1 and HO2) in proestrus females was significantly higher compared with males following trauma and hemorrhage, which correlated with elevated HO1 protein expression in proestrus females. The markedly elevated HO1 activity and expression correlated with decreased liver tissue damage (lower ALT level) and maintained hepatocellular function (unaltered bile flow and lower portal pressure) in proestrus females following trauma and hemorrhage. Consistent with previous studies, we demonstrated increased expression of HO1 in male rats following trauma and hemorrhagic shock.5 It has been shown that endogenous CO has protective effects following trauma and hemorrhage in male animals.6 However, that study used a less severe trauma-and-hemorrhage model compared with ours. Despite the doubled HO1 activity in males following trauma and hemorrhage, no protective effects of HO1 were observed in males. However, the markedly increased activity found in proestrus females improved liver function, and...
Female proestrus females maintained immune function reportedly compared with males following trauma and hemorrhage, whereas vehicle-treatment of testosterone-releasing pellets in female mice resulted in decreased levels of 17\textbeta-estradiol. Studies have also demonstrated that implantation of testosterone-releasing pellets in male mice. Furthermore, Eckhoff et al\textsuperscript{19} have shown that estradiol administration significantly reduced injury after ischemic reperfusion to the liver; this effect was mainly receptor mediated and was associated with increased serum nitric oxide levels, decreased levels of tumor necrosis factor \( \alpha \), and a decreased number of neutrophils in liver biopsy specimens. Our recent study\textsuperscript{17} has shown that estradiol administration following trauma and hemorrhage improves splanchic perfusion even after the induction of sepsis.

Estradiol is the predominant circulating sex hormone in females. It has been demonstrated that female rodents have the highest circulating estradiol levels in their proestrus phase compared with the other phases.\textsuperscript{31} The peak level of estradiol is reached in the morning hours, followed by a gradual decrease. On the basis of the observed salutary effects of estradiol on the proestrus state of the female animals following trauma and hemorrhage,\textsuperscript{13,16,20,29,32} we used proestrus female and male animals in our studies. Previous results from our laboratory have shown that whereas cardiac output, heart performance parameters, and hepatocellular function are significantly depressed in male animals following trauma and hemorrhage, these functions are maintained or even enhanced in proestrus females in those conditions.\textsuperscript{16,19,31} In contrast, ovariectomized females with decreased levels of 17\textbeta-estradiol have significantly reduced hepatocellular function and in-

**Mean Arterial Blood Pressure Following Trauma and Hemorrhagic Shock**

<table>
<thead>
<tr>
<th>Window</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>103.9 ± 7.2</td>
<td>122.7 ± 2.4</td>
</tr>
<tr>
<td>Trauma and hemorrhage</td>
<td>118.5 ± 2.6</td>
<td>120.4 ± 1.7</td>
</tr>
</tbody>
</table>

**SnPP, mm Hg**

<table>
<thead>
<tr>
<th>Window</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>105.9 ± 5.4</td>
<td>117.2 ± 5.3</td>
</tr>
<tr>
<td>Trauma and hemorrhage</td>
<td>79.7 ± 3.1†</td>
<td>94.5 ± 3.4†</td>
</tr>
</tbody>
</table>

Abbreviation: SnPP, tin protoporphyrin IX.

\*Data are presented as mean ± SEM for 6 or 7 animals per group. Zero minutes represents the beginning of the experiment; 5 hours, the time point after resuscitation.

†\( P<.05 \) compared with sham animals.
increased ALT activity following trauma and hemorrhage. Furthermore, Jarrar et al have shown that depressed cardiovascular and hepatocellular functions were restored in animals receiving 17β-estradiol following trauma and hemorrhage. These results collectively indicate that cardiovascular and immunological functions are maintained in proestrus females but not in ovariectomized females, males, or females in the nonproestrus stages of the estrus cycle following trauma and hemorrhagic shock. Therefore, male animals served as controls for proestrus females in this study. Further studies are required to determine the possible differences in HO1 expression in female animals in the different stages of the estrus cycle in such conditions.

Although the HO pathway was originally described in connection with the degradation of the heme molecule to biliverdin, recent findings have suggested that HO may be an important vasoactive effector through the formation of CO, activation of guanylate cyclase, and subsequently increased formation of cyclic guanosine monophosphate. It has been shown that HO1 has cytoprotective effects following hepatic ischemic reperfusion injury via the generation of CO. Furthermore, Suematsu et al have shown that CO can function as an endogenous modulator of vascular perfusion in the liver in an isolated perfusion model. Hepatic stellate cells, or Ito cells, have been identified as liver-specific pericytes influencing sinusoidal circulation. CO has been shown to increase the formation of cyclic guanosine monophosphate in Ito cells, indicating the low importance of HO in maintaining the liver microcirculation in normal conditions.

Because the expression of HO1 reaches its maximum around 5 hours after resuscitation in male animals following trauma and hemorrhage, we selected this time point for our study. Furthermore, studies indicate the importance of maintaining liver circulation in the early phase of circulatory disturbances following trauma and hemorrhage. Additional studies are required to determine the exact time course of HO expression following trauma and hemorrhage in proestrus females and to clarify whether different estradiol concentrations (baseline estradiol levels in other phases of the estrus cycle) or those of other female sex hormones (eg, progesterone or prolactin) have any salutary effects on HO1 expression.

