

Unintended consequences; fluid resuscitation worsens shock in an ovine model of endotoxemia

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Abstract

Background: Fluid resuscitation is widely considered a life saving intervention in septic shock however recent evidence has questioned both its safety and efficacy in sepsis.

This study sought to compare fluid resuscitation with vasopressors with vasopressors alone in a hyperdynamic model of ovine endotoxemia.

Methods: Endotoxemic shock was induced in sixteen sheep after which they received fluid resuscitation with 40mls/kg of 0.9% saline or commenced haemodynamic support with protocolized noradrenaline and vasopressin. Microdialysis catheters were inserted into the arterial circulation, heart, brain, kidney and liver to monitor local metabolism. Blood samples were recovered to measure serum inflammatory cytokines, creatinine, troponin, ANP, BNP and hyaluronan. All animals were monitored and supported for 12 hours after fluid resuscitation.

Results: After resuscitation animals that received fluid resuscitation required significantly more noradrenaline to maintain the same mean arterial pressure in the subsequent 12 hours (68.9mg vs. 39.6mg $p=0.04$). Serum cytokines were similar between groups. Atrial natriuretic peptide increased significantly after fluid resuscitation compared to animals managed without fluid resuscitation (335ng/mL (256-382) vs. 233ng/mL (144 – 292) $p 0.04$). Cross-sectional time series analysis showed the rate of increase of the glyocalcyx glycosaminoglycan hyaluronan was greater in the fluid resuscitated group over the course of the study ($p=0.02$)

Conclusion: Fluid resuscitation resulted in a paradoxical increase in vasopressor requirement. Additionally, It did not result in improvements in any of the measured microcirculatory or organ specific markers measured. The increase in vasopressor requirement seen may have been due to endothelial/glycocalyx damage secondary to ANP mediated glycocalyx shedding.

Introduction

Conservative estimates place the annual incidence of sepsis at 19 million cases with global estimates of costs upwards of 16 billion dollars (USD)^{1,2}. Mortality remains high with estimates of greater than 20% in the developed world³.

Fluid resuscitation (FR) is widely regarded as an essential life-saving intervention in sepsis with early FR recommended in all iterations of the international surviving sepsis guidelines^{4,5}.

The onset of sepsis is thought to result in pathological tissue hypoperfusion which if untreated leads to progressive organ dysfunction⁶. To this end FR is employed to increase cardiac output to reverse pathological hypoperfusion, preventing organ injury^{4,7}. Whilst there has been widespread adoption there is emerging evidence that calls into question both the safety and efficacy of fluid resuscitation⁸⁻¹⁰. Importantly, prior to 2011 there were no randomised control trials (RCTs) directly examining the impact of fluid resuscitation on mortality in sepsis.

The “Fluid Expansion as Supportive Therapy” (FEAST) trial was published in 2011 and remains the only RCT of fluid resuscitation in sepsis¹¹. Children with severe sepsis were randomized to received FR with either 0.9% saline, 4% albumin or supportive care. The

trial demonstrated harm with FR with both resuscitation arms having a 45% relative increase in mortality¹¹. Similarly, Andrews et al. conducted a RCT of early protocolized resuscitation with fluids and vasopressors in Zambian patients presenting with sepsis and found increased mortality in the resuscitated patients suggesting that there might be harm associate with the traditional resuscitation paradigm⁸. The results of these trial are in direct contrast to more than 50 years of medical theory and call into question the safety this common intervention and suggest it may in fact be harmful.

In a secondary analysis of the FEAST trial authors demonstrated that the excess mortality in the FR arms was due to delayed cardiovascular collapse, a potentially novel mechanism of harm¹². Consequently, we aimed to examine the effectiveness of FR in the treatment of septic shock in an animal model of endotoxemia. Our hypothesis was fluid resuscitation would not result in substantial improvements in endotoxemic shock and would lead to increased need for cardiovascular support after FR. The primary outcome of the study was vasopressor requirements in the post FR period. Additionally, as fluid resuscitation is used to maintain tissue perfusion we used tissue microdialysis of the vital organs to assess for pathological tissue hypoperfusion. Serum pro-inflammatory cytokines and biochemistry were measured throughout the study in to assess for a putative mechanistic connection between fluid resuscitation and the inflammatory state and the development of organ failure. Given the observation of delayed cardiovascular collapse in the FEAST study plasma atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), troponin and glycocalyx products were measured to assess for potential cardiac and endothelial damage.

