[Frontiers in Bioscience 12, 4670-4675, May 1, 2007]

Lethal and edema toxins of anthrax induce distinct hemodynamic dysfunction

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1. ABSTRACT

Fatalities due to anthrax are associated with severe hypotension suggesting that the toxins generated from *Bacillus anthracis*, lethal toxin (LeTx) and edema toxin (EdTx), have cardiovascular effects. Here, we demonstrate the effects of these toxins and characterize their effects by echocardiography. LeTx leads to a significant reduction in ejection fraction, decreased velocity of propagation (diastolic dysfunction), decreased velocity of circumferential fiber shortening (decreased contractility), and increased LV systolic area (pathophysiology). EdTx leads to a significant reduction in left ventricular volumes and cardiac output (reduced stroke volume) but does not cause significant change in ejection fraction or contractility. These results indicate that LeTx reduces left ventricular systolic function and EdTx reduces preload but does not have direct myocardial effects. Together, these findings suggest that LeTx and EdTx exert distinct hemodynamic dysfunction associated with anthrax infection.

2. INTRODUCTION

Bacillus anthracis causes infection in both humans and animals and due to a high fatality rate, this agent can be used in biological warfare. However, the mechanism of death due to anthrax is not fully understood. *Bacillus anthracis* vegetative bacteria secrete three proteins—protective antigen (PA), lethal factor (LF), and edema factor (EF) which combine to form anthrax lethal toxin (LeTX;PA and LF) and anthrax edema toxin (EdTX; PA and EF). PA binds cells and self-assembles into a heptameric core that binds three LF or EF. LF and EF both enter cytosol after endocytosis. EF, a calmodulin-dependent adenylate cyclase, elevates intracellular levels of cAMP, and induces altered cell physiology or cell death (1). LF, on the other hand, leads to proteolysis of mitogen-activated protein kinases (MAPK), alters cell function and leads to cell death (2, 3).

The experience gained from the inhalation anthrax in the bioterrorism of 2001 included three detailed

Table 1. LeTx ejection fraction response, 48h post injection

	Normal Ejection Fraction N (%)	Low Ejection Fraction N (%)	All
Group			
LeTx	3 (21)	11 (79)	14
Control	7 (88)	1 (12)	8
All	10	12	22

Fisher's exact test: P value = 0.006 N= Number of animals

case reports of fatalities that were associated with severe hypotension. Borio, *et al.*, reported two fatal cases that progressed to refractory hypotension and respiratory failure requiring mechanical ventilation (4). However, these reports did not include data to determine whether the anthrax induced hypotension was due to hypovolemia, tamponade or resulted from myocardial malfunction. Later, in 2002, Mina, *et al.*, (5) reported a fatal case of inhalational anthrax. The respiratory and hemodynamic status of the patient deteriorated rapidly. The first echocardiographic analysis and right heart hemodynamics indicated that patient initially experienced hypovolemic shock. However, further echocardiographic analysis showed cardiac tamponade to be the cause of refractory hypotension. Using LeTx in a rat model, Cui, *et al* reported that circulatory shock and death were not due to excessive inflammatory cytokine or release of NO (6). Together, these initial findings suggest that anthrax associated shock has a complex pathophysiology. Here, we used echocardiography to further characterize the hemodynamic effects of the primary anthrax toxins.

3. MATERIALS AND METHODS

3.1. Generation of LeTx and EdTx

Recombinant PA, LF and EF were produced and purified as previously described (7-10). The toxins were stored at -80^{o}C in phosphate buffered saline, pH 7.4 (PBS). Immediately prior to injection, toxin components were thawed and mixed in vehicle, PBS, and with 1 mg/mL rat plasma.

3.2. Experimental protocol

The experimental procedures were performed in accordance with guidelines of the National Institutes of Health and American Association for the Accreditation of Laboratory Animal Care (AAALAC), and approved by the Scott and White Memorial Hospital/Texas A and M University System Health Science Center Institutional Animal Care and Use Committee, Temple, TX. Sixteen Sprague-Dawley rats were injected intraperitoneally with LeTx (100 μg/kg PA and 50 μg/kg LF) in 500 μL of vehicle. Sixteen rats were injected intraperitoneally with EdTx (200 μg/kg PA and 100 μg/kg EF) in 500 μL of vehicle. Sixteen rats were injected with vehicle alone as controls. Total volume of injection was less than 1 mL. The rat group assignment and sequence of injection were randomized. The systolic and diastolic functions were assessed by echocardiography (11). Baseline echocardiography was recorded 48 hours prior to injection. Following injection, the initial echocardiography was performed twelve hours after injection, and repeated every 6 hours for a total of 48 hours. Animals were sacrificed and myocardial tissues were collected, when ejection fraction decreased more than 30% from the pre-treatment value.

