SALUTARY EFFECTS OF ANDROSTENEDIOL ON CARDIAC FUNCTION AND
SPLANCHNIC PERFUSION FOLLOWING TRAUMA-HEMORRHAGE

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Running head: Androstenediol after trauma-hemorrhage

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Abstract:

Recent studies have shown that dehydroepiandrosterone (DHEA) administration following trauma-hemorrhage (T-H) improves cardiovascular function and decreases cytokine production in male animals. Although androstenediol, one of the metabolites of DHEA, is reported to have estrogen-like activity, it remains unknown whether androstenediol per se has any salutary effects on cytokines and cardiovascular function following T-H. To examine this effect, male Sprague-Dawley rats underwent laparotomy and were bled to and maintained at a mean arterial blood pressure of 35-40mmHg for ~90min. The animals were resuscitated with 4 times the volume of maximal bleedout volume in the form of Ringer’s lactate. Androstenediol (1mg/kg BW intravenously) or vehicle were administered at the end of resuscitation. Twenty-four hrs after resuscitation, cardiac function and organ blood flow were measured by using Sr^{85}-microspheres. Circulating levels of nitrate/nitrite and IL-6 were also determined. Cardiovascular function and organ blood flow were significantly depressed after T-H. However, these parameters were restored by androstenediol treatment. The elevated plasma IL-6 levels following T-H were also lowered by androstenediol treatment. In contrast, plasma levels of nitrate/nitrite were the highest in the androstenediol-treated T-H animals. Since androstenediol administration following T-H decreases cytokine production and improves cardiovascular function, this agent appears to be a novel and useful adjunct for restoring the depressed cardiovascular function and for cytokine production in male following adverse circulatory conditions.

Key Words: Hemorrhagic shock, nitric oxide, NOS, adiol, 5-androstene-3β 17β-diol.
INTRODUCTION

Hemorrhagic shock results in a rapid decrease in cardiac output and organ blood flow. Furthermore, studies have shown that intestinal perfusion remains depressed even after the recovery of cardiac output by fluid resuscitation (29). This splanchnic hypoperfusion after hemorrhagic shock also activates the inflammatory cascade. The depressed cardiovascular function and excess production of inflammatory mediators play an important role in the development of multiple organ failure following hemorrhagic shock (20).

Previous work from our laboratory has shown that left ventricular performance, cardiac output and organ blood flow in the liver, small intestine and kidney decreased significantly following trauma-hemorrhage (3, 4, 25) and plasma levels of IL-6 were elevated under those conditions in male animals (1, 25). However, clinical and laboratory studies have shown that gender differences exist in the organ and immune function following hemorrhagic shock (13, 23, 31). In this regard, our previous studies have shown that male sex steroids have deleterious effects and female sex steroids produce beneficial effects on cardiovascular functions following trauma-hemorrhage (5, 16, 31). In particular, studies have shown that administration of a single dose of estrogen after trauma and hemorrhagic shock, improved cardiovascular and hepatocellular functions (18, 25). In addition, dehydroepiandrosterone (DHEA) is the most abundant steroid hormone in plasma and is an intermediate in the pathway for the synthesis of testosterone and estrogen. DHEA treatment following trauma-hemorrhage has been reported to improve organ functions and normalize cytokine production after circulatory stress (9, 15).

Androstenediol (adiol or 5-androstene-3β, 17β-diol) is one of the metabolites of DHEA. Androstenediol has been reported to have greater protective effects than DHEA against lethal bacterial infections and endotoxin shock (7). Furthermore, androstenediol has also been reported
to produce protective effects following ionizing radiation in mice (21, 34). These studies have shown a significant improvement in survival of mice treated with androstenediol after whole-body ionizing irradiation with gamma rays. The estrogen-like activities of androstenediol are observed at physiological concentration in breast cancer cells. Androstenediol causes an increase in estrogen receptor-dependent beta-galactosidase activity in yeast (26). Furthermore, Schmidt et al. reported the conversion of DHEA to downstream steroid hormones in macrophages. These investigators suggested that the conversion of DHEA leads to an increase of downstream effector hormones in macrophages which may play an important role in local immunomodulation (30).

