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# Aromatase Blockade Is Associated With Increased Mortality in Acute Illness in Male Mice

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**Context:** The increase in circulating estrogen levels with acute illness in humans is accompanied by increased aromatase expression in adipose tissue and increased peripheral aromatization of estrogens to androgens. Animal studies indicate that estrogen may be beneficial in acute illness.

**Objective:** We hypothesized that blockade of aromatase in acute illness would decrease survival.

**Design:** Prospective sham controlled.

**Setting:** Maine Medical Center Research Institute animal facility.

**Animals:** Six- to 8-week-old male black 6 mice.

**Intervention:** Mice underwent cecal ligation and puncture (CLP) to induce acute illness and were administered letrozole to block aromatase or saline. Mice undergoing sham surgery with or without letrozole served as controls. Adipose and cardiovascular tissue was harvested for preliminary evaluation of aromatase expression.

**Main outcome measurements:** Survival was the main outcome measurement. Evidence for aromatase expression in tissue samples was assessed using western blot and/or immunohistochemistry.

**Results:** With aromatase blockade, survival in CLP mice was decreased ($P = 0.04$). The presence of aromatase in adipose tissue was observed by western blot in CLP but not control mice. Similarly, the presence of aromatase was observed in cardiac tissue of CLP but not in control mice.

**Conclusions:** The decreased survival during sepsis with aromatase blockade suggests that this response to acute illness may be important both physiologically and clinically. The preliminary observation of aromatase expression in adipose and cardiovascular tissue during acute illness in this mouse model indicates that this model has parallels to human physiology and may be useful for further studying the aromatase response to acute illness.



**Freeform/Key Words:** acute illness, aromatase blockade, survival

Estrogen exerts various functions beyond its traditional roles in sexual development and reproduction, including influences on cardiovascular function [1–3]. Animal studies suggest that during acute illness estrogen has beneficial effects on the immune system and on hepatic,

Abbreviations: CLP, cecal ligation and puncture; SS, sham surgery.

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renal, pulmonary, cardiac, and vascular function [4–11] and possibly improved survival [12, 13]. At the onset of severe illness in men and women, circulating estrogen levels rise [14–17]. Estrogens are synthesized from androgens via the P450 aromatase enzyme [2]. In humans, the rise in serum estrogen levels during acute illness is accompanied by enhanced peripheral aromatase activity and increased aromatase expression from adipose tissue [18]. In addition to the ovaries and adipose tissue, aromatase expression has been reported in vascular tissue and the myocardium among other tissues [1, 2, 19–23].

In this study we used a mouse model of acute illness to further explore the aromatase response to illness and its possible adaptive significance. Our primary goal was to determine if blockade of aromatase would decrease survival. Such an effect would provide impetus for additional studies of aromatase and estrogen physiology and effect during nonendocrine illnesses. A secondary goal was to confirm that in mice, similar to in humans [18], increased aromatase expression in adipose tissue accompanies acute illness. Finally, we obtained preliminary data to investigate whether an increase in aromatase expression may also occur in myocardial and arterial tissue that might support cardiovascular function during illness. Together, this evidence can help establish an animal model to study this aromatase response, as well as provide preliminary assessment of the likelihood that the response is clinically important.

## 1. Materials and Methods

### *A. Induction of Illness in Mice*

Seven- to 8-week-old young adult male black 6 mice (Jackson Laboratory, Bar Harbor, ME) were used. Acute illness was induced with cecal ligation and puncture (CLP) [24] with sham surgery (SS) serving as a control procedure. In our preliminary trials, the gauge of the needle and the number of punctures were adjusted to achieve 50% mortality rate over 2 to 5 days. This was accomplished with one puncture with an 18-g needle. CLP consisted of a 1-cm medial abdominal incision with exposure of the cecum that was ligated 1 cm from the tip of the cecum below the ileocecal valve. Puncture was produced with an 18-g needle, and a droplet of intestinal content was extruded into the abdominal cavity. The incision was closed in two layers and 1 mL of normal saline was injected subcutaneously. SS was identical except that the cecum was not ligated or punctured. The protocol was guided by previously established humane end points for shock in animal research models [25] and approved by the Maine Medical Center Research Institute Institutional Animal Care and Use Committee.

