

# EA-230 Renal Protection in Porcine Peritonitis

Goldfarb RD<sup>1</sup>, Cinel I<sup>1</sup>, Leighton A<sup>2</sup>, Fraimow H<sup>1</sup>, Knob C<sup>1</sup>, Cinel L<sup>1</sup>, Parrillo JE<sup>1</sup>, Dellinger RP<sup>1</sup>

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<sup>1</sup>Department of Medicine, Robert Wood Johnson School of Medicine, Camden, PA and <sup>2</sup>Exponential Biotherapies, Inc., McLean, VA.

## ABSTRACT

**Background:** EA-230 is a tetrapeptide which has exerted renal protective effects in small animal studies. It is being developed for renal protection in septic shock among other conditions. Severe sepsis and septic shock are associated with consumptive coagulopathy, thrombosis, and hemorrhage in multiple organ systems. In the present study, we looked for an effect of EA-230 on organ function and histology in a porcine model of peritonitis with emphasis on renal injury. Urinary neutrophil gelatinase-associated lipocalin (NGAL) is an early marker of renal injury and appears to stimulate regeneration of the renal tubular epithelium. Consequently, it is a marker of compensation for damage as well as damage itself.

**Methods:** Effects of EA-230 were assessed in a highly fatal peritoneal Gram negative bacteria autologous clot implant into female pigs as previously described. There were three sham animals (minilaparotomy without clot implantation), and three study arms, saline control (n=4); 2-dose EA230 group, 30 mg/kg EA-230 at 15-30 minutes after completion of infected clot implantation and again at 6-6.25 hours after the infected clot implantation (n=7); and a 3-dose group, 30 mg/kg EA-230 at 1, 3 and 6 hrs after clot implantation (n=5). Cardiovascular data was recorded continuously and urine samples were taken hourly for measurement of neutrophil gelatinase-associated lipocalin (NGAL). At the end of the experiment the kidneys were removed, sectioned, stained with H & E, and examined under a microscope.

**Results:** Light microscopy showed the kidneys of EA-230 treated animals to be less damaged than those of untreated controls. Protection from thrombosis and edema in the 3-dose EA-230 treated animals was associated with the highest NGAL levels. There was no evidence of untoward effects of EA-230.

**Conclusions:** The combination of elevated NGAL and preserved renal histology in the 3-dose treatment group, and the lack of deleterious effects on cardiovascular parameters, indicate potential utility of EA-230 as a renoprotective agent in septic shock.

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## INTRODUCTION

Systemic sepsis is a syndrome that affects over 500,000 Americans each year, killing about 40% of patients. Sepsis is followed either by recovery of cardiovascular stability, usually due to removal or eradication of the septic focus, or development of cardiovascular collapse characterized by hypotension, multiple organ failure, cardiac and vascular dysfunction. Numerous pathophysiological pathways have been reported to be activated by sepsis such as NF $\kappa$ B, inflammatory cytokines, apoptosis, toll-receptor pathways as well as many others.

Acute renal failure occurs in about 19 percent of patients with moderate sepsis, 23 percent with severe sepsis, and 51 percent with septic shock when blood cultures are positive. Similarly, a progressive increase in acute respiratory distress syndrome (ARDS) is associated with sepsis severity. The combination of sepsis and renal failure is associated with 70% mortality vs 45% mortality in patients with acute renal failure alone. Thus sepsis with acute renal failure is a particularly serious medical problem.

EA-230 has been shown to increase survival and protect renal function in a number of nonclinical models, including ischemia reperfusion and sepsis models. It was also shown that a single treatment with EA-230 up to 24-hr after high dose LPS injection inhibited septic shock in BALB/c mice. In another *in vivo* experiment EA-230 blunted the increase in the number of macrophages and granulocytes and of pro-inflammatory cytokines. In brief, it is suggested that EA-230 modulates the inflammatory process by preventing release of pro-inflammatory cytokines from activated macrophages/granulocytes and by regulating the proliferation and differentiation of immune cells, and that these effects of EA-230 are particularly effective for renal protection in a number of acute animal models.

