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Prompt Administration of Antibiotics and Fluids in the Treatment of Sepsis: A Murine Trial

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Abstract

Objective—Sepsis, the acute organ dysfunction caused by a dysregulated host response to infection, poses a serious public health burden. Current management includes early detection, initiation of antibiotics and fluids, and source control as necessary. Although observational data suggest that delays of even a few hours in the initiation of antibiotics or intravenous fluids is associated with survival, these findings are controversial. There are no randomized data in humans, and prior animal studies studied time from experimental manipulation, not from the onset of clinical features of sepsis. Using a recently developed murine cecal ligation and puncture (CLP) model that precisely monitors physiologic deterioration, we hypothesize that incremental hourly delays in the first dose of antibiotics, in the first bolus of fluid resuscitation, or a combination of the two at a clinically relevant point of physiologic deterioration during polymicrobial sepsis will shorten survival.

Design—Randomized laboratory animal experimental trial.

Setting—University basic science laboratory

Subjects—Male C57BL/6J, female C57BL/6J, aged (40–50 weeks) male C57BL/6J, and BALB/C mice

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Interventions—Mice (n=200) underwent biotelemetry-enhanced CLP and were randomized after meeting validated criteria for acute physiologic deterioration. Treatment groups consisted of a single dose of imipenem/cilastin, a single bolus of 30 mL/kg fluid resuscitation, or a combination of the two. Mice were allocated to receive treatment at the time of meeting deterioration criteria, after a 2-hour delay, or after a 4-hour delay.

Measurements and Main Results—Hourly delays in the initiation of antibiotic therapy led to progressively shortened survival in our model ($p<0.001$). The addition of fluid resuscitation was unable to rescue animals, which received treatment four hours after meeting enrollment criteria. Systemic inflammation was increased and host physiology was increasingly deranged with hourly delays to antibiotics.

Conclusions—We conclude that antibiotic therapy is highly time-sensitive, and efforts should be made to deliver this critical therapy as early as possible in sepsis, perhaps extending into the first point of medical contact outside the hospital.

Keywords

sepsis; animal model; antibiotics; resuscitation; telemetry

Introduction

Sepsis is common and deadly (1–4). In the US alone, more than 1 million patients are hospitalized with sepsis annually, and sepsis accounts for nearly 1 out of every 2 hospital deaths (1–4). Despite advances in our understanding of the complex biology of sepsis, many precision approaches to sepsis, including biologic therapeutics, have not meaningfully improved outcomes in randomized clinical trials (5). Thus, the cornerstones of treatment remain antibiotics, resuscitation with intravenous fluids, and provision when needed of vital organ support, such as vasopressors and mechanical ventilation. International guidelines, large cohort studies, and state and national quality metrics all support the administration of antibiotics and fluids as early as possible (6).

Despite preclinical and observational data that suggest early antibiotics are associated with improved outcomes, no randomized trial has tested this strategy to provide causal inference (7–11). Further, a recent meta-analysis has cast some doubt on the association between antibiotic delays and clinical outcomes (12). Previous preclinical work in a monomicrobial murine sepsis model found that delaying antibiotics until after the onset of hypotension resulted in significantly greater mortality rates (13). However, the use of fixed time points to administer treatments in animal studies represents a significant discordance with clinical medicine, where patients' physiologic changes serve as trigger thresholds for therapy administration. As such, a recent animal model established that the moment of physiologic decompensation could be identified in real time during experiments to guide testing of potential therapeutics (14).

The dissemination of more intense strategies to promote early antibiotics or fluids, especially given the diagnostic challenges at the onset of shock, likely requires stronger evidence, such as randomized trials of very early vs. usually timed antibiotics. Such trials in

turn require justification with stronger causal inference regarding whether incrementally small, hourly advancements in the delivery of therapy improve outcomes. To address this knowledge gap, we conducted a murine trial testing the effects of an early vs. a delayed single dose of antibiotics and/or fluid bolus on measures of physiology, organ dysfunction, and survival curves in a validated, physiology-based model of polymicrobial sepsis.