Nitric oxide is another gaseous mediator that acts as a vasodilator through the activation of soluble guanylate cyclase. In normal conditions, minimal nitric oxide–mediated vasodilatation is observed; however, increased nitric oxide activity has been described after endotoxic shock. Although increased inducible nitric oxide synthase activity occurs in vascular hyperactivity states such as trauma and hemorrhage, Nishida et al and Wang et al showed reduced nitric oxide release from aortic rings following trauma and hemorrhage and resuscitation. Thus, the role of endothelium-derived relaxing factor (nitric oxide) remains unclear in the regulation of systemic vascular tone and hepatic sinusoidal circulation following trauma and hemorrhage.

In the liver, Kupffer cells are the only cell population expressing HO1 constitutively. Bauer at al have shown that HO is increased in male rats following trauma and hemorrhage. Our findings extend these results by showing that the induction of HO activity following trauma and hemorrhage is significantly greater in proestrus females than in males. Moreover, HO1 is the isoform responsible for the observed increase in enzyme activity. Blockade of HO with SnPP showed that in normal conditions, the HO-CO system has little effect on the macrocirculation of the liver. In contrast, trauma and hemorrhage resulted in elevated portal pressure in males but not in proestrus females. Treatment with SnPP attenuated this sex-specific difference and increased the portal pressure in both males and females. The observed sex-specific differences suggest that CO is protective against hepatic circulatory disturbances and that the increased induction of HO in females may be partly responsible for the better-preserved hepatocellular function in proestrus females compared with males following trauma and hemorrhage.

Our study focused on the circulatory aspects of the HO system and CO. However, other potential mechanisms might be responsible for the observed effects. Besides its vasoactive properties, CO possesses functional and immunomodulatory properties such as anti-inflammatory effects. Furthermore, CO and HO1 have been shown to modulate various cellular functions such as cytokine production, cell proliferation, and apoptosis. In addition, it has been shown that the induction of HO1 may protect the cell against oxidative injury by producing biliverdin (an antioxidant). Biliverdin and bilirubin, considered harmful substances in vivo, have been shown to scavenge reactive oxygen species following ischemic reperfusion injury and endotoxin administration. Further studies are needed to determine whether there are any additional effects of CO, biliverdin, and bilirubin following trauma and hemorrhagic shock.

Recent studies using immunohistochemistry techniques have demonstrated that the distribution of the HO enzyme has distinct topographic patterns: HO1, the inducible form, was observed only in Kupffer cells; HO2, the constitutive isoform, was distributed in the parenchymal cells (but not in the Kupffer cells) in normal conditions. In contrast, neither of the 2 isoforms was detectable in hepatic stellate cells or sinusoidal endothelial cells. Hemorrhagic shock led to a different cell type–specific expression pattern of HO1. A marked upregulation was detected with immunohistochemistry in parenchymal and sinusoidal lining cells as well as in periporal hepatocytes and sinusoidal lining cells in the periportal region. However, such studies have been performed only in male animals. Additional studies are needed to determine the localization and possible differences in HO1 expression in proestrus female rats following trauma and hemorrhagic shock.

The perturbation of hepatic circulation (microcirculation and macrocirculation) and the resulting decrease in energy charge following trauma and hemorrhage and SnPP treatment are associated with decreased total bile production, an indicator of hepatic secretory function. Although the observed decrease was moderate in female rats, males exhibited significantly depressed bile flow following trauma and hemorrhage. These findings may in-
di cate that the protective effects of CO are manifested not only by preventing tissue damage but also by maintaining hepatocellular function in proestrus females.

In summary, our study provides evidence that female sex hormones influence the expression of H01 following trauma and hemorrhagic shock. Thus, the higher level of H01 and the generated CO in proestrus females may help to preserve portal pressure and secretory function (bile flow) after trauma and hemorrhage. These results lead us to conclude that the HO-CO pathway may be partly responsible for the previously observed attenuation of hepatocellular damage following trauma and hemorrhage in proestrus females.

CONCLUSIONS

Trauma and hemorrhagic shock with accompanying multiple organ failure is the major cause of morbidity in patients 39 years and younger. Several experimental and epidemiological studies have shown that female animals tolerate the consequences of trauma better than males. Although some epidemiological data do not support sex-related effects on outcome following trauma, those studies do not take into account the hormonal status of the women. In contrast, experimental studies show that the high levels of 17β-estradiol in the proestrus state are essential to observe the beneficial effects. This could explain the difference between the experimental and clinical studies. Future clinical studies should include the hormonal status of the subjects. In this study, we attempted to find a possible explanation that could help to clarify the mechanism of salutary effects observed in proestrus female following trauma and hemorrhage. We examined liver activity and expression of the HO system in male and proestrus female rats following trauma and hemorrhage. The results demonstrate that the expression of H01 is twice as high in proestrus females as in males and that this is associated with decreased portal pressure, normal bile production, and decreased ALT levels in proestrus females following trauma and hemorrhage. Because the cycle stops after trauma and the precise menstrual cycle of women who undergo trauma may not be known, administration of a single dose of estradiol in women and particularly in postmenopausal women following trauma would be expected to prevent the elevation of portal pressure, maintain normal bile production, and decrease hepatic injury in those conditions.

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Corresponding author and reprints: Irshad H. Chaudry, PhD, Center for Surgical Research, University of Alabama at Birmingham, 1670 University Blvd, Volker Hall, Room G094, Birmingham, AL 35294-0019 (e-mail: Irshad.Chaudry@ccc.uab.edu).

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