ORIGINAL ARTICLE

# Attenuation of Vascular Endothelial Dysfunction by Testosterone Receptor Blockade After Trauma and Hemorrhagic Shock

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**Hypothesis:** The salutary effects of the testosterone receptor antagonist flutamide on the depressed immune and cardiovascular functions after hemorrhage and resuscitation are related to improved endothelial cell function, which can subsequently lead to an increase in organ blood flow, oxygen delivery, and tissue oxygen consumption.

**Design, Interventions, and Main Outcome Measures:** Male adult rats underwent a 5-cm midline laparotomy (ie, trauma) and were bled to and maintained at a mean systemic arterial pressure of 40 mm Hg until 40% maximal blood-out volume was returned in the form of Ringer lactate). The animals were then resuscitated with 4 times the total volume of shed blood with Ringer lactate for 60 minutes. Flutamide (25 mg/kg) or an equivalent volume of the vehicle propanediol was injected subcutaneously 15 minutes before the end of resuscitation. At 20 hours after resuscitation, aortic rings (approximately 2.5 mm in length) were isolated and mounted in an organ chamber. Dose responses for an endothelium-dependent vasodilator (acetylcholine chloride) and endothelium-independent vasodilator (nitroglycerine) were determined. Organ blood flow was measured using strontium 85–labeled microspheres. Total hemoglobin and oxygen content in the femoral artery and portal, hepatic, and renal veins were determined. Oxygen delivery and consumption in liver, small intestine, and kidneys were calculated.

**Results:** Administration of flutamide after trauma-hemorrhage attenuated the depressed endothelial function. Furthermore, flutamide treatment restored the reduced blood flow and oxygen delivery and consumption in all organs tested after trauma-hemorrhage and resuscitation.

**Conclusion:** Flutamide appears to be a useful adjunct for improving vascular endothelial function and regional hemodynamics after trauma-hemorrhage and resuscitation.

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PREVIOUS STUDIES have shown that vascular endothelial cell dysfunction occurs during hemorrhagic shock and persists despite fluid resuscitation.[1-3] Vascular endothelial cells play a critical role in the maintenance of tissue perfusion.[4] It is therefore important to investigate potential therapeutic approaches for maintaining endothelial cell function after hemorrhagic shock. In this regard, studies have been conducted to examine the role of sex hormones in the pathophysiology of trauma and severe hemorrhage (hereafter referred to as trauma-hemorrhage).[5-8] It has been demonstrated that proestrus female animals show a normal or even enhanced immune response after trauma-hemorrhage, whereas male animals exhibit a depressed immune response.[6] Furthermore, since gonadectomy before induction of trauma-hemorrhage in male animals prevents the occurrence of immune depression,[7] male sex hormones might play an inhibitory role in posttraumatic immune responses. Furthermore, flutamide, a nonsteroidal testosterone receptor antagonist, has been shown to restore depressed immune function to normal in male subjects after trauma-hemorrhage.[5,8] With regard to organ function, testosterone receptor blockade after trauma-hemorrhage has been shown to improve depressed cardiac, hepatic, and adrenal functions in male rats.[9,10] However, it remains unknown whether the salutary effects of this agent are related to the attenuation of depressed endothelial cell function (ie, the production of vascular endothelium-derived nitric oxide [EDNO]) and the subsequent improvement in tissue perfusion and oxygen use in male subjects under such conditions. The aims of this study, therefore, were to determine (1) whether testosterone receptor blockade with flutamide after trauma-hemorrhage attenuates depressed endothelium-dependent vascular relaxation and (2) whether this agent also improves blood flow in, oxygen delivery to, and oxygen consumption in the liver, small intestine, and kidneys in male rats after trauma-hemorrhage and resuscitation.