Materials and Methods

Ethics Statement

All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol no. 13021581) in accordance with guidelines established by the National Institutes of Health.

Experimental Design

This is a randomized trial to test the ramifications of changes in the timing of two components of the Surviving Sepsis Campaign guidelines: 1) “administration of antimicrobials be initiated as soon as possible...” and 2) “at least 30 mL/kg of IV crystalloid fluid be administered within 3 hours” on survival using a physiology-based murine polymicrobial sepsis model. Secondary outcomes of interest included host physiologic trends, systemic inflammation, organ dysfunction, and blood gas parameters of shock.

Our group previously developed and validated criteria that define acute physiologic deterioration after cecal ligation and puncture (CLP): 1) 10% decline of heart rate from its peak value and 2) a scaled 10% drop in core temperature calculated as: $10\% \times (\text{peak core temperature} - 25^{\circ}\text{C})$ (14). Each animal was continuously monitored, and at the point of acute physiologic deterioration, each animal was assigned to a treatment group.

The experimentation consisted of three parallel trials: 1) a single dose of antibiotics given at the time of meeting criteria, two hours, or four hours later; 2) a single dose of antibiotics and a single bolus of fluid resuscitation, given at the time of meeting criteria, two hours, or four hours later; and 3) a single bolus of fluid resuscitation only, given at the time of meeting criteria or at a two hour delay. We did not conduct a trial arm consisting of mice given only fluid resuscitation at a 4-hour delay due to ethical concerns of treatment futility.

For each group described above, experimentation was carried out in two phases: survival experiments carried out until death of the animal or 7 days, and non-survival experiments which consisted of sacrificing animals 24 hours after trial randomization. The non-survival experiments tested plasma cytokines and biomarkers of organ dysfunction at the time point of 24 hours, a point which we have previously established results in reliable prevalence of septic shock and organ dysfunction (15).

The intervention of antibiotics consisted of a single intraperitoneal (IP) dose (25 mg/kg) of imipenem/cilastatin administered in a small volume of sterile 0.9% saline (0.15 mL) to minimize lavage of the peritoneal cavity or any potential contribution to additional fluid resuscitation. Fluid resuscitation consisted of a single bolus of 30 mL/kg 0.9% normal saline, injected subcutaneously. Our testable hypothesis was that small delays in the

administration of the first dose of antimicrobial or the first bolus of IV fluids worsened outcome. Thus, we chose to administer a single dose of antibiotics and/or a single bolus of volume resuscitation, rather than continued administration of either, to maximize our ability to discern differences in these biological, physiological and clinical outcomes in the context of the timing of treatment.

Experimental Animals

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) aged 8 to 12 weeks (mass 25–30 grams) were used for all experiments. In addition, sensitivity analyses were performed using male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) aged 40–50 weeks, female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) aged 8 to 12 weeks (mass 25–30 grams), and male BALB/C (Jackson Laboratories, Bar Harbor, ME) mice aged 8 to 12 weeks (mass 25–30 grams) to determine the conservation of any observations across age, gender and species. Mice were housed in specific pathogen-free rooms under 12 hour light/12 hour dark conditions with an ambient temperature of $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Mice were allowed to acclimate to their new surroundings for one week prior to any experimentation. Animals were given ad libitum access to water and LabDiet Prolab Isopro RMH 3000 diet pellets (LabDiet, St. Louis, MO). To account for circadian variation we initiated experiments in the morning between 0700 and 1000.