The experiment tested the hypothesis that EA-230 treatment would protect renal function and/or improve cardiac function and thereby attenuating the natural history of severe porcine peritonitis.

## METHODS

**Pigs:** Yorkshire pigs weighing between 30 and 35 kg were pre-anesthetized with ketamine HCl (20 mg/kg) and glycopyrolate (0.004-0.01 mg/kg) I.M. Each animal was moved to preparation area where its abdomen was cleaned and shaved, then moved to the operating area. Anaesthesia was induced by IV administration of Propofol 1-3 mg/kg and maintained at 5-10 mg/kg/hr. Fentanyl was administered in a dose of 0.02-0.05 mg/kg IV. Fentanyl was used in conjunction with propofol because propofol can cause hypotension and has no analgesic effect. When fentanyl was added to propofol anaesthesia the combination allows one to use a lesser amount of propofol to get the same level of anaesthesia. IV access was maintained through an ear vein and constant drip of normal saline was initiated and continued for the procedure's length. Arterial blood gases were monitored by pulse oxymeter on an ear lobe. A heating pad was placed under the animal to maintain body temperature at 37 °C.

Through an incision in the groin a catheter was introduced into the descending aorta through a femoral artery for administration of fluids and measurement of arterial blood pressure and collection of arterial samples for blood gas analysis. A thermofluid catheter was introduced into the pulmonary artery via femoral vein. Through this catheter, pulmonary artery pressure, cardiac output and core temperature were recorded. The bladder was catheterized and urine collected.

**Cardiovascular data acquisition and analysis:** All recordings were analyzed using software provided by DISS, Inc. Measured variables recorded were continuous cardiac output (Edwards Scientific) pulmonary artery and arterial blood pressures (PAP and AOP). From these measured variables, the following were calculated: heart rate (HR), cardiac output, stroke volume, systemic and pulmonary vascular resistances. Echocardiographic measurements of myocardial function were performed using and Acuson Cypress System (Siemens Medical Solutions). Images were obtained from a trans-thoracic position. Fractional shortening (FS) was calculated from standard m-mode measurements. LV volumes were calculated by measuring LV area from 2-D images and ejection fraction (EF) calculated in the usual manner.

**Microbiology:** E. coli O111:B4 was used in these studies. The strain used is sensitive to a wide variety of antibiotics, including penicillin. Stored bacteria was inoculated on trypticase soy agar containing 5% sheep's blood and grown overnight at 37°C. Several well formed colonies were used to inoculate 500 ml of BHI broth which was grown overnight to stationary phase with shaking (150 RPM) at 37 °C. The bacterial cells were harvested by centrifugation and were washed twice in sterile saline. The washed bacterial harvest was re-suspended in sterile saline to a final volume of 10<sup>10</sup> ml. Concentration of the cell suspension was estimated by direct microscopic cell counts and by optical density in a spectrophotometer. Actual concentration was determined through serial dilution cell plating onto blood agar plates. The clot was prepared from 100 ml of a 1% sterile solution of bovine fibrinogen. An appropriate amount of bacterial suspension solution was added to achieve a dose based on colony forming units and animal weight (CFU/Kg). After mixing, 100 units of human thrombin was added to form a bacteria-laden clot. This fibrin clot was implanted employing sterile surgical techniques and time of surgery was defined as Time = 0. Sterile clots for sham animals were prepared on the day of the experiment by adding 100 units of human thrombin to 100 ml of 1% sterile bovine fibrinogen solution.

**Endpoints:** Each animal was observed for up to 8 hrs post implant and then euthanized. If during this observation period, cardiac output dropped to less than 40% of baseline, that animal was euthanized at that time. This occurred once in the control group, once in the dual dose group and not in the triple dose group. All animals were implanted with infected clots and three groups created: vehicle group, dual or triple administration of EA-230 group as described below.