### *B. Aromatase Blockade and Survival*

To block aromatase activity, letrozole (provided by Novartis®, Basel, Switzerland) was administered daily subcutaneously at a dose of 10 μg [26] for 7 days prior to surgical procedures in groups of CLP and SS mice and continued afterward for the 5 days of observation. In control groups of CLP and SS mice, vehicle was administered subcutaneously daily as previously mentioned. Fifty mice underwent CLP with 30 receiving letrozole and 20 receiving vehicle. Thirty mice underwent SS with 20 receiving letrozole and 10 receiving vehicle. Survival or death and rating of illness severity were observed on a daily basis in all 80 mice over the 5 days of the protocol or until death (if death occurred prior to the fifth day of observation). However, in 17 mice that died before the end of the observation period (nine CLP with vehicle and eight CLP with letrozole), 29 observations of severity of illness were not recorded prior to death. Thus, severity of illness was not compared between groups unless reflected by death. In several of the animals that died, the hour of death was not clear. Thus, survival analysis was performed with mice censored if alive at day 5. The log-rank test was used to assess statistical significance of differences in survival curves. A $P$ value of $< 0.05$ was considered significant.

### *C. Evaluation of Aromatase Expression in Adipose and Cardiac Tissue*

In a separate protocol, tissue from 12 mice that underwent CLP and eight that underwent SSwere evaluated for aromatase expression. Cardiac and adipose tissues were harvested

24 to 72 hours following CLP when mice exhibited signs of illness. SS mice were killed simultaneously with CLP mice. CLP mice had moderate to severe illness at the time of euthanasia, whereas SS mice appeared healthy. Severity of illness was scored according to the following criteria with 1 assessed as healthy, 2 and 3 as moderately ill, and 4 as severely ill: (1) no illness, (2) weight loss and ruffled fur, (3) weight loss, ruffled fur and decreased activity, and (4) all of the above plus ocular secretions and hypothermia [25]. Myocardial and peritoneal adipose tissue samples were collected immediately after euthanasia and flash frozen in liquid nitrogen.

The presence of aromatase protein in adipose and cardiac tissue was assessed by western blots. In addition, immunohistochemistry studies on cardiac tissue samples were performed in six CLP animals to confirm the presence of aromatase. For western blots, protein was extracted from harvested adipose and cardiac tissues using 5X passive lysis buffer (Sigma-Aldrich, St. Louis, MO) with complete mini protease inhibitor. Protein was identified with an antiaromatase rabbit polyclonal antibody (Promega, Madison, WI). Protein was extracted from ovaries of healthy female black 6 mice to serve as a positive control.

For immunohistochemistry evaluation, harvested tissues were fixed in 4% paraformaldehyde and dehydrated in serial concentrations of ethanol. Tissues were embedded in paraffin and sectioned at a thickness of 5 μm. Sections were treated with citrate buffer as an antigen retrieval. The antiaromatase rabbit polyclonal antibody (Abcam, RRID: AB_444718) was used to localize aromatase expression. Hematoxylin was used as a counterstain. All samples were stained following exactly the same protocol starting from the fixation, processing, sectioning, and staining (concentration of antibody, time of staining, washing, *etc.*). Controls were run at all times to ensure that the staining obtained was actually due to the antibody used. No primary controls were run to make sure that there was no background staining.

## 2. Results

### *A. Survival With Aromatase Blockade*

No mice died in the SS groups either with vehicle or with letrozole. Mortality rates in mice following CLP with or without letrozole are listed in Table 1 and displayed in Fig. 1. The mortality rate following CLP was greater in mice receiving letrozole (83%) than in those receiving vehicle (55%) ($P < 0.04$).

### *B. Adipose Tissue Aromatase*

Aromatase protein was observed by western blots in adipose tissue samples in all 12 mice undergoing CLP but in none of the eight mice undergoing SS (Fig. 2). With minimal or no aromatase evident in the SS mouse samples, statistical comparison was not undertaken.

**Table 1. Mortality in Different Cohorts**

| | Cumulative Deaths by Days After CLP or SS | | | |
|---|---|---|---|---|
| **Group** | **Day 1** | **Day 2** | **Day 3** | **Day 4** |
| SS + V n = 10 | 0 | 0 | 0 | 0 |
| SS + L n = 20 | 0 | 0 | 0 | 0 |
| CLP + V n = 20 (%) | 1 (5) | 5 (25) | 7 (35) | 11 (55) |
| CLP + L n = 30 (%) | 4 (13) | 14 (47) | 23 (77) | 25 (83)[a] |

Abbreviations: L, letrozole; V, vehicle.
[a] $P < 0.04$ vs CLP +V.