Limited haemodynamic and microdialysis data from the control group (NR) has been published previously¹³.

Materials and Methods

This study was approved by the Animal Research and Ethics Committee of the Queensland University of Technology and The University of Queensland (Approval no. 1400000032) and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 8th Edition 2013 of the National Health and Medical Research Council (NHMRC)¹⁴.

Animal preparation

Sixteen Merino ewes (43.5 ± 6.2 kg) were fasted overnight for the study. Detailed description of animal care, data capture, anesthetic and surgical technique including placement of microdialysis catheters and microdialysis technique was in accordance with previously published methodology¹⁵. At the commencement of the study all animals were anesthetized with midazolam 0.5mg/kg (Pfizer (Perth) Bentley, WA, Australia), buprenorphine 300mcg (Reckitt Benckiser Healthcare, Hull, U.K.) and alfaxalone 3mg/kg (Jurox, Rutherford, NSW, Australia). Anesthesia was maintained with an infusion of alfaxalone 6 mg/kg/hr (Jurox, Rutherford, NSW, Australia), midazolam 0.25 mg/kg/hr (Pfizer (Perth) Bentley, WA, Australia), fentanyl 15 mcg/kg/hr (Hameln Pharmaceuticals, Hameln, Germany) and ketamine 10 mg/kg/hr (Troy Laboratories, Glendenning NSW, Australia). All anesthetic and analgesic medications were titrated to maintain adequate surgical anesthesia.

A central venous catheter (Arrow International, Reading, PA, USA), facial artery arterial catheter (Vygon arterial leadercath, Ecouen, France), pulmonary artery catheter (Swan-

Ganz CCombo, Edwards Lifesciences, Irvine, CA, USA) were inserted to facilitate drug administration and cardiovascular monitoring.

Microdialysis catheters (CMA 63 & 70 MD probes Kista, Sweden) were surgically inserted into the femoral artery, brain, heart, liver and left kidney.

Throughout the study all animals received protocolized ventilation to maintain SaO₂ >94% and ETCO₂ between 35-45mmHg.

Experimental protocol

After completion of surgical instrumentation, a 60 minute interval was allowed prior to commencement of the experimental protocol for physiological stabilization of the animal.

To avoid profound pulmonary hypertension, the lipopolysaccharide infusion (LPS; *E. coli* serotype O55:B5) was commenced at 0.5 mcg/kg/hr and escalated to 4mcg/kg/hr over 4 hours (figure 1). Resuscitation occurred in the last hour of the endotoxin infusion. All animals had a mean arterial pressure (MAP) below 60mmHg prior to resuscitation. Animals received either fluid resuscitation with 40mls/kg of normal saline (Fluid resuscitation (FR) n=8) given over 1 hour or commenced vasopressor support (No fluid resuscitation (NR) n=8). After fluid resuscitation noradrenaline 60 mcg/ml in 5% dextrose (Hospira, Lake Forest, IL, USA) was commenced in order to maintain a MAP between 60-65 mmHg. In both groups the starting noradrenaline rate was 5mcg/min and it was titrated every 5 minutes to maintain the MAP in the target range. If the noradrenaline dose reached a pre-determined 20 mcg/min, vasopressin (PPC, Richmond Hill, ON, Canada) was

commenced at 0.8 units/hr and increased to a maximum of 1.6 units/hr if hypotension persisted. Controlled administration of noradrenaline and vasopressin was achieved using a Gemini infusion pump (Alaris medical systems San Diego, CA, USA) and the administration protocol was the same for both groups. Animals were monitored for 12 hours after the end of the endotoxin infusion and received a total of 13 hours of haemodynamic support (figure 1).