Anesthesia was induced with 4% isoflurane combined with 3 L/min oxygen. Then, a mixture of 0.025 mL of xylazine (10 mg/mL) and 0.025 mL of ketamine (100 mg/mL) was administered at a minimal dose of 0.05 mL intramuscularly. The animals were placed on a warming table to maintain normothermia. Echocardiography was then performed using Agilent Sonos 5500, Hewlett Packard, Palo Alto, CA with a 12-MHz probe. Anesthetized rats were allowed to breathe spontaneously with nose cone supplemental oxygen during echocardiography. Using American Society of Echocardiography guidelines, electrocardiographic, and transthoracic echocardiographic parameters or calculations were recorded (12). All measurements were made online and optimal digital images were selected from more than 10 cardiac cycles. Left ventricular (LV) end-systolic and end-diastolic areas were traced in a single-plane apical 4-chamber view and online Simpson's rule ejection fraction was calculated using the modified single-plane method. Systolic blood pressure was recorded by tail cuff manometry just prior to each echocardiogram. Heart rate was recorded at the beginning of echocardiography from the ECG monitor. Standard formulas were used for echocardiographic calculations (13). There are no prior published echocardiographic studies of anthrax toxin treated animals.

3.3. Statistical Analysis

Data were averaged and shown as means ± standard error of mean (S.E.). Fisher's exact probability test was applied to categorical tabulations. Echocardiographic parameters were analyzed by Students unpaired t-test for differences of means of data for controls and LeTx treatment, and by Students paired t-test of rats' baseline vs. eighteen-hour echocardiograms. Results were considered significant if p value was < 0.05.

4. RESULTS AND DISCUSSION

All control rats survived during the 48 hour experiment and, only in one control rat, there was a 30% decrease in ejection fraction. These findings show that anesthetic stress does not significantly alter cardiac hemodynamics. From 16 animals treated with LeTx, one died 8 hours after injection and before the initial echocardiography. The second animal died 45 hours after injection and after six cycles of anesthesia-echocardiography. Thus, only one death could reliably be attributed to LeTx injection. At least a 30% reduction in ejection fraction was observed in eleven (responders) of the 14 rats that survived 48 hr after LeTx injection (Table 1, Figure 1). There was an acute increase in left ventricular end systolic area. The primary endpoint of reduced ejection fraction first occurred, 18 hours post injection. Within the same time frame, there were significant changes of reduced