Previous studies indicate that vascular endothelial cell dysfunction occurs early after trauma-hemorrhage and may contribute to further alterations in tissue perfusion and cellular function (33). Angele et al. demonstrated that L-arginine (i.e., the substrate for constitutive nitric oxide synthase, cNOS) restored the depressed cardiac output and organ blood flow and decreased plasma levels of IL-6 (2). Recently, androstenediol has been reported to increase nitric oxide synthase (NOS) activity in the vagina of ovariectomized rabbits (32).

Although androstenediol has been reported to produce the above-mentioned salutary effects, it remains unknown whether this metabolite or its parent compound is responsible for producing the salutary effects following trauma-hemorrhage. We hypothesized that androstenediol itself is a useful adjunct for improving the depressed cardiovascular function and cytokine production following trauma-hemorrhage. To test this hypothesis, we examined whether administration of androstenediol has any salutary effects on cardiovascular function and cytokine production in males following trauma-hemorrhage. In addition, plasma levels of nitrate/nitrite were determined to evaluate systemic nitric oxide production.
MATERIALS AND METHODS

Animals: Adult male (275-325g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) 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 approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Experimental procedures: A non-heparinized model of trauma-hemorrhage in the rat, as previously described, was used in this study (31). Briefly, male Sprague-Dawley rats (275-325g) were fasted overnight before the experiment, but allowed water ad libitum. The rats were anesthetized by isoflurane (Attane, Minrad Inc., Bethlehem, PA) inhalation before the induction of soft tissue trauma (i.e., 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, Sparks, MD]. The animals were then restrained in a supine position and the wounds were bathed with 1% lidocaine (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 pressure (MAP) of 35-40 mmHg within 10 min. The time at which the animals could no longer maintain a MAP of 35-40 mmHg without infusing some fluid was defined as maximum bleed-out volume. The rats were maintained at this MAP until 40% of the shed blood was returned in the form of Ringer’s lactate. The animals were then resuscitated with 4 times the volume of shed blood with Ringer’s lactate over 60 min.

Following resuscitation, the catheters were removed, the vessels were ligated and skin incisions closed with sutures. Sham-operated animals underwent the same groin dissection, which included the ligation of the femoral artery and catheters were placed in the femoral vein to administer agents; however, neither trauma-hemorrhage nor resuscitation was carried out. The
animals were returned to their cages and were allowed food and water *ad libitum* until sacrifice. The animals were sacrificed at 24h after the end of resuscitation.

In the treatment group, 1mg/kg of body weight androstenediol (Steraloids, Inc., Newport, RI) was administered intravenously at the end of the resuscitation. In the vehicle-treated group (control-group) rats received the same volume of vehicle (Intralipid, 1mL/kg BW, Sigma, St. Louis, MO).

**Measurement of plasma levels of nitrate/nitrite:** Blood samples were obtained and placed in microcentrifuge tubes at 24h after the end of resuscitation or sham operation and plasma separated by the centrifugation, immediately frozen and stored at –80°C until assayed. The production of systemic nitric oxide was evaluated by measuring plasma nitrate/nitrite levels using a commercially available colorimetric assay kit (Cayman Chemical, Ann Arbor, MI).

**Measurement of plasma levels of IL-6:** Plasma levels of IL-6 were determined using ELISA kits (Pharmingen, San Diego, CA) according to the manufacturer’s instructions.

**Measurement of heart performance, cardiac output and organ blood flow:** In an additional set of animals, heart performance was evaluated at 24h after resuscitation or sham operation, as previously described (4). Briefly, rats were anesthetized with pentobarbital sodium (~30 mg/kg body wt) and a PE-50 catheter was inserted into the left ventricle via right carotid artery. The maximal rates of left ventricular pressure increase (+dP/dt$_{max}$) and decrease (–dP/dt$_{max}$) were determined with a heart performance analyzer.