## MATERIALS AND METHODS

### ANIMAL MODEL OF TRAUMA-HEMORRHAGE

We used a nonheparinized model of trauma-hemorrhage and resuscitation in the rat, as previously described,[11] with minor modifications. Briefly, Harlan Sprague-Dawley male rats (weight, 275-325 g) were fasted overnight before the experiment but were allowed water ad libitum. The animals were anesthetized using 2% isoflurane and oxygen inhalation and underwent a 5-cm ventral midline laparotomy to induce tissue trauma before the onset of hemorrhage. The abdominal incision was then closed in layers. Both femoral arteries and 1 femoral vein were cannulated with polyethylene-50 tubing for bleeding, monitoring of mean arterial pressure, or fluid resuscitation. All incisions were closed and bathed with 1% lidocaine hydrochloride to provide analgesia throughout the experiment. The animals were then bled to a mean arterial pressure of 40 mm Hg (ie, severe hypotension) within 10 minutes. The blood pressure of 40 mm Hg was maintained by removing more blood in increments of 0.2 mL until the animal was no longer able to keep blood pressure at that level (ie, maximum bleed-out). At that point, the blood pressure was maintained thereafter by infusing Ringer lactate intravenously in 0.2-mL bolus increments until 40% of the shed blood volume was returned in that form. Following this, the animals were resuscitated with 4 times the volume of maximum bleed-out with Ringer lactate for 60 minutes at a constant rate. The sham-operation group underwent the same surgical procedure but were not bled or resuscitated. The time required for maximum bleed-out was approximately 45 minutes, the volume of maximum bleed-out was approximately 60% of the calculated circulating blood volume,[12] and the total hemorrhage time was approximately 90 minutes. The experiments described herein were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals. This project was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

### EXPERIMENTAL PROTOCOL

At 15 minutes before the end of resuscitation, the rats received subcutaneous flutamide (Schering Plough, Kenilworth, NJ), 25 mg/kg of body weight (hemorrhage-flutamide group),[9,10] or an equal volume (approximately 0.3 mL) of the nontoxic vehicle propanediol (hemorrhage-vehicle group). The catheters were then removed, the vessels were ligated, and skin incisions were closed with the use of sutures. The rats were returned to their cages and allowed food and water ad libitum. At 20 hours after the completion of fluid resuscitation, the animals were reanesthetized using pentobarbital sodium, and aortic ring endothelial cell function was determined. Organ blood flow and tissue oxygen delivery and consumption were determined in additional groups of animals.

### AORTIC RING PREPARATION AND DETERMINATION OF VASCULAR REACTIVITY

Immediately after the animals were killed with an overdose of isoflurane at 20 hours after resuscitation, the chest was opened and thoracic aortas were rapidly removed. Each thoracic aorta was immediately immersed in Krebs-Ringer bicarbonate ($HCO_3$) solution (composition, 118.3mM sodium chloride, 4.7mM potassium chloride, 2.5mM calcium chloride, 1.2mM magnesium sulfate, 1.2mM potassium phosphate, 25.0mM sodium bicarbonate, 0.026mM calcium-EDTA, and 1.11mM glucose),[13,14] which was aerated with a mixture of 95% oxygen and 5% carbon dioxide (pH, 7.4; $P_{O_2}$, 580 mm Hg). The thoracic aorta was dissected with care to prevent any damage to vascular endothelial cells and was cut into rings approximately 2.5 mm in length. The aorta rings were carefully mounted on 2 specimen holders and placed in glass organ chambers containing 20 mL of aerated Krebs-Ringer $HCO_3$ solution at a temperature of 37°C. One holder was stationary and the other was connected to an isometric force-displacement transducer (model FT03; Astro-Med, Inc, West Warwick, RI) coupled to a polygraph (model 7D; Astro-Med, Inc). The vessels were incubated for 60 minutes at a tension of 1000 mg, during which the organ chamber was rinsed every 15 minutes with the aerated Krebs-Ringer $HCO_3$ solution. When basal tension was stable, a submaximal contraction (approximately 75% of the maximal contraction) was induced by $2\times10^{-7}$M norepinephrine bitartrate (Sigma-Aldrich Corp, St Louis, Mo). An endothelium-dependent vasodilator, acetylcholine chloride (concentration range, $10^{-8}$M to $10^{-5}$M) (Sigma-Aldrich Corp), or an endothelium-independent vasodilator, nitroglycerine (concentration range, $5\times10^{-9}$M to $5\times10^{-6}$M) (American Regent Laboratories, Shirley, NY), was applied cumulatively thereafter. After each series of agent additions, the ring preparations were washed with Krebs-Ringer $HCO_3$ solution and allowed to reequilibrate for at least 30 minutes.