Statistical analysis

Data are presented as mean (SD) for normally distributed variables and median [IQR] for non-normally distributed variables. Only the mean arterial pressure, serum lactate, base excess and serum hyaluronan were normally distributed and values at each time point were compared with a two-sample T-test. All other variables were not normally distributed and values at individual time points were compared using the Wilcoxon rank-sum test.

A $p < 0.05$ was considered statistically significant.

Repeated measures regression analysis was performed for hyaluronan, noradrenaline and vasopressin infusion rate over time to test for differences between groups. Fixed effect modeling was used throughout. Confirmation of the validity of this methodology was assessed using the Hausman test. Independent variables used were resuscitation group (FR or NR) and time in hours and all models were tested for significant interactions between the independent predictors. Model diagnostics included testing the error terms (residuals) for normality using the Shapiro-Wilk test and homoscedasticity using the Breusch-Pagan test. Outliers and high leverage points were

identified using leverage to squared residual plots while influential points were identified using Cook's distance method.

Statistical analyses were performed using STATA™ (StataCorp, TX, USA) statistical software package (version 13). One animal in the FR group was euthanized 90 minutes before the end of the experiment as it was deemed unsupportable by the investigators due to metabolic acidosis and refractory hypotension (pH <6.9 and MAP <60mmHg despite 240mcg/min of noradrenaline).

Results

Hemodynamic variables

At baseline hemodynamic variables were similar in both groups. Endotoxemia produced a similar reduction in blood pressure in both groups with mean MAP between 44-49mmHg prior to resuscitation (table 1 & figure 2A). Endotoxemia produced a temporary decrease in cardiac index (CI) one hour post-infusion that recovered to normal or supra-normal levels prior to resuscitation (table 1 figure 2B). Fluid resuscitation produced a significant increase in cardiac index increasing from a median of 3.42 IQR (2.52-3.72) to 4.94 (3.91-5.62) (p=0.005). Systemic vascular resistance index (SVRI) was similar between groups at baseline and prior to resuscitation (figure 2C). Fluid resuscitation resulted in a significant decrease in SVRI from 1110(950-1230) to 714(630-807) (p=0.02) associated with a relatively large increase in cardiac output and the concomitant modest increase in blood pressure.

Hemodynamic support

Vasopressor use differed significantly between groups. Noradrenaline requirements rose for both groups throughout the monitoring period with a statistically significant difference between groups at both 8 and 12 hours of the monitoring period (figure 2D). The median noradrenaline rate 8 hours after resuscitation in the FR group was 140mcg/min (96.5-172.5) and 62mcg/min (31.0-110.0) in the NR groups ($p=0.03$). At the end of the monitoring period the difference between groups had increased and was 240.0mcg/min [104.0, 270.0] in the FR group compared to 73.0mcg/min [33.0, 137.5] in the NR group ($p=0.02$). Repeated measures regression analysis demonstrated that the rate of rise of was significantly greater for the FR compared to the NR group (Beta of 18.74mcg/min/hr ($p=0.001$) vs. 6.51 mcg/min/hr ($p=0.001$) with the group time interaction term $p<0.001$) (figure 2D). With the exception of noradrenaline dose, no interactions were noted and the regression error terms were normally distributed and homoscedastic. All animals required full dose vasopressin (0.03units/min), though there was no significant difference in vasopressin use between groups (figure 2E).

Biochemistry

Serum lactate increased in both groups throughout the study however no statistically significant difference was observed at any point (figure 2F), though the mean lactate did diverge over the course of the monitoring period with the final mean lactate in the FR arm being 11.22 (+7.65) and 5.07 (+2.02) in the NR resuscitation group ($p=0.09$). Serum base excess decreased in both groups throughout the study (figure 2G) and was

greater in the FR group compared to NR ($p=0.048$). This effect persisted throughout the study with the serum base excess being $-12.52 (+2.82)$ in the FR group and $-6.26(+4.94)$ at the end of the monitoring period ($p=0.02$).

Microdialysis

The lactate/pyruvate (L/P) ratios of both resuscitation strategies were similar across all organs (figure 3). For the arterial, brain, heart and kidney microdialysis samples there were no statistically significant differences between groups at any time points. The liver microdialysis samples did demonstrate a statistically significant difference after resuscitation however the groups had large differences in baseline L/P ratios prior to resuscitation (figure 3E). None of the other timepoints in the liver microdialysis demonstrated significant differences.