Cecal Ligation and Puncture with Biotelemetry Monitor Implantation

A 1 cm ligation, 21-gauge double puncture model of cecal ligation and puncture (CLP) under anesthesia was performed as previously described (14). At the time of CLP, an HD-X11 wireless biotelemetry device (Data Sciences International, St. Paul, MN) capable of continuously measuring heart rate, core temperature, and activity was implanted into the peritoneal cavity in accordance with manufacturer instructions. At the conclusion of the procedure all mice received a subcutaneous injection of warmed 0.9% normal saline (30 mL/kg) to compensate for intraoperative fluid losses and recreate the hyperdynamic state associated with early sepsis (16). This administration of fluid occurred concomitantly with CLP as a component integral to the model; it was delivered far in advance of the development of sepsis, and thus was not considered a therapeutic intervention. Analgesia was achieved with buprenorphine (0.1 mg/kg SQ Q12H). After surgery each animal was placed in an individual cage on a heating pad until full emergence from anesthesia (animal upright, mobile, foraging activity). The cage was then removed from the heating pad, and biotelemetry monitoring commenced, capturing data each minute. Data collection and analysis was performed using Ponemah 5.20 (Data Sciences International, St. Paul, MN). For animals in non-survival study experiments, euthanasia was carried out at the specified time point with blood obtained from cardiac puncture.

Inflammatory Cytokine and Organ Dysfunction Analysis

Heparinized blood was centrifuged at 2000g for 15 minutes, and the plasma was frozen at -80°C for future analysis. Plasma concentrations of IL-6, IL-10, and TNF- α were analyzed by ELISA (R&D Systems, Minneapolis, MN). Acute kidney injury was quantified by measuring Cystatin C concentrations with ELISA (R&D Systems).

Blood Gas Parameters of Shock

Heparinized blood collected at the time of animal euthanasia was analyzed via venous blood gas using an i-STAT handheld device (Abbott, Princeton, NJ).

Statistical Analysis

We analyzed continuous data by nonparametric Wilcoxon rank-sum or Kruskal-Wallis tests. Cuzick's nonparametric test for trend was applied to investigate trends across ordered groups. Survival data were modeled using the Kaplan-Meier method and curves were analyzed using Cox Proportional Hazards. Physiologic data were analyzed using fractional polynomial panel techniques. All tests were two-sided with $\alpha = 0.05$. Statistical analysis was conducted using Stata 14 (StataCorp, College Station, TX).

Results

The time from enrollment to randomization (i.e., from CLP until acute physiologic deterioration) did not significantly differ between any of the treatment groups. In the fluids-only subcohort, the two treatment groups had similar times to randomization (median 474 vs. 462 minutes, $p=0.57$). Mice treated with antibiotics early, at a 2-hour delay, or at a 4-hour delay experienced similar baseline times to acute physiologic deterioration (median 464 vs. 467 vs. 451 minutes, respectively, $p=0.96$). Mice receiving fluids and antibiotics at early, 2- and 4-hour delays were also similar in time to reaching acute physiologic deterioration (median 479 vs. 438 vs. 438 minutes, $p=0.20$).

Early vs. late administration of fluids does not significantly improve outcomes

After meeting criteria for acute physiologic deterioration, mice received a single bolus of fluid resuscitation 1) early at the point of acute physiologic deterioration or 2) after a 2-hour delay. There was no significant difference in time to death after physiologic deterioration (median 1583 vs. 1809 minutes, early versus 2-hour delay, $p=0.14$, Figures 1A and 1B). Both groups had similar rates of bradycardia and hypothermia (Figure 2A). We observed no differences in venous blood gas parameters of shock (Figure 3) or plasma cytokine concentrations and renal dysfunction (Figure 4). Compared to control mice receiving no treatment (median survival 1156 minutes after meeting criteria for acute physiologic deterioration), both early fluids and fluids after a 2-hour delay prolonged survival ($p=0.005$, Figures 1A & 1B).