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## RESULTS

### ALTERATIONS IN ACETYLCHOLINE- AND NITROGLYCERINE-INDUCED VASCULAR RELAXATION

The data presented in **Table 1** indicate that the aortic ring weights and norepinephrine-induced contractions were not altered in any of the groups. As demonstrated by **Figure 1**, the acetylcholine-induced maximal relaxation was significantly depressed compared with sham-operation animals at acetylcholine concentrations of $5\times10^{-7}$M to $5\times10^{-5}$M at 20 hours after trauma-hemorrhage and resuscitation ($P\leq.004$). In hemorrhage-flutamide animals, however, the depressed acetylcholine-induced relaxation was maintained almost at sham-operation levels. There was no significant difference in nitroglycerine-induced relaxation among various groups at any nitroglycerine concentration in this study (**Figure 2**).

### ALTERATIONS IN ORGAN BLOOD FLOW

As shown in **Figure 3**, blood flow in the liver (A), small intestine (B), and kidneys (C) at 20 hours after hemorrhage were significantly lower than in the sham-operation group ($P<.05$). In contrast, the decrease in blood flow in those organs was not evident in hemorrhage-flutamide animals.

### MEASUREMENT OF ORGAN BLOOD FLOW

At 20 hours after resuscitation or sham operation, the animals were anesthetized with pentobarbital sodium and an additional polyethylene-50 catheter was inserted into the left ventricle via the right carotid artery. The position of the catheter tip in the left ventricle was confirmed by means of the left ventricular pressure, and its exact position in the left ventricle was verified at the autopsy. Organ blood flow was determined by using a radioactive microsphere technique, as previously described by us.[15] Briefly, strontium 85–labeled microspheres (DuPont/NEN, Boston, Mass) were suspended in 10% dextran containing 0.01% polysorbate 80 (Tween 80, Sigma-Aldrich Corp) to prevent aggregation. The microspheres were dispersed with a vortex shaker for 3 minutes before infusion. A 0.2- to 0.25-mL suspension of microspheres with an activity of approximately 4 μCi (approximately 500 000 cpm) was injected manually into the left ventricle in each rat via the left ventricle catheter for 20 seconds at a constant rate. The reference blood sample was withdrawn from the femoral arterial catheter into a 3-mL syringe beginning 20 seconds before microsphere infusion and continuing for an additional 60 seconds at a rate of 0.7 mL/min using a pump (Harvard Apparatus, Holliston, Mass). Isotonic sodium chloride solution was infused manually at the rate of 0.7 mL/min immediately after microsphere infusion to replace the volume of blood loss. The rat was then killed using an overdose of isoflurane inhalation. Various organs were then harvested, weighed, and placed in 1 or more test tubes, and organ radioactivity was counted using an automatic γ-counter (1470 Wizard; Wallac, Gaithersburg, Md). The reference blood sample was transferred from a syringe into a test tube for radioactivity measurement. The remaining microspheres, which were left in the syringe after injection, were also counted. Organ blood flow was calculated according to following equation[15]:

$$\text{Organ Blood Flow} = [(RBF \times C_t)/C_r] \times 1/100,$$

where *RBF* is the reference blood sample withdrawal rate (0.7 mL/min), $C_t$ is counts per minutes per gram of tissue, and $C_r$ is counts per minute in reference blood sample.