Inflammatory cytokines

Endotoxemia produced large increases in all the measured serum cytokines (figure 4). TNF- α , IL-1 β , IL-8 and IL-10 all exhibited a similar pattern of response with peak levels reached at or near the end of the endotoxin infusion before decreasing to near baseline values. IL-6 increased during endotoxemia and remained near maximal values throughout the monitoring period. The pattern of cytokine response between groups was remarkably similar with no significant differences at any of the time points for TNF α , IL-1 β , IL-6 and IL-10. IL-8 values were similar between groups throughout the

experiment however final values at the end of the monitoring period were significantly different 1779pg/ml (759-5051) in the NR group compared to 5903pg/ml (3519-14591) in the FR group ($p=0.04$) (figure 4).

Cardiac, Endothelial and Renal Biomarkers

Brain natriuretic peptide (BNP) rose during endotoxemia in both groups reaching maximum values at 3 hours. There were no significant differences in BNP values between groups at any time points (figure 5A). Endotoxemia resulted in a large increase in serum atrial natriuretic peptide (ANP) in both groups (Figure 5B). In the NR group, ANP peaked at a median of 297ng/ml (227-331) 3 hours into the endotoxin infusion then declined throughout the rest of the experimental period. In the FR group ANP reached a similar value after 3 hours 274ng/ml (142-418) however increased further after fluid resuscitation to 335ng/ml (256-382). There was a significant difference between median ANP between the two groups after resuscitation 335ng/mL (256-382) in the fluid resuscitation group vs. 233ng/mL (144 292) in the non-fluid resuscitated group ($p=0.02$). There was a large difference in high sensitivity serum troponin at the end of the study with median values of 8.60ng/ml (5.85-15.50) in the NF group compared to 52.5ng/ml (18-104) in the FR group ($p=0.03$) (figure 5E).

The glyocalyx glycosaminoglycan (GAG) hyaluronan rose in both groups throughout the study (Figure 5C). Maximal mean values of 10734 (+8940) in the non-resuscitated group and 16681 (+13903) ($p=0.33$) were reached at the end of the experiment. The values between groups were not significantly different at any of the measured timepoints (figure 5C). Univariate time series cross-section analysis demonstrated that the rate of increase was significantly greater in the FR compared to the NR group

($p=0.02$). Similarly multivariate regression analysis confirmed that FR was a predictor of an increased rate of rise of hyaluronan.

Serum creatinine rose throughout the study in both groups reaching maximal median values of 263 $\mu\text{mol/L}$ (191-335) in the FR group and 208 $\mu\text{mol/L}$ (148-240) in the NR group ($p=0.12$) at the end of the study (figure 5D).

Discussion

Fluid resuscitation is promoted as a critical life saving intervention in sepsis and has been strongly recommended as the first line resuscitative therapy in all iterations of the international surviving sepsis guidelines⁴. It is thought that adequate fluid resuscitation in the early phase of sepsis improves shock and results in reduced vasopressor requirements later in a patients illness⁷. The key finding of this study was that fluid resuscitation given for endotoxemic shock produced short-lived haemodynamic improvements which were then followed by significantly increased vasopressors requirements in the subsequent 12 hours. These results challenge the current paradigm of resuscitation and warrant detailed discussion.

Firstly, our study has a number of important differences to previous animal models demonstrating benefit of fluid resuscitation in sepsis and endotoxemia. Our model was developed to produce hyperdynamic/distributive shock with progressive decline in SVRI while maintaining near normal cardiac output at the initiation of resuscitation. These are the changes most often encountered in both human septic shock and endotoxemia¹⁶⁻¹⁹. Previous studies that supported fluid resuscitation in animals have