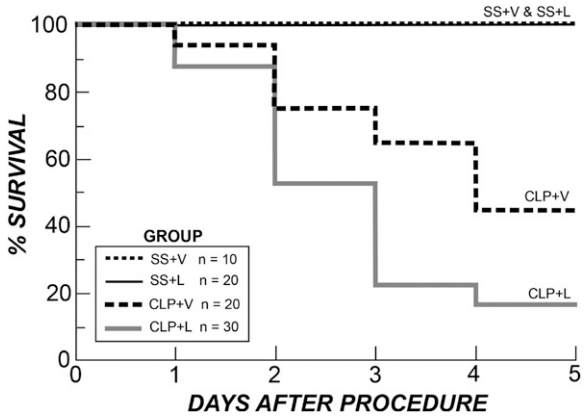


**Figure 1.** Survival curve in the four cohorts of mice. Percent mice surviving in each cohort for the 5 days after procedures were performed. Mortality was significantly greater in CLP mice receiving letrozole ($P$ = 0.04). L, letrozole; V, vehicle.

### *C. Myocardial Tissue Aromatase*

Aromatase protein was evident in myocardial tissue samples by western blots in all 12 mice undergoing CLP but in none of the eight mice undergoing SS (Fig. 2). With minimal or no aromatase evident in the SS mouse samples, statistical comparison was not undertaken. In six of these 12 mice, immunohistochemistry was also performed and confirmed the presence of aromatase in cardiac tissue (Fig. 3).

## 3. Discussion

The decreased survival with aromatase blockade in acutely ill mice observed in this study provides information pointing to the potential physiological and clinical importance of the aromatase response to acute illness. Serum estrogen concentrations rise in acute illnesses ranging from sepsis and myocardial infarction to cardiac surgery [14–18]. The rise in serum estrogen levels is accompanied by increased aromatase expression in adipose tissue and increased peripheral aromatase activity, indicating that the source of the increase in circulating estrogens is an increased rate of aromatization of androgens to estrogens by aromatase in adipose tissue [18]. Aromatization in adipose tissue is a major source of circulating estrogens, particularly in men and postmenopausal women [1, 2]. Several lines of evidence suggest that increased estrogen production may be beneficial in illness. Estrogens exert diverse effects in a variety of tissues that appear to be beneficial, including the heart and blood vessels [1, 3, 21]. Furthermore, estrogen has been reported to be beneficial in animal models of trauma and sepsis [4–13]. However, whether the aromatase response has clinical significance has not yet been established.

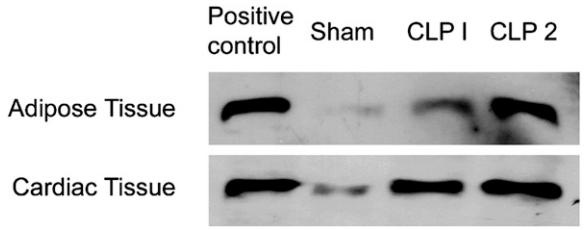


**Figure 2.** Western blot analysis of adipose and cardiac tissue. Aromatase protein was evident by western blot in adipose and cardiac tissue samples in all 12 mice undergoing CLP but to a minimal degree or not at all in the eight mice undergoing SS. Blots from the sham mouse and the CLP mice in this figure are typical of the groups.