### DETERMINATION OF OXYGEN DELIVERY AND CONSUMPTION

At 20 hours after resuscitation, a 3.5F umbilical vessel catheter (Sherwood, St Louis, Mo) was placed in the hepatic vein though the jugular vein for hepatic venous blood sampling. The exact position of the hepatic venous catheter tip was confirmed at the autopsy. A 1-mL heparinized syringe with a 22-gauge needle was inserted into the portal vein, secured to prevent blood leakage and used for portal venous blood sampling. The same technique was used for renal blood sampling. Blood samples (approximately 0.15 mL each) were collected immediately after microsphere infusion from the femoral artery and vein and hepatic and portal veins simultaneously with the aid of an assistant to minimize the effects of multiple-site blood sampling. To avoid the oversampling that may cause adverse effects on systemic hemodynamics, the renal venous blood samples were taken from additional experimental groups. Those additional groups were used for obtaining the ratio of blood oxygen content and blood-gas measurements between the renal and systemic venous blood. Such ratios were used to obtain a more accurate assessment of oxygen content and blood-gas measurements in the renal venous blood samples. The blood-gas measurements were determined using a blood-gas machine (ABL5; Radiometer, Copenhagen, Denmark). Total hemoglobin and oxygen content were determined using a hemoximeter (OSM; Radiometer). Oxygen delivery was calculated by multiplying arterial oxygen content by blood flow. Oxygen consumption was determined by calculating the difference in oxygen content between arterial and venous blood, multiplied by blood flow. The oxygen consumption in the small intestine was calculated by the difference in oxygen content between the systemic arterial and portal venous blood. Hepatic oxygen consumption was determined by calculating the difference in oxygen content between the hepatic inflow (ie, hepatic arterial and portal venous blood) and hepatic outflow blood (hepatic venous blood). The calculation of renal oxygen consumption was performed by determining the difference in oxygen content between systemic arterial and renal venous blood.

### STATISTICAL ANALYSIS

All data are presented as mean±SE. One-way analysis of variance (ANOVA) and Tukey test were used for the comparison between hemorrhage-vehicle, hemorrhage-flutamide, and sham-operation groups at 20 hours after resuscitation, and the differences were considered significant at $P \leq .05$.

## ALTERATIONS IN TOTAL HEMOGLOBIN AND OXYGEN CONTENT AND OXYGEN DELIVERY AND CONSUMPTION

As shown in **Table 2**, the total hemoglobin and systemic arterial oxygen content in the hemorrhage-vehicle and hemorrhage-flutamide groups decreased similarly (by approximately 60%) at 20 hours after resuscitation compared with the sham-operation group. Table 2 also shows that oxygen delivery and consumption in the liver, small intestine, and kidneys were significantly decreased after hemorrhage. However, administration of flutamide significantly attenuated the decreased oxygen consumption and delivery to those organs compared with the hemorrhage-vehicle group (Table 2).

## COMMENT

Endothelial cells cover the entire vasculature and form the single cell layer of the capillaries, which play a key role in control of vascular smooth muscle tone. Furthermore, endothelial cell damage has now been implicated in several vascular diseases, such as hypoxic pulmonary hypertension, thrombosis, acute renal failure, atherosclerosis, arterial spasm, anaphylactic disorders, and circulatory shock.[16] Studies from our laboratory have shown that endothelial cell dysfunction (ie, decreased acetylcholine-

**Table 1. Weight of Aortic Rings and Initial Contraction Induced by Norepinephrine***

| Groups | Ring Weight, mg | Initial Contraction, mg |
|---|---|---|
| Sham-operation | 2.3 ± 0.1 | 1052 ± 57 |
| Hemorrhage-vehicle | 2.2 ± 0.2 | 1034 ± 72 |
| Hemorrhage-flutamide | 2.2 ± 0.2 | 1023 ± 33 |

*Data are given as mean ± SE (n = 6). Groups are described in the "Animal Model of Trauma-Hemorrhage" and "Experimental Protocol" subsections of the "Materials and Methods" section.