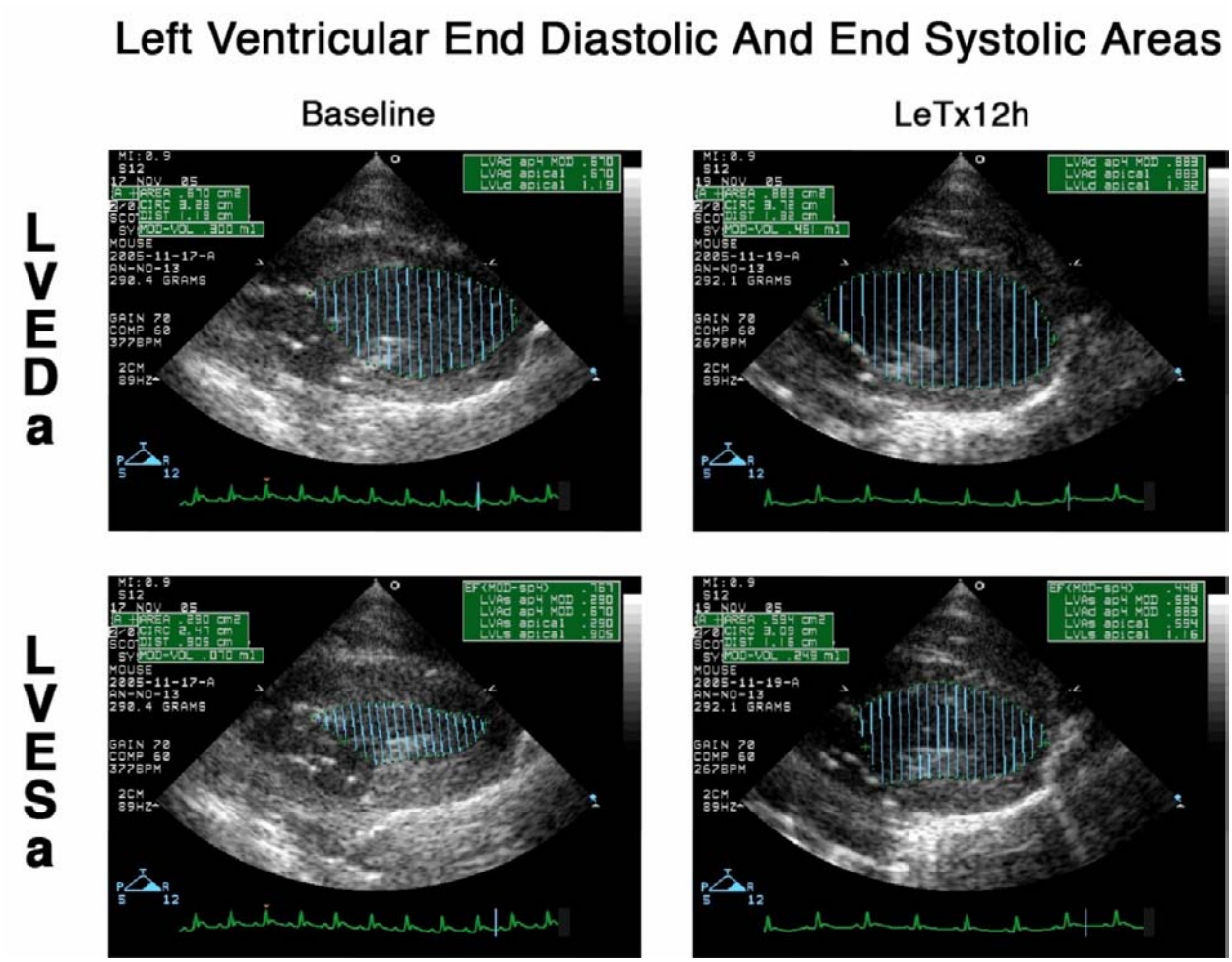

Figure 1. Example of modified Simpson rule tracings at baseline compared to 12 h after injection in LeTx injected rat. End diastolic area increased from 0.67 cm^2 to 0.88 cm^2, end systolic area increased from 0.29 cm^2 to 0.59 cm^2, and ejection fraction decreased from 77 to 45 percent.

systolic blood pressure and echocardiographic parameters including primary end point of reduced ejection fraction. (Figure 2-3). The reduced ejection fraction is due to the increased left ventricular end systolic area (LVAs). No significant increase in the left ventricular end diastolic area was observed. Heart-rate-corrected-velocity of circumferential fiber shortening (vcfc) was decreased in the LeTx injected rats indicating decreased left ventricular contractility. Color M-mode velocity of propagation (Vp) was decreased suggesting diastolic dysfunction. Some of the echocardiographic changes induced by EdTx are consistent with fluid loss due to rapid extravasation of fluid in intestinal lumen as reported by Firoved, *et al* (14). Two animals treated with EdTx died within 24 hours before inital echocardiography. The rat-tails were pale and cool and blood pressure could no longer be obtained 12 hours after injection of EdTx. Consistent with these findings and within the same time period, there were significant decreases in left ventricular end-diastolic (LVAd) and end-systolic areas (LVAs) (Figure 4). The changes left ventricular area imply similar changes in left ventricular volumes. There was also a significant decrease in cardiac output without a significant change in heart rate likely due to a decrease in stroke volume (Figure 5). Consistent with an acute loss of intravascular volume, there were also decreased in passive pulmonary vein velocities (Figure 6). EdTx does not seem to have direct myocardial effects since there were no significant differences in weight, ejection fraction, fractional shortening fraction, corrected velocity of circumferential fiber shortening, mitral valve inflow diastolic parameters and tissue Doppler E' between the controls and EdTx injected rats.

The findings reported here show distinct hemodynamic effects of EdTx and LeTx and suggest that these effects might play a role in death induced by anthrax toxins. However, other causes appear to contribute to death including hypovolemia due to rapid fluid loss (14), respiratory failure due to central nervous system malfunction (15), and terminal anoxia of the central nervous system (16). Further studies with the rodent model should help establish the significance of these hemodynamic effects.