used hypodynamic models of illness with rapid and severe reductions in cardiac output being the dominant cause of hypotension²⁰⁻²³. In a porcine model of endotoxemic shock, Oi et al. demonstrated improved mortality with fluid resuscitation with hypertonic saline-dextran. However after the administration of endotoxin untreated animals developed a profound and persistent low cardiac output state with a mortality of 77% at 5 hours (the end of the experiment). Similarly Bressack et al. were able to show improved outcomes with fluid resuscitation in a porcine model of septic shock, however animals in the study also developed severe cardiogenic shock. As hyperdynamic shock appears to be the most commonly seen presentation in human sepsis¹⁷⁻¹⁹, the rapid development of a life-threatening low cardiac output state in these models makes extrapolation of their findings to the clinical arena difficult. The incremental increase in rate of endotoxin infusion minimized the cardiac dysfunction which has hampered other models and allowed our model to generate a hyperdynamic response similar to that seen in clinical sepsis. We believe therefore that this model better reflects the actual effect of fluid resuscitation in the clinical setting.

Additionally, previous studies of fluid resuscitation have not had an alternative hemodynamic support strategy and have compared fluid resuscitation to untreated shock²⁰⁻²³. The relevance of such models is a concern as clinically fluid resuscitation is one element of a larger resuscitation strategy and short-term studies of it in isolation do not reflect how it is used clinically and may miss important interactions with other therapies. The advantage of this study is that it compares fluid resuscitation as part of a resuscitation strategy that is similar to current guideline recommendations with a viable alternative. This model better addresses the question of what is the likely impact of fluid resuscitation on current patients treated with septic shock and helps inform clinical studies of alternative strategies to management of septic shock.

As expected the fluid resuscitated animals had a large increase in cardiac output immediately after the fluid bolus was administered. While MAP improved over the hour of resuscitation, the magnitude of the increase was modest compared to the increase in cardiac output. This was due to a concurrent decrease in the SVRI of the resuscitated animals. This observation of fluid resuscitation induced vasodilation has been reported clinically in a number of studies. Monge Garcia et al described the immediate effects of fluid bolus resuscitation on arterial load in 81 septic patients²⁴. 67% of the patients had an increase in cardiac output with fluid resuscitation, however this only resulted in an increase in MAP in 44% of those patients. Overall fluid resuscitation resulted in a decrease in SVRI that was most marked amongst patients that increased their cardiac output. In a similar observational study in 51 patients with sepsis Pierrakos et al found that for those that had a significant increase in their cardiac output fluid resuscitation resulted in a decrease in their SVRI²⁵. The underlying mechanism of this vasodilation is unclear but it is thought due to occur due to a combination of baroreceptor mediated reduction in sympathetic tone and flow mediated vascular relaxation secondary to endothelial nitric oxide release²⁴.

The novel finding of this study was the divergent trends in vasopressor requirement after resuscitation. Interestingly, this was not apparent in the immediate post resuscitation period with noradrenaline requirements similar between groups for the first 3-4 hours. After 3-4 hours the noradrenaline requirement diverged, plateauing in the non-fluid resuscitated animals and increasing rapidly in the fluid resuscitated animals. This was reflected in a statistically significant difference in between the groups in the repeated measures linear regression analysis and differences between groups at

both 9 and 12 hours post resuscitation. This result has some parallels to the exploratory analysis of increased mortality seen in the landmark FEAST trial²⁶. In the post hoc analysis of the excess mortality the authors noted that the largest contributor to the excess mortality seen with fluid resuscitation was delayed cardiovascular collapse occurring between 2-11 hours. This mode of death alone accounted for more than half of the additional deaths seen in the fluid resuscitated groups²⁶. The results of our study support the hypothesis that fluid resuscitation produces delayed vascular injury that manifests as vascular hypo-reactiveness hours after the initial bolus is given.

One might expect the above differences seen in the progression of shock to be attributable to alterations in inflammatory signalling and increased systemic inflammation due to fluid resuscitation. However, the measured serum cytokines do not support this hypothesis. While endotoxemia produces large increases in all the measured cytokines, fluid resuscitation had little additional effect with both groups having very similar cytokine profiles over the entire course of the study.