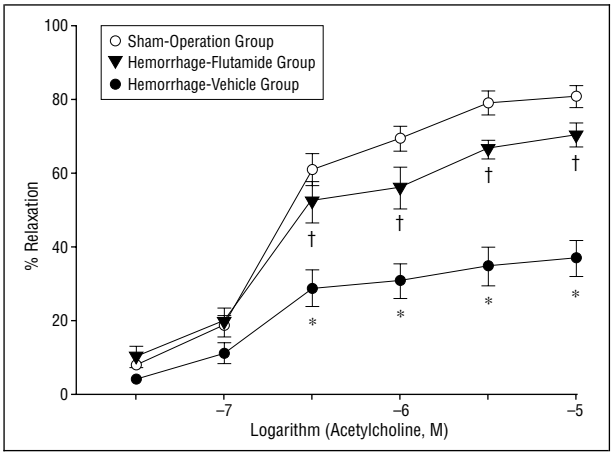


**Figure 1.** Alterations in acetylcholine-induced vascular relaxation at 20 hours after sham operation, hemorrhage with vehicle administration, and hemorrhage with flutamide treatment. Data are expressed as mean ± SE and compared by 1-way analysis of variance and Tukey test. Asterisk indicates $P<.05$ vs sham-operation group; dagger, $P<.05$ vs hemorrhage-vehicle group.

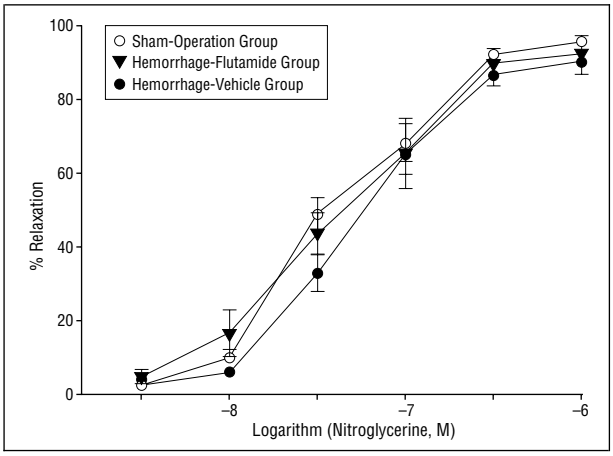


**Figure 2.** Alterations in nitroglycerine-induced vascular relaxation at 20 hours after sham operation, hemorrhage with vehicle administration, and hemorrhage with flutamide treatment. Data are expressed as mean ± SE and compared by 1-way analysis of variance and Tukey test. Differences between groups were not significant.

induced vascular relaxation) occurs very early after trauma-hemorrhage and persists despite fluid resuscitation.[2] Recently, it has been shown that flutamide, a testosterone receptor antagonist, restores the depressed immune function in male mice after trauma-hemorrhage.[5,8] Flutamide treatment also prevents vasoconstriction by testosterone[17,18] and improves cardiac, hepatic, and adrenal