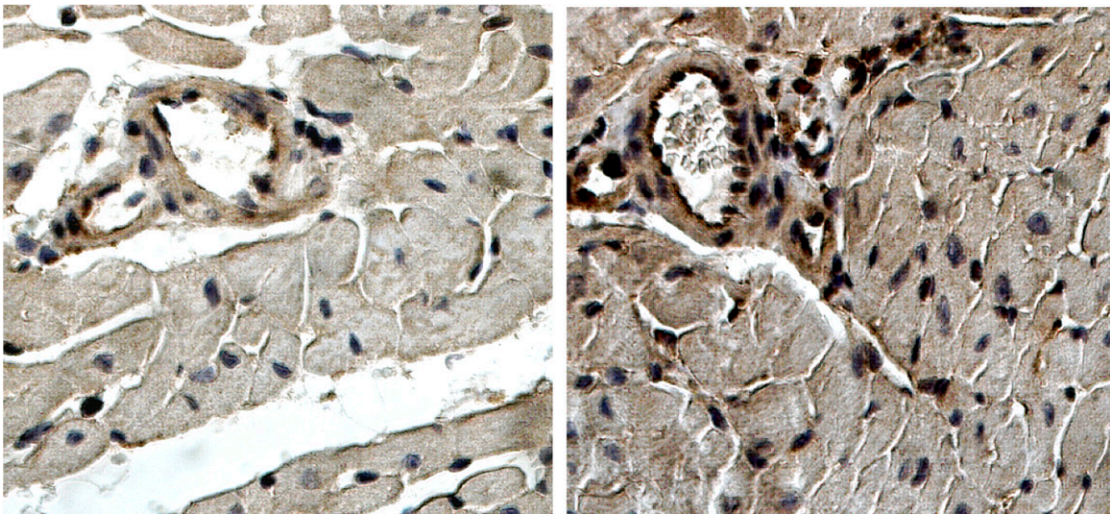

**Figure 3.** Immunohistochemistry staining of cardiac tissue. Immunohistochemistry staining of the heart demonstrated denser aromatase expression in the myometrium from the CLP animal on the right compared with the SS animal on the left indicated by the increased brownish tone. Aromatase expression is also suggested in the vessel on the right panel.

Our data provide evidence that the aromatase response may have physiological significance in combatting illness. Previous reports that survival is decreased in mice with decreased circulating estrogen due to oophorectomy and increased with 17$\alpha$-ethynylestradiol-3-sulfate treatment following severe blood loss have indirectly suggested that increased aromatization, by providing greater amounts of estrogens, may positively affect disease outcome [12, 13].

We observed aromatase protein in adipose tissue of all CLP mice but to a minimal degree or not at all in SS mice consistent with an increase in aromatase expression in the adipose tissue of these animals with acute illness. This indication of increased aromatase expression in adipose tissue accompanies acute illness in this mouse model provides important context for the decreased survival of the animals receiving aromatase blockade. That is, aromatase expression is increased in acute illness and its blockade of aromatase activity seems to be deleterious for survival. The increased expression of adipocyte aromatase also provides an important parallel to humans further supporting this model as relevant to potential future clinical research. Furthermore, the conservation of this aromatase response across species further suggests an adaptive advantage in evolution [29]. At least one study, an evaluation of the relationship of aromatase genetic variation to global outcome after traumatic brain injury, suggests that aromatase activity may influence the course of acute illness in humans [30].

Our data also provide observational evidence for an increase in aromatase expression in myocardial tissue with acute illness. Estrogen promotes cardiac contractility and vasodilation [31–33]. Studies in animal models of acute illness and trauma indicate that cardiovascular function is improved with greater systemic levels of estrogen or increased presence of estrogen receptors [4, 9, 28]. Thus, increased local production of estrogen in cardiovascular tissue via a local increase in aromatase expression could enhance local estrogen effects beyond those derived from systemic estrogens. Because increased estrogen stimulation of the intima results in vasodilation, increased intimal aromatase expression may benefit the response to acute illness by increasing blood flow to the heart and other tissues. However, the array of myocardial estrogen actions during acute illness may be complex with studies in aromatase knockout mice or aromatase-overexpressing mice, indicating that cardiac aromatase activity in a mouse model of ischemia is inversely related to myocardial recovery [19, 23, 34].

Our findings support this mouse model of acute illness as useful for studying the physiology and significance of the aromatase response. As in humans, aromatase expression is enhanced in adipose tissue during illness. The decreased survival with blockade of letrozole and the

previously reported positive actions of estrogen during trauma and sepsis both indicate that estrogen may have beneficial effects during sepsis in rodents [4–11]. The potential influence of aromatase expression and estrogen production, as well as estrogen therapy on improving clinical outcomes, can be evaluated further in this model. Measurement of serum and tissue estrogen levels was beyond the scope of this project but can provide valuable information in future studies. This model also permits the further study of the physiological aspects of the aromatase response in illness, such as changes in aromatase expression and estrogen production in specific tissues and its modulation by factors such as cytokines. The availability of aromatase knockout mice (C57Black6 X J129) and aromatase-overexpressing mice [23, 27, 35] also provides additional unique opportunities to evaluate the aromatase response to acute illness produced by CLP in mice. Finally, this study involved only male mice. Additional studies will be valuable to assess sex differences in the aromatase response and its effects in acute illness in ovariectomized female mice or the more complex model of cycling female mice.

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