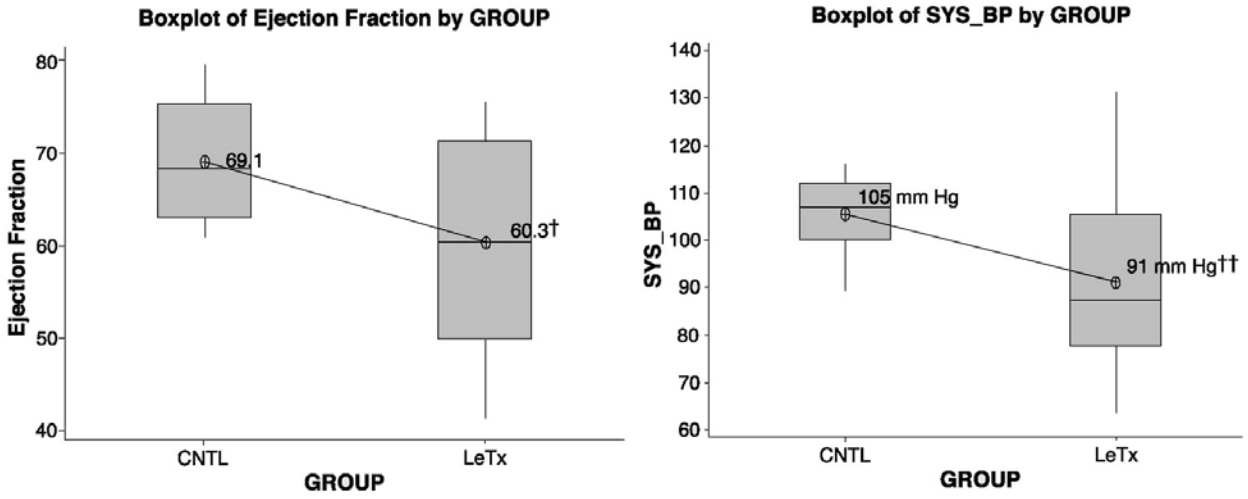

Figure 2. Box plots at 18 hours post LeTx injection compared to controls of ejection fraction and systolic blood pressure. The box represents the middle 50% of the data. The line through the box represents the median. The lines (whiskers) extending from the box represent the upper and lower 25% of the data. The line on each plot connects the means of the sample. The end-systolic pressure-volume point has been shifted to the right and downward. SYS_BP = systolic blood pressure. CNTL=Controls (n=8). LeTx= Anthrax Lethal Toxin injected (n=13). † p= 0.035. ††p=0.034