**Data calculations:** Primary cardiovascular data obtained in these experiments included central core temperature, arterial and pulmonary artery vascular pressures and cardiac output via continuous thermal dilution technique. Data was recorded in digital form via DISS, Inc. hardware and software. From these data, heart rate (HR), stroke volume (CO/HR), systemic and pulmonary vascular resistances were calculated.

**Statistical Analysis (Cardiovascular):** Time and treatment effects in each cardiovascular parameter were compared by 2 way analysis of variances (ANOVA) for repeated measures with p values less than 0.05 being considered significant. Variation at each time point was performed using Dunnett's test (SigmaStat).

**Histological samples:** Samples of heart, lung and kidney were obtained at termination of each experiment. The specimens were fixed in 10% formalin for 24 h, and standard dehydration and paraffin-wax embedding procedures were used. Sections (5 mm) were cut in a microtome, adhered to glass slides with polylysine. Hematoxylin and eosin-stained slides were prepared by using standard methods and evaluated by light microscopy.

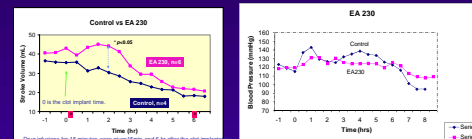
**Lung weights:** Following completion of the experiment, one lung from each animal was excised, dried of surface material and weighed. It was then placed into a drying oven (80°C) until its weight was stable for 24 hrs. The ratio of wet to dry weight was then calculated.

**Laboratory assessments: NGAL:** Neutrophil gelatinase-associated lipocalin (NGAL) was measured by a research enzyme-linked immunosorbent assay (ELISA) by the methods described in (2). Amounts of NGAL were calibrated by 12 calibration standards (NGAL range 0-1925 ng/ml). NGAL measurements by ELISA and by TrageAR(NGAL) Device (Biosite Incorporated).

## RESULTS

### a. Cardiovascular data

Figures 1-3 present stroke volume during the course of these experiments of the vehicle control and 2-dose group. Stroke volume (CO/HR) generally declined in all animals after infection due to increased heart rate and decreased cardiac output. In the initial phase, cardiac output goes up (hyperdynamic sepsis) followed by a marked decrease (hypodynamic) leading to either death or recovery, in this model, at 24-48 hrs (1).

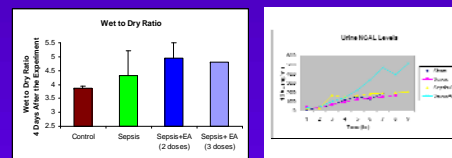


**Figure 1.** Stroke volume of treated vs 2 dose EA-230 treated group. The EA-230 treated group exhibited a significantly higher stroke volume at 2 hrs post implant. However, final stroke volumes were equal. Green arrow represents implant of an infected clot

**Arterial blood pressure (ABP):** ABP was monitored throughout the experiments. In vehicle group, it remained at basal values for the initial 5 hrs post implant. At 7 hrs post implant, it fell 20 mmHg. In the EA230 double treated group, ABP rose after the first drug treatment (+15 min after implant). This was a transient increase, as ABP was similar to vehicle treated values at 90 min post implant. The second EA230 infusion did not induce any changes in ABP (Fig 2, below).

**Echocardiographic data:** Fractional shortening and ejection fraction were measured at baseline, immediately before and after EA-230 in three of the three-dose animals. As the peritonitis progressed, there was a consistent decrease in fractional shortening, confirming previously observed results (1). There was no statistically significant change in fractional shortening as a result of EA-230 infusion.

**b. Lung wet-to-dry ratios:** The wet-to-dry weight ratio of lungs excised from these animals is presented below (Figure 3). Treatment with EA-230 did not prevent accumulation of fluid in the lungs of these animals.