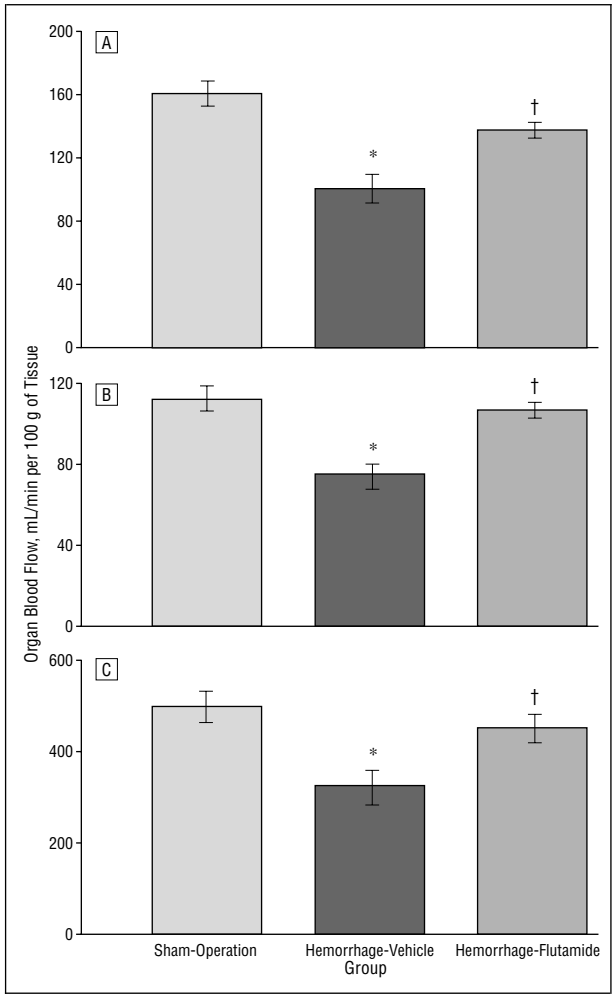


**Figure 3.** Alterations in blood flow in liver (A), small intestine (B), and kidneys (C) at 20 hours after sham operation, hemorrhage with vehicle administration, and hemorrhage with flutamide treatment. Data are expressed as mean ± SE and compared by 1-way analysis of variance and Tukey test. Asterisk indicates $P<.05$ vs sham-operation group; dagger, $P<.05$ vs hemorrhage-vehicle group.

function in male rats after trauma-hemorrhage.[9,10] We therefore hypothesized that testosterone receptor blockade by flutamide after trauma-hemorrhage and resuscitation would also improve the depressed endothelial cell function and consequently increase organ blood flow, tissue oxygen delivery, and oxygen use. In view of this, we conducted the present study to determine the effect of flutamide on endothelial cell function and oxygen use in male animals at 20 hours after trauma-hemorrhage and resuscitation.

The results of the study indicate that although the aortic ring weight and norepinephrine-induced contraction were not altered in any of the groups of animals at 20 hours after trauma-hemorrhage and resuscitation, acetylcholine-induced relaxation (endothelium-dependent) was significantly depressed. However, in the hemorrhage-flutamide group, depressed acetylcholine-induced relaxation was significantly improved. In contrast, there was no significant difference in nitroglycerine-induced relaxation (endothelium-independent) among the various groups at any nitroglycerine concentration in this study. We have shown that flutamide treatment after hemorrhage and resuscita-

**Table 2. Alterations in Hemoglobin and Oxygen Content and Oxygen Delivery, Consumption, and Extraction***

| | Groups | | |
|---|---|---|---|
| | Sham-Operation | Hemorrhage-Vehicle | Hemorrhage-Flutamide |
| Total Hb, g/100 mL | 15.03 ± 0.17 | 6.07 ± 0.33† | 5.97 ± 0.35† |
| Systemic oxygen content, % | 19.80 ± 0.34 | 8.05 ± 0.47† | 7.75 ± 0.55† |
| Oxygen delivery, mL/min per 100 g | | | |
| Liver | 19.62 ± 1.96 | 5.11 ± 0.44† | 7.13 ± 0.37†‡ |
| Small intestine | 22.31 ± 1.35 | 6.02 ± 0.54† | 9.79 ± 0.68†‡ |
| Kidney | 105.0 ± 8.65 | 26.83 ± 2.62† | 41.67 ± 2.76†‡ |
| Oxygen consumption, mL/min per 100 g | | | |
| Liver | 14.48 ± 1.72 | 2.48 ± 0.10† | 3.70 ± 0.30†‡ |
| Small intestine | 10.03 ± 1.00 | 2.29 ± 0.26† | 4.88 ± 0.51†‡ |
| Kidney | 33.83 ± 7.23 | 8.04 ± 1.24† | 12.48 ± 1.24†‡ |

*Data are given as mean ± SE (n = 6). Data were measured at 20 hours after resuscitation and compared by 1-way analysis of variance and Tukey test. Groups are described in the "Animal Model of Trauma-Hemorrhage" and "Experimental Protocol" subsections of the "Materials and Methods" section. Hb indicates hemoglobin.

†$P<.05$ vs sham operation group.

‡$P<.05$ vs hemorrhage-vehicle group.

tion improved the depressed endothelial cell function. This improvement does not seem to be due to changes in oxygen content, since the total hemoglobin and systemic oxygen content decreased by the same percentage (60%) in the hemorrhage-vehicle and hemorrhage-flutamide groups. Thus, our data clearly demonstrate that testosterone receptor blockade with flutamide attenuates the decreased endothelial cell function in male animals after trauma-hemorrhage and resuscitation.