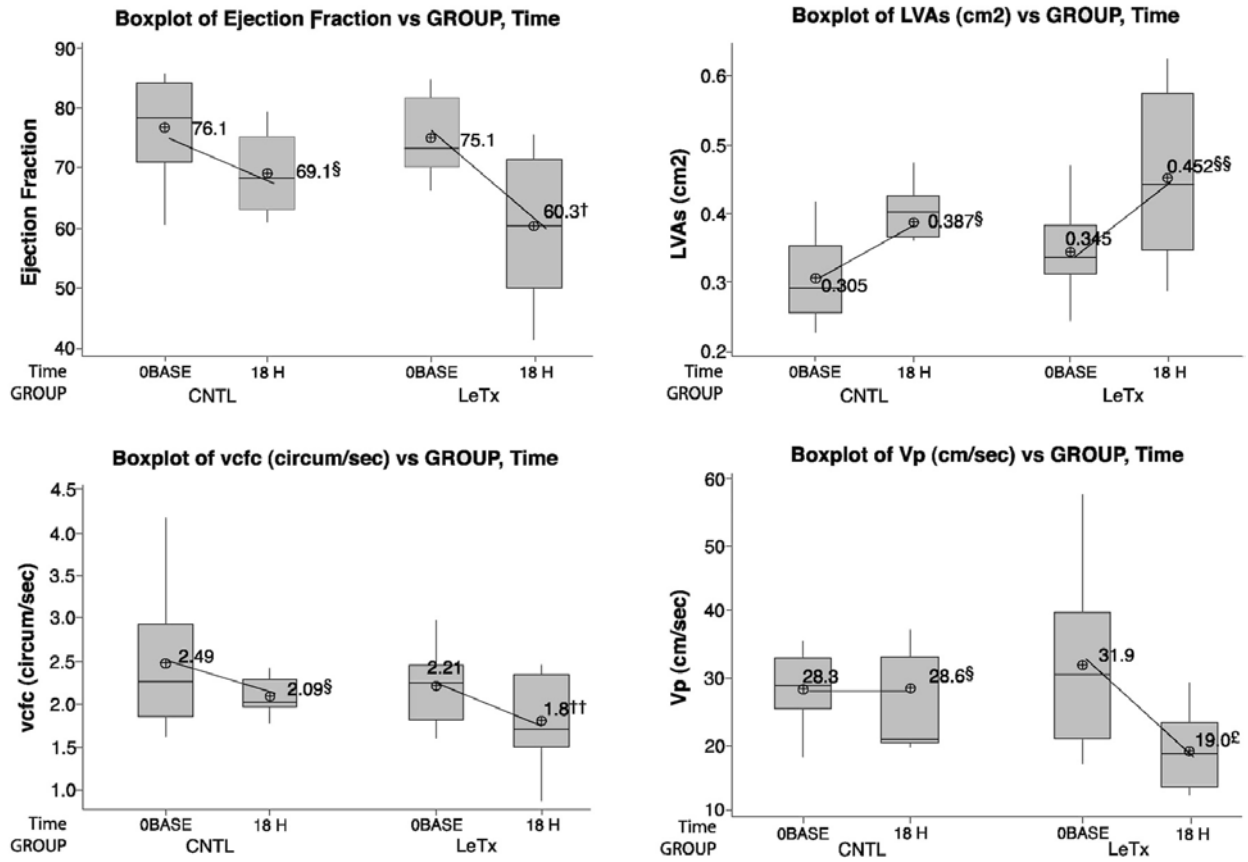

Figure 3. Paired t test of baseline to 18-hour echocardiograms of controls and LeTx injected. §= not significant. †P-Value=0.00047. §§P-Value = 0.002. ††P-Value = 0.006. £ P-Value = 0.002. See Legend Figure 2 for Box plot symbols. LVAs = left ventricular end systolic area. Vcfc = heart-rate-corrected-velocity of circumferential fiber shortening. Vp = color m-mode velocity of propagation.

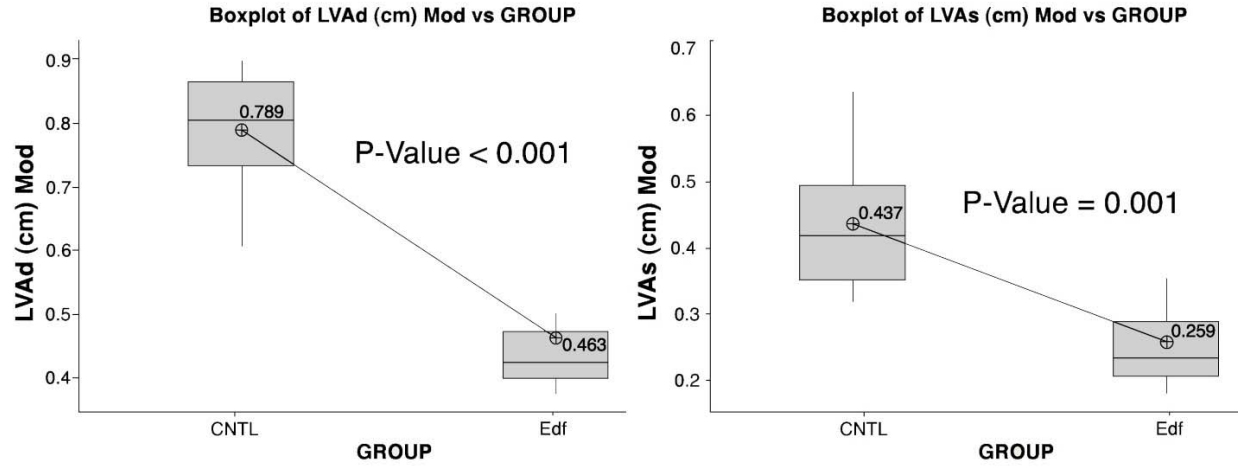

Figure 4. EdTx 12 hours post injection resulted in decreased left ventricular end diastolic (LVAd) and end systolic areas (LVAs).