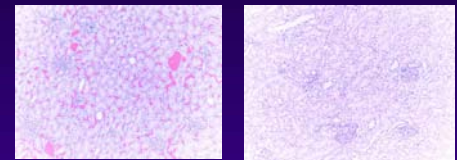
**Figure 3** Wet to dry weight ratio of lungs from EA-230 pigs and controls (Left). Two non-septic animals are on the left; lungs from septic but untreated animals (N=4) demonstrated an increased weight. Lung weights from EA-230 treated animals were higher, but not significantly, from vehicle treated septic animals.

Urine NGAL in four experimental groups (Right). Neutrophil Gelatinase-Associated Lipocalin was measured in hourly urine samples. Clot implant was performed after sample 1. The four groups were sham (n=3), untreated sepsis (n=4), EA230, 2 doses (n=5) and EA230, 3 doses (n=5).

**Histology** Histological examination of sections of heart and kidney from untreated animals uniformly revealed pathophysiological alterations. In the 2 dose treated group, 2 of the six animals showed few pathophysiological changes in heart and kidney specimens, while the other 4 animals exhibited significant signs of injury. Renal sections of four of six treated animals and all controls demonstrated congestion and focal hemorrhage. Cardiac sections of 4 of 6 treated animals demonstrated PMN infiltration supporting acute inflammation, congestion and hemorrhage, while myocardial sections of the other 2 treated animals appeared normal.

In the 3 dose EA230 group, sections demonstrated no histological alterations; these sections were indistinguishable from sections obtained in sham (sterile clot) implanted animals. These findings were uniform in all 5 animals treated with 3 doses of EA230. This was in sharp contrast to untreated peritonitis pigs in which cardiac and renal sections demonstrated severe congestion and neutrophil infiltration.

### Renal Histopathology



**Figure 4:** Representative renal histopathology (H&E, X100) slides from vehicle treated septic animals (left) and EA230 treated animals. Note that the kidney sections demonstrated severe congestion and focal hemorrhage. Renal sections of EA230 animals appeared normal (2 of 6 dual dose and 4/4 triple dose).

Histological examination of cardiac sections indicated that administration of EA-230 in sepsis (2 dose) was associated with few pathophysiological changes in heart in 2 animals and kidney specimens in two animals but significant changes in 3 animals. In the 3 dose group, no histological alterations were observed; these sections were indistinguishable from sham samples. In untreated septic animals, all cardiac and renal sections exhibited significant signs of injury.

The administration of EA-230, in 2 or 3 doses, following implant of an infected clot did not alter the cardiovascular trajectory of peritonitis. Although transient support of cardiac output was noted in some animals, there was no consistent pattern of vascular support nor was there a persistent positive effect over time. In instances where cardiac output appeared to rise or the slope of decline decreased during and subsequent to EA-230 administration, cardiac output resumed its post infection decline prior to second EA-230 infusion. Since the protocol required animals to be euthanized at a defined time, survival rate was not a variable that could be measured.

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Urine NGAL rose in all septic groups; it was most highly elevated in the 3 dose group – the group which showed no histological changes. Urine NGAL rose moderately in the other three groups (sham, untreated peritonitis and D2 groups), which is consistent with previous reports (2) which found in animal and human models, urine NGAL goes up moderately with stressful situations such as sham surgery or peritonitis. It has been reported that NGAL may be expressed by the damaged tubule to induce re-epithelialization (2). The D3 group consistently induced NGAL. That would explain good sense, since NGAL is well known to be a protective agent, and might even explain the histological protection observed in this group (2). An alternative explanation would be that EA230 (in 3 doses) is protective enough to the kidney by other mechanisms, thereby allowing it to maximally express endogenous regenerative and repair mechanisms such as NGAL induction.

## Conclusions

The combination of elevated NGAL and preserved renal histopathology in the 3 dose treatment group, and the lack of deleterious cardiovascular parameters, indicate potential utility of EA-230 as a renal protective agent in septic shock.

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