Recently the role of the endothelial glycocalyx in pathophysiology of sepsis has received increased attention. The glycocalyx being a thin gel structure lining the vascular endothelium thought to play a critical role in maintaining plasma oncotic pressure, prevention of leukocyte adhesion, inhibition of coagulation activation and vascular responsiveness^{27,28}. It has been demonstrated to be shed in both sepsis and endotoxemia with circulating levels of glycocalyx fragments being associated with mortality^{29,30}. ANP has been shown to be a potent trigger for glycocalyx shedding in experimental models of exogenous ANP administration^{31,32}. Similarly transgenic mice with knock-out inactivation of the endothelial guanylyl cyclase-A ANP receptor

demonstrated decreased vascular permeability compared to controls and did not have the same immediate increase in haematocrit from vascular leak in response to exogenous ANP³³. Furthermore, hypervolemia has been shown to be a potent trigger for glycocalyx shedding potentially mediated by ANP release³⁴. In our model, endotoxemia was associated with large increase in ANP (more than 14 fold in both groups). Fluid resuscitation resulted in a further rise in ANP whilst levels in the non-fluid resuscitated group had already begun to decline. This additional increase in ANP may be potentially pathological and cause increased glycocalyx shedding that may exacerbate vascular dysfunction. This is supported by the increased rate of rise of the glycosaminoglycan hyaluronan in the fluid resuscitated animals.

One of the main aims of fluid resuscitation in sepsis is the reversal of pathologic tissue hypoperfusion preventing the onset of tissue ischemia and organ dysfunction^{6,7}. We aimed to use tissue microdialysis to detect potentially favourable effects of fluid resuscitation on the microcirculation of the various vital organs. The lactate/pyruvate ratio is a sensitive marker of the redox state of a tissue with high levels associated with hypoperfusion and impaired oxidative metabolism³⁵. It would be expected that if fluid resuscitation had reversed or improved pathological hypoperfusion this would be reflected in a comparatively lower L/P ratio in the respective organ. This was not demonstrated in arterial circulation, heart, brain or kidney with remarkably similar L/P ratios throughout the experimental period. The liver results are notable for the large difference in baseline values prior to resuscitation with the hepatic L/P ratio for the NR group nearly 3 fold higher than that of the FR group. There was a significant difference in the hepatic L/P ratios after resuscitation however the significance of this must be questioned given the large differences in baseline values. The authors are not able to

explain the discordant baseline values given the identical treatment of the experimental subjects prior to resuscitation. It is notable however that the hepatic L/P ratio in the NR decreased throughout the observation period and had approximated the FR group by the end of the study.

Similarly if fluid resuscitation improved perfusion and prevented organ injury one might expect to see a difference in serum biomarkers over time between groups (creatinine and troponin). In this study there was no difference in serum creatinine between groups throughout the experiment with both groups developing evidence of acute kidney injury at a similar rate. Of note, serum troponin was similar between groups for the majority of the study however there was a dramatic late rise seen in the fluid resuscitated group. This finding is of interest as increases in serum troponin have been observed in septic patients without obstructive coronary disease and are associated with increased mortality³⁶. The mechanism underlying this phenomena is unclear but a number of potential contributing mechanisms have been proposed including mitochondrial oxidative stress, microvascular thrombi and increased sarcolemma membrane permeability^{36,37}. Catecholamines have been shown to increase myocardial oxidative stress in experimental models,³⁸ and in a murine model of sepsis Haileselassie et al demonstrated that increased oxidative stress was a central factor in development of septic induced myocardial dysfunction. The observed increase in troponin is a concerning find as it suggests the increased vasopressor requirement is not a benign epiphenomena and may produce secondary cardiac injury.

Arterial lactate has been shown to be associated with increased mortality in sepsis³⁹. Furthermore a serial decrease in arterial lactate during treatment is associated with

improved outcomes⁴⁰. In this study, fluid resuscitation did not result in a significantly lower serum lactate in the immediate post resuscitation period or result in a significantly lower arterial lactate at any timepoint. Contrary to what would be expected there was a trend towards higher arterial lactate in the FR group as the monitoring period progressed with the final median lactate in the FR group of 9.1mmol/L compared to 4.9mmol/L in the NR group. Similarly the metabolic acidosis was worse in the FR sheep throughout the post-resuscitation period. Neither of these results support the conclusion that fluid resuscitation had favourable effects on the progression of metabolic derangements associated with endotoxemia.