Cardiac output and organ blood flow in the small intestine, liver, spleen, kidney, pancreas, adrenal grand, brain, heart and lung were determined by using a radioactive microsphere technique as previously described (4). Briefly, strontium$^{85}$-labeled microspheres (~500,000 cpm; DuPont NEN, Boston, MA) were injected manually into the left ventricle. The reference blood
sample was withdrawn from the femoral arterial catheter for 60sec at a rate of 0.7ml/min. Isotonic sodium chloride solution was infused at the same rate to replace the volume of blood lost. The animals were sacrificed and abdominal organs were then harvested and weighed. The radioactivity in the organs and reference blood sample were counted with an automatic gamma counter (1470 Wizard; Wallac, Gaithersburg, MD). Cardiac output and organ blood flow were calculated according to the following equations: cardiac output = [(RBF×CT)/Cr] ×100/BW, organ blood flow = [(RBF×Ct)/Cr] ×100; where RBF is the reference blood sample withdrawal rate (0.7ml/min); CT is counts per minute of total injected dose; Cr is counts per minute in the reference blood sample; BW is body weight (g) and Ct is counts per minute per gram of tissue.

**Statistical Analysis:** Data are presented as means ± SEM. Statistical differences between groups were determined by one way analysis of variance (ANOVA) followed by Fisher’s LSD as a post hoc test. The differences were considered significant if p<0.05.
RESULTS:

Alterations in hemodynamic parameters. There was no significant difference in the hemodynamic parameters between androstenediol treated and non-treated sham animals. As shown in Fig.1, cardiac output significantly decreased in vehicle treated rats following trauma-hemorrhage. Androstenediol treatment following trauma-hemorrhage increased cardiac output and the values were similar to those observed in shams (Fig. 1, panel A). Furthermore, the positive and negative dP/dt_{\text{max}} shown in panel B and C, were also significantly depressed at 24h after trauma-hemorrhage. Treatment of rats with androstenediol prevented the decrease in positive dP/dt_{\text{max}} and negative dP/dt_{\text{max}}, however, negative dP/dt_{\text{max}} was still significantly lower than shams (Fig. 1, panel B and C).

Mean blood pressure also decreased significantly in rats following trauma-hemorrhage (Fig. 2, panel A). Treatment of rats with androstenediol following trauma-hemorrhage did not significantly improve mean blood pressure compared to vehicle-treated trauma-hemorrhaged animals. Furthermore, no significant difference in heart rate was observed among the different group of animals (Fig. 2, panel B).

Alteration in organ blood flow. There was no difference in organ blood flow between androstenediol- and vehicle-treated sham groups (Table 1). Trauma-hemorrhage markedly decreased blood flow in the small intestine, liver, spleen, kidney, pancreas and adrenal grand of the vehicle-treated rats. Androstenediol treatment following trauma-hemorrhage prevented the decrease in blood flow in the small intestine and liver. Furthermore, although androstenediol treatment following trauma-hemorrhage increased blood flow in the spleen, kidney and pancreas; however, it was not restored to normal in these organs (Table 1). Androstenediol treatment did not improve blood flow in the adrenal gland. However, blood flow in the brain increased
significantly in both the vehicle- and androstenediol-treated rats following trauma-hemorrhage. There was no significant difference in blood flow in the heart and lung among the different group of animals (Table 1).

**Plasma levels of nitrate/nitrite.** Plasma levels of nitrate/nitrite increased significantly at 24h after trauma-hemorrhage. Androstenediol treatment further increased nitrate/nitrite levels in rats following trauma-hemorrhage (Fig. 3) but it did not influence the nitrate/nitrite levels in the sham rats.

**Plasma IL-6 levels.** Plasma IL-6 levels were significantly elevated following trauma-hemorrhage compared to sham in the vehicle treatment group. However, androstenediol treatment markedly decreased the levels of IL-6 following trauma-hemorrhage (Fig. 4).
DISCUSSION:

The present study showed that administration of androstenediol following trauma-hemorrhage improved left ventricular performance, cardiac output and organ blood in the small intestine, liver, spleen and kidney. This improvement of cardiovascular function was associated with increase of plasma nitrate/nitrite levels. Furthermore, elevated circulating levels of IL-6 following trauma-hemorrhage were significantly reduced by androstenediol treatment.