Earlier vs. late antibiotics improves survival, host physiology, and inflammation

Mice that received early antibiotics survived longer compared to those receiving antibiotics after a 2- or a 4-hour delay: 3969 vs. 3485 vs. 2619 minutes ($p < 0.001$, Figures 1A & 1C). We observed that mice administered early antibiotics exhibited preserved physiology as evidenced by less bradycardia and hypothermia as compared to 2- or 4-hour delays (Figure 2B). We did not observe significant differences in parameters of shock, such as pH, lactate, or base excess (Figure 3). We observed that the early antibiotic group had less systemic inflammation compared to a 2- or 4-hour delay, as measured by plasma IL-6 concentration (median 8672 vs. 13224 vs. 39599 pg/mL, respectively, $p < 0.01$, Figure 4B). The differences for serum TNF- α and IL-10 concentrations were not significantly different (Figures 4A, 4B

and 4C). Renal dysfunction, as measured by Cystatin C, was modestly increased comparing early vs 4-hour delay (median 865 vs. 777 vs. 1084 ng/mL, $p=0.11$, Figure 4D).

Combination antibiotic and fluid treatment is time-dependent

Mice receiving antibiotics and fluids early or after a 2-hour delay survived longer than the 4-hour delay group (median survival 3946 vs. 4468 vs. 2520 minutes, $p=0.046$, Figures 1A and 1D). Mouse physiology was similar for early vs. 2-hour delays, while the 4-hour delay group experienced more pronounced bradycardia and hypothermia (Figure 2C). A 2-hour delay in fluids and antibiotics was associated with a trend towards worse shock: HCO_3^- ($p=0.06$) and base excess ($p=0.07$, Figures 3B and 3C). We found a relative increase in inflammatory biomarkers and Cystatin C for groups with delayed combination therapy but these did not achieve statistical significance (Figure 4).

The response to a delay in antibiotics in aged, female and BALB/C mice

To determine the degree to which these responses were conserved, we compared early antibiotics against a 4-hour delay in aged, female and BALB/C mice. As shown in Figure 5, for each group survival was prolonged for animals receiving antibiotics early, and this difference was significant for aged (HR 3.03, 95% CI 1.17 to 7.87, $p=0.02$) and BALB/C (HR 2.39, 95% CI 1.00 to 5.72 $p=0.049$) mice. For each cohort, early antibiotics attenuated the development of hypothermia and bradycardia, by comparison to the delivery of antibiotics after a 4-hour delay (Figure 5).

Discussion

Using a biotelemetry-enhanced CLP model of sepsis, we found that short delays in antibiotic therapy and combination treatment with antibiotics and fluid resuscitation shortened survival, disrupted host physiology, and increased inflammation. When we administered a single dose of antibiotics to mice at the moment of physiologic deterioration rather than after a 2- or 4-hour delay we observed stepwise increases in the duration of survival, reduced bradycardia and hypothermia and more modest inflammatory response with earlier treatment. The preservation of homeostasis with early antibiotics was also observed in aged, female and BALB/C mice, supporting a time dependence for antimicrobial therapy independent of age, gender and species of mice. Notably, the addition of fluid resuscitation offset the poor outcomes seen in mice receiving antibiotics alone at a 2-hour delay. These findings extend earlier findings in murine models using only fixed time points for sepsis treatment and provide preclinical data for causal inference on the importance of timely antibiotic and fluids at the moment of physiologic deterioration. In contrast to previous studies that focused on antibiotic delays after the onset of septic shock, treatment in this study was timed to earlier and less severe physiologic changes that more generally represent the septic population at-large (7, 9, 13).

There are a variety of mechanisms by which prompt antibiotics and fluids may improve murine outcomes in polymicrobial sepsis. The host inflammatory response, such as IL-6 release, may be modified with timely antibiotic therapy, and an exaggerated immune response is postulated to underlie the development of hemodynamic instability during sepsis,

resulting in greater mortality (17–22). We found similar evidence in this study, where animals receiving a dose of antibiotics early manifested less bradycardia and hypothermia, correlating with lower plasma concentrations of IL-6 and prolonged survival. Although we did not observe significant differences in organ dysfunction at 24 hours, previous data has linked increases in systemic inflammation with organ dysfunction in sepsis, and sequential organ failure leading to death is a pathophysiologic hallmark in sepsis (17, 23–26). Our time-point of assessing organ injury may have been too early in the progression of disease in this model of polymicrobial sepsis to detect a separation in organ dysfunction with delays in therapy. Thus, earlier control of pathogen growth via antibiotic administration may mitigate an excessive immune response and lead to improvements in survival.