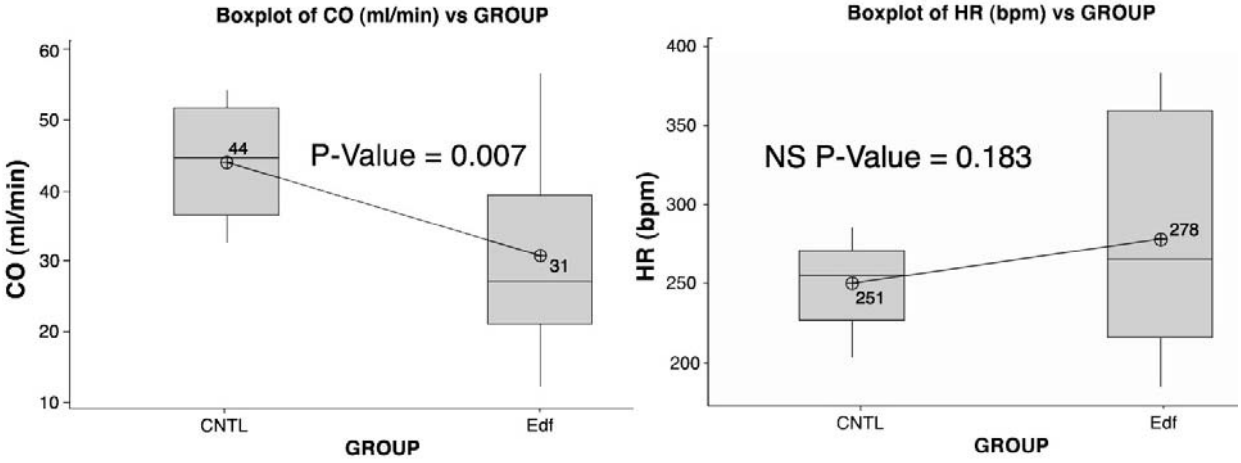

Figure 5. EdTx 12 hours post injection reduced cardiac output (CO) without reducing heart rate (HR).

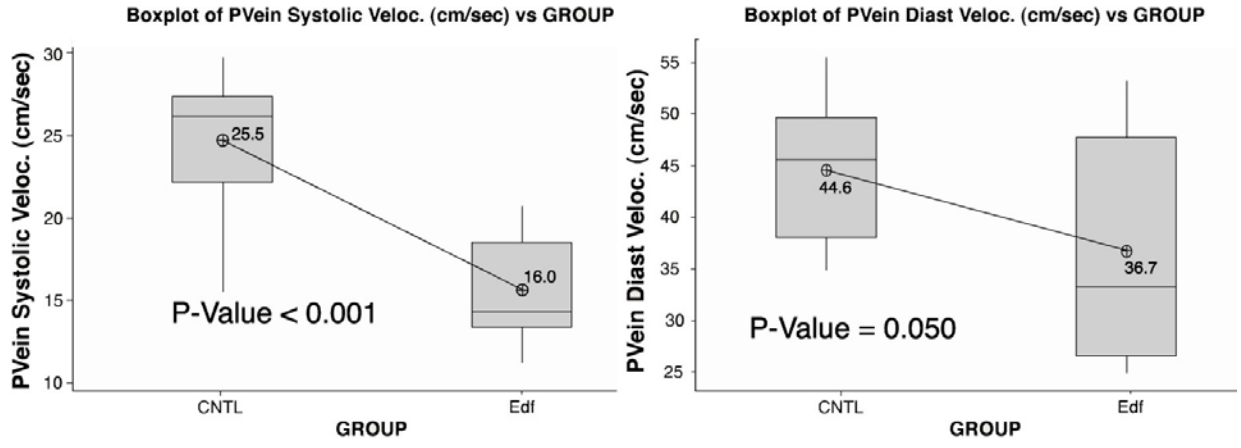

Figure 6. EdTx 12 hours post injection reduces both systolic and diastolic pulmonary vein Doppler velocities.

6. ACKNOWLEDGMENTS

We thank Shihui Liu for contributing the reagents and for continuous discussion of this work. We also thank James Mullis, Jr., ASCPT, RDCS for his contribution to sonographic data. This work was supported by National Institutes of Health R01 CA76178 (PI, A. Frankel), R01 CA90263 (PI, A. Frankel), R01 HL68838 (PI, D. Dostal). The non-sporogenic avirulent strain, LL44, was obtained from Dr. Steve Leppla at the National Institute of Allergy and Infectious Disease, Bethesda, MD.

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Abbreviations: LeTx, lethal toxin; EdTx, edema toxin; PA, protective antigen; EF, edema factor; LF, lethal factor; MAPK, mitogen-activated protein kinases; PBS, phosphate buffered saline; AAALAC, American Association for the Accreditation of Laboratory Animal Care; LV, left ventricular; LVA, left ventricular end systolic area; Vp, velocity of propagation; LVAd, left ventricular end-diastolic

Key Words: Anthrax lethal toxin, Anthrax edema toxin, Echocardiography, Ventricular Dysfunction, Left; Shock

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