Gender dimorphism was observed in the cardiac function and tissue perfusion following trauma-hemorrhage. Previous studies from our laboratory have shown that proestrus female rats, which have high circulating levels of estrogen and progesterone, maintain cardiac output and higher splanchnic perfusion compared to male rats after trauma-hemorrhage (3). Our previous studies have also shown that administration of a single dose of estrogen after trauma-hemorrhage improved cardiovascular and hepatocellular functions (18, 25). Alternatively, testosterone receptor blockade following trauma-hemorrhage by flutamide in normal male rats restored the depressed cardiac function, blood flow, oxygen delivery and consumption in all organs tested (5). Additionally, Jarrar et al. demonstrated that DHEA administration following trauma-hemorrhage restored the depressed cardiac and hepatocellular functions in male rats (15). DHEA is the most abundant steroid hormone in plasma and is an intermediate in the pathway for the synthesis of testosterone and estrogen. The salutary effects of DHEA were not observed if the estrogen receptor antagonist ICI 182780 was administered with DHEA. However, plasma levels of 17β-estradiol and testosterone were not significantly altered in animals receiving DHEA (15). Thus, the above studies collectively suggest that gender dimorphism does exist in the cardiac function and tissue perfusion following trauma-hemorrhage.
In this study, we examined the effect of androstenediol on cardiac functions following trauma-hemorrhage since androstenediol is one of the metabolites of DHEA. This agent has been reported to have protective effects against lethal bacterial infections and endotoxin shock. The results presented in this manuscript clearly suggest that treatment of rats with single dose of androstenediol (1mg/kg iv) following trauma-hemorrhage significantly improved cardiac function, prevented the elevation in IL-6 and further increased nitrate/nitrite levels under those conditions. Whether androstenediol increases eNOS and whether this increase is responsible for the elevated nitrate/nitrite levels remains to be determined. It should be pointed out, however, that the dose of androstenediol that was used in this study did not restore blood flow in some organs. Whether androstenediol at higher doses or repeat treatment would restore blood flow to those organs remains unknown. We did not use a higher dose of androstenediol or repeat treatment since a number of potential risk factors associated with higher dose and long-term androstenediol use have been reported (34). Long-term oral supplementation with androstenediol appears to adversely affect blood lipids (i.e., decrease of HDL-C) and increase the risk of pancreatic cancer or prostate cancer (8). Nonetheless, our findings suggest that administration of a single dose (1mg/kg iv) of androstenediol as an adjunct to resuscitation following trauma-hemorrhage is effective for restoring and maintaining cardiovascular function and cytokine production following trauma-hemorrhage.

The precise mechanism responsible for producing the beneficial effects of androstenediol on cardiac function and organ blood flow is not known. Nonetheless, several studies have shown that hemorrhagic shock induces a cascade of pro-inflammatory cytokines which are associated with immunosuppression (12), hemodynamic depression and organ dysfunction (6). In this regard, studies by Mizushima et al. have shown that administration of estrogen following
trauma-hemorrhage reduced circulating levels of IL-6 (25). Knoferl et al. also reported that administration of DHEA following trauma-hemorrhage significantly reduced the elevated circulating IL-6 levels, suggesting an attenuation of the inflammatory response under those conditions (17). Furthermore, Kuebler et al. have demonstrated that progesterone treatment following trauma-hemorrhage also significantly reduction plasma IL-6 levels (19). Consistent with these findings, the present findings also indicate that the elevated circulating levels of IL-6 following trauma-hemorrhage were significantly reduced by androstenediol treatment. Since elevated levels of IL-6 have been correlated with cardiac dysfunction in many injury conditions (14, 22) as well as following trauma-hemorrhage (17, 19, 25), it is likely that attenuation of IL-6 production could be a factor responsible for androstenediol salutary effect in preventing cardiac dysfunction following trauma-hemorrhage.