Fluid resuscitation administered early did not prolong survival compared to a 2-hour delay, but was superior to no treatment at all. As such, the benefit of fluid resuscitation in the absence of concomitant antibiotics does not appear to be as time-sensitive, at least for the time points we tested. One explanation may lie in the augmentation of circulating volume in the absence of antibiotics or infectious source control. Earlier fluids without concomitant antibiotic therapy may speed the systemic distribution of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These PAMPs and DAMPs may then act to further increase the inflammatory response (27–31). This mechanism may also underlie the absent physiologic differences between the two fluids-only groups, though remains to be studied.

Similarly, a reduced time to the delivery of both interventions, fluids and antibiotics together, prolonged survival, though the time interval of significance was broadened. When antibiotics and fluids were combined, the effects of fluid administration appeared to reduce the effects of a 2-hour delay in antibiotic therapy alone. This observation should not diminish the importance of early antibiotic therapy; rather it suggests a synergy between treating the offending infection and resuscitating the circulating volume. However, as the delay progressed to four hours, fluid resuscitation no longer compensated, and survival was reduced. Differences in median biomarker concentrations were smaller and failed to achieve statistical significance at the 24-hour time point, again supporting the hypothesis that at early time points the provision of fluid resuscitation offsets some of the negative effects associated with antibiotic delays.

These data may have important clinical implications. The increased mortality with delays in antibiotic therapy strengthen the available preclinical data, which previously focused on delays in antibiotic delivery that occurred after the onset of shock (13). Herein, our model is more broadly generalizable to the sepsis population at-large, rather than confined to patients in septic shock. There may be additional implications from the fluid resuscitation data, which suggest that the benefits of fluid resuscitation are less time-sensitive than antibiotics but play a strong supportive role. Ultimately, strategies to advance the timing of sepsis therapies require human studies in a randomized clinical trial setting. These data support the development of such a trial, especially in the face of current equipoise on the subject of antibiotic timing and fluids (12).

This study is not without limitations. Preclinical animal data do not always translate to human trials. However, using physiologic entry criteria to enroll animals, we created a preclinical murine model of sepsis that better aligns with human clinical trial design, testing therapies based upon physiologic inclusion criteria, rather than at fixed time intervals (14, 32). We acknowledge sample sizes in this study are smaller than its human trial counterpart, which may support an elevated prevalence of Type II errors. We utilized imipenem due to its broad antimicrobial activity and standardized use in CLP models (33–35). This drug may or may not be feasible for use in a human trial, and alternate antibiotics may need to be tested. We chose 30mL/kg of fluid as a “starting point” in accordance with the SCC guidelines. Evidence suggests that larger volumes (e.g., 40 mL/kg) may be needed to achieve euvolemia in mice, and thus under-resuscitation may explain our inability to observe an association between delays in volume resuscitation and survival (36–39). Therefore, caution must be exercised in interpreting our data, and we do not propose that fluids are ineffective. In this vein, for both antimicrobials and fluids a single dose was chosen. Our objective was to test the hypothesis that incremental delays in the initiation of the first dose of early sepsis therapy worsened outcome, and we perceived *a priori* that subsequent management may mask our ability to discern subtle differences in the ramifications of differential treatment. However, these experimental interventions differ from clinical practice and the referenced trials in which volume resuscitation continues until clinical evidence of euvolemia is achieved, and antimicrobial therapy is continued after source control. This difference likely underlies our observation that though survival was prolonged, all animals died within the period of observation, which raises the issue of therapeutic value. Future studies are needed to assess whether or not earlier administration of antibiotics and fluids when provided in the context of full support (i.e., continued antibiotics and volume resuscitation with reexploration and cecectomy for source control) increases absolute survival. We administered fluids via a subcutaneous route, which though a validated method of resuscitation in murine models of sepsis, differs from the intravenous route used in clinical medicine (16, 40). Intravenous fluid resuscitation is possible in mice but requires longer handling times (provoking the stress response) as well as tail warming to promote vasodilation and facilitate injection, which may introduce bias (41). Our endpoints of analysis were necessarily limited for feasibility, and we could not incorporate analyses of all the known biological mechanisms underlying sepsis-related tissue dysfunction and death (i.e., DAMPs). Future investigation should focus on the causal mechanisms (e.g., reduced DAMPs, enhanced reduction in bacterial burden) by which early therapies improve outcomes.