It has been shown that EDNO, a potent endogenous vasodilator, is identical to endothelium-derived relaxing factor in its pharmacological and chemical properties.[4,19] A number of studies have demonstrated a decreased release of EDNO from vascular endothelial cells[20] and up-regulation of the inducible isoform of nitric oxide synthase[21] after hemorrhagic shock. In contrast, it has been reported that administration of EDNO donors or L-arginine improves vascular endothelial function, restores the depressed cardiac output and organ blood flow, and decreases plasma levels of interleukin 6 after various circulatory conditions.[22-27] The present results also show that flutamide treatment attenuated the depressed endothelial cell function after hemorrhagic shock and restored blood flow and oxygen delivery and consumption in various organs, such as the liver, small intestine, and kidneys, under such conditions. Therefore, the vascular endothelial cell dysfunction after hemorrhagic shock may contribute to further alterations in tissue perfusion and organ or cellular function. In this regard, our present finding that flutamide attenuates the decreased endothelial cell function in male animals after trauma-hemorrhage and resuscitation may explain why flutamide can also restore depressed immune function and improve cardiac, hepatic, and adrenal function in male rats after trauma-hemorrhage.[5,8-10] Similarly, our previous studies have shown that administration of pentoxifylline or adenosine triphosphate–magnesium chloride after trauma-hemorrhage and resuscitation significantly improves endothelial function and has many other beneficial effects on experimental animals.[1,3,28-39]

The precise mechanism responsible for the beneficial effects of flutamide on endothelial function remains unknown. Previous studies have shown that plasma testosterone levels are not significantly altered after trauma-hemorrhage and resuscitation.[7] Therefore, the effect of flutamide on endothelial function may not be mediated via alterations in levels of testosterone, but may be due to decreased testosterone receptor activity and its signal transduction mechanisms or enhanced specific cellular effects of other sex steroids such as estradiol. Recent studies have indicated that testosterone increases vascular smooth muscle thromboxane $A_2$ receptor levels in the aorta.[18] It has also been shown that testosterone treatment enhances vasoconstriction in response to thromboxane $A_2$ in coronary circulation,[18,40] which can be blocked by administration of flutamide.[40] In addition, it has been shown that testosterone treatment inhibits the synthesis of prostacyclin by rat aortic smooth muscle cells in culture.[41] It is therefore possible that the inhibition of thromboxane $A_2$ and/or the enhancement of prostacyclin is the mechanism by which flutamide increases organ perfusion and subsequently improves endothelial function. Moreover, it has been shown that flutamide is capable of inducing estrogen receptors.[42] However, it remains unknown whether the beneficial effects of flutamide observed after hemorrhagic shock are the result of up-regulation of estrogen receptors.

Studies have also indicated that up-regulation of tumor necrosis factor α (TNF-α) production may be responsible for vascular endothelial cell dysfunction.[43-45] Our previous work has demonstrated that administration of TNF-α in vivo and in vitro significantly depresses endothelium-dependent vascular relaxation.[46] Because circulating levels of TNF-α are elevated after trauma-hemorrhage and resuscitation,[47] and because TNF-α produces vascular endothelial cell dysfunction,[43-46] the up-regulation of TNF-α production appears to be responsible for the decreased EDNO levels after hemorrhage and resuscitation. Recently, it has been shown that flutamide restores the depressed immune function and down-regulates TNF-α production in male mice after trauma-hemorrhage.[5,8]

Therefore, this down-regulatory effect of flutamide on TNF-α production may also be responsible for the improved endothelial function.

## CONCLUSIONS

The results indicate that administration of flutamide during resuscitation after trauma and hemorrhagic shock significantly attenuated the depressed endothelial function. Furthermore, blood flow and oxygen delivery to the liver, small intestine, and kidneys were significantly elevated by flutamide treatment. In addition, oxygen consumption also significantly increased in all the tested organs compared with those of the vehicle-treated animals. These results, taken together, suggest that flutamide is a useful adjunct for improving endothelial cell function in male animals after trauma-hemorrhagic shock and resuscitation.

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