In addition to IL-6, we also found an increase in nitrate/nitrite production following trauma hemorrhage and treatment with androstenediol further elevated systemic nitrate/nitrite levels. Eckhoff et al. have shown that estrogen administration significantly reduced hepatic injury after ischemia-reperfusion to the liver and this effect was associated with increased serum nitric oxide metabolites (11). Our result showed further induction of nitrate/nitrite in the androstenediol-treated rats. Although we have not determined whether the increase in nitrate/nitrite levels is due to inducible or constitutive nitric oxide synthase (NOS), it is likely that the increase in NOS, especially eNOS activity in androstenediol-treated animals may contribute to the improvement of cardiac output and splanchnic perfusion following trauma-hemorrhage.

Several studies have shown salutary effects of androstenediol treatment on immune functions after injury such as endotoxin shock, sepsis, and radiation injury (7, 21, 34). Ben-
Nathan et al. reported that androstenediol had greater protective effects on survival rate than DHEA against lethal bacterial infections and endotoxin shock (7). In contrast, Padgett et al. have shown that androstenediol had little influence on the secretion of LPS-induced pro-inflammatory cytokine (IL-6, TNF-α and IL-1) from macrophages in vitro (28). Whether the difference in the results of Ben-Nathan et al. (7) and Padgett et al. (28) is due to in vivo versus in vitro use of DHEA remains unclear.

Although the precise mechanism responsible for the beneficial effects of androstenediol on cytokine production remains unknown, it is possible that androstenediol mediates its action via the estrogen receptor(s). In this regard, several studies have shown that estrogens have protective effects on the cardiovascular system via the rapid nongenomic and long-term genomic mechanisms (10, 24). Furthermore, studies have shown that administration of a single dose of estrogen after trauma-hemorrhage improved cardiovascular, hepatocellular functions and splanchnic perfusion (18, 25). Recent study from our laboratory has also shown that estrogen attenuated the production of IL-6 by Kupffer cells from both sham and trauma-hemorrhage animals in a dose-dependent manner (35). Since Kupffer cells have been reported to be the major source of IL-6 production following trauma-hemorrhage (27), it is likely that similar to estrogen, androstenediol treatment down-regulates the production of IL-6 from Kupffer cells. However, further studies are needed to determine such a paradigm.

In summary, our study indicates that androstenediol administration following trauma-hemorrhage improves cardiac function, organ blood flow and decreases cytokine production. This improvement of cardiovascular function may be associated with attenuation in IL-6 levels and an increase in plasma nitrite/nitrate levels. Since androstenediol produced the above-mentioned salutary effect, these results lead us to conclude that androstenediol appears to be a
useful adjunct for restoring the depressed cardiovascular functions and attenuating cytokine production following trauma-hemorrhage.

Acknowledgement

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References


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Organ blood flow measurements were performed at 24h after sham operation or trauma-hemorrhage. Data are mean ± SEM of 6-7 animals per group. For further details, see “Materials and Methods”. *p<0.05 vs. other groups, #p<0.05 vs. sham.
Figure Legends:

**Figure 1.** Effects of androstenediol on [A] cardiac output and maximal rate of pressure [B] increase (+dP/dT\text{max}) and [C] decrease (-dP/dT\text{max}) in the left ventricle at 24h after sham operation or trauma-hemorrhage. Data are presented as mean ± SEM (n=6 animals/group).

*p*<0.05 vs. other groups, #p*<0.05 vs. sham-Adiol. Adiol, androstenediol. T-H, trauma-hemorrhage.

**Figure 2.** Effects of androstenediol on [A] mean blood pressure and [B] heart rate at 24h after sham operation or trauma-hemorrhage. Data are presented as mean ± SEM (n=6 animals/group):

*p*<0.05 vs. sham. Adiol, androstenediol. T-H, trauma-hemorrhage.

**Figure 3.** Plasma levels of nitrate/nitrite at 24h after sham operation or trauma-hemorrhage. Data are presented as mean ± SEM (n=6 animals/group). *p*<0.05 vs. other groups, #p*<0.05 vs. sham. Adiol: androstenediol. T-H, trauma-hemorrhage.

**Figure 4.** Effects of androstenediol on plasma IL-6 levels at 24h after sham operation or trauma-hemorrhage. Data are mean ± SEM (n = 6 animals/group). *p*<0.05 vs. other groups. Adiol, androstenediol. T-H, trauma-hemorrhage.
Figure 1
Figure 2
Figure 3
Figure 4