The collective body of literature supports the concept of delivering antibiotic therapy as early as possible in sepsis. Our study demonstrates that incremental treatment delays for sepsis *prior* to the development of a shock state negatively affect survival. (7–11, 13). If we are to provide therapy at the earliest possible time, then prehospital delivery offers the greatest time advantage, with the opportunity to intervene hours in advance of the current care paradigm in sepsis (1, 10, 11, 42). Emergency medical services have proven capable of initiating care protocols for other time sensitive conditions, such as stroke and myocardial infarction (42–44). It is our perspective that we should ‘arm’ prehospital EMS with the same capacity to deliver life-saving therapies to patients at risk for sepsis. To that end, there are

several international trials currently planned, although all three focus on the septic shock subcohort (ClinicalTrials.gov ID: NCT03068741, NCT01988428, & NCT02473263).

This study is the first to our knowledge to use physiologic changes in mice as entry criteria for a therapeutic trial. Employing previously validated criteria for murine physiologic deterioration, we demonstrate that hourly treatment delays can significantly alter inflammation, host physiology, and survival. These data support the concept of an “earlier is better” approach to the treatment of sepsis, perhaps moving therapy to the point of first medical contact.

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Figure 1. Survival curves

Proportion of animals (n=10 in each sub-cohort) surviving, plotted using the Kaplan-Meier method as function of time after meeting criteria for acute physiologic deterioration, which served as the enrollment point for each trial. Plots are divided by treatment: all cohorts (A), fluid resuscitation only (B), antibiotic treatment only (C), or combination therapy with antibiotics and fluid resuscitation (D).

Figure 2. Animal physiology after trial enrollment

Core temperature (Celsius) and heart rate (beats per minute) are plotted as a function of time after meeting criteria for acute physiologic deterioration using fractional polynomial panel analysis to generate a mean curve for each sub-cohort. Plots shown are mean with 95% confidence intervals. Plots are divided by treatment: fluid resuscitation only (A), antibiotic treatment only (B), or combination therapy with antibiotics and fluid resuscitation (C). n=10 animals for each sub-cohort.

Figure 3. Parameters of shock

Mixed venous blood gas analysis was performed 24 hours after trial enrollment. Sub-cohorts are separated by treatment and by treatment timing. Parameters shown include pH (A), bicarbonate concentration (B), base excess (C), and lactate (D). Data is represented as median with individual data points.

Figure 4. Systemic inflammation and organ injury

Biomarkers of inflammation (tumor necrosis factor-alpha, [A]; interleukin-6, [B]; interleukin-10, [C]) and organ dysfunction (Cystatin C, [D]) measured 24 hours after trial enrollment. Sub-cohorts are separated by treatment and by treatment timing as indicated. Data is represented as median with individual data points.

Figure 5. Survival curves and animal physiology in aged, female and BALB/C mice

Plots are divided by treatment: aged mice (A), female mice (B), and BALB/C mice (C). Top panel represents proportion of animals surviving, plotted using the Kaplan-Meier method as function of time after meeting criteria for acute physiologic deterioration. Middle and bottom panels represent core temperature (celsius) and heart rate (beats per minute) plotted as a function of time after meeting criteria for acute physiologic deterioration using fractional polynomial panel analysis to generate a mean curve for each sub-cohort. Plots shown are mean with 95% confidence intervals. (n=10 in each cohort)