It is important to recognize the limitations of this study. The observed association between increased ANP, increased hyaluronan and a need for increased vasopressors is interesting given the current understanding of the function of the glycocalyx in vascular function. However, this study is not able to quantify the degree to which the observed differences in both ANP and hyaluronan account the differences in vasopressor requirements. Future studies exploring this phenomena with either ANP inhibition or exogenous ANP are required to establish the degree to which changes in glycocalyx function are responsible for changes in vascular reactivity. Additionally, as the trial was investigating the harms seen in the FEAST study the same fluid bolus dose (40mls/kg) was used. This is more than is recommended by the surviving sepsis guidelines⁴ and the same effects may not be seen with different volumes or rates of administration.

Conclusion

In conclusion, we found fluid resuscitation had a detrimental effect on endotoxemic shock with an increased vasopressor requirement in the 12 hours after resuscitation. This did not appear to be attributable to increased inflammatory signalling with the inflammatory cytokine results suggesting a non-inflammatory mechanism for the difference seen. Fluid resuscitation was associated with an increase in plasma ANP and the glycocalyx glycosaminoglycan hyaluronan suggesting it resulted in iatrogenic damage to the glycocalyx. This may have been a causative factor in the need for higher doses of vasopressor after resuscitation. Additionally we were not able to demonstrate any beneficial effects of fluid resuscitation with respect to individual organ metabolism or any of the measured markers of injury or function.

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Competing financial interests

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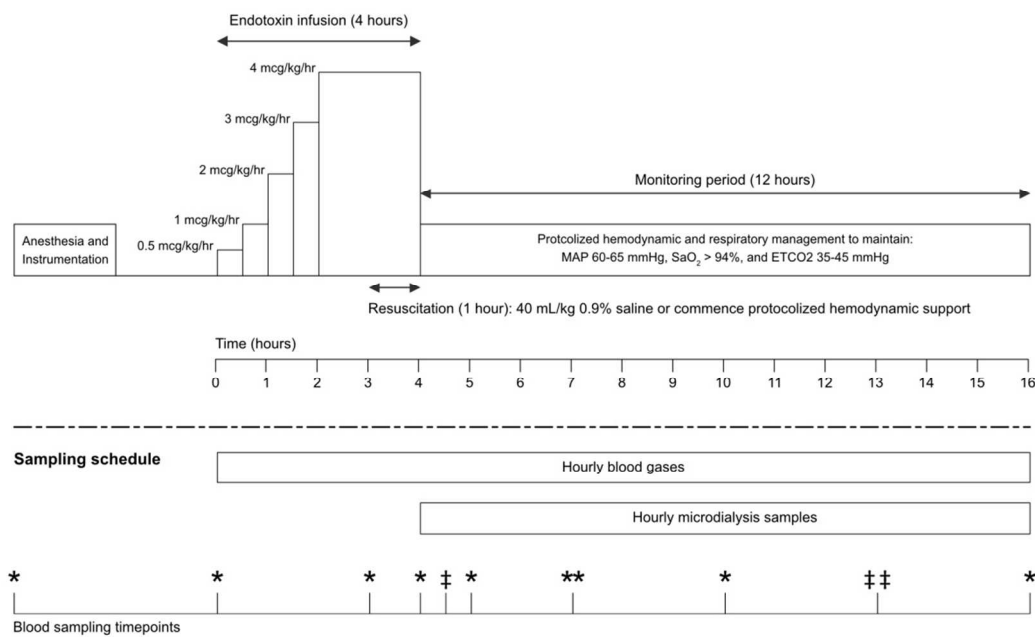
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Table 1: Systemic hemodynamic variables at baseline and throughout the experimental period in the fluid resuscitated (FR) and non-fluid resuscitated animals (NR)

Timepoint	Mean arterial pressure mmHg (mean and SD)			Cardiac index L/min/m ² (median and IQR)			Systemic vascular resistance index dynes · sec/cm ⁵ (median and IQR)		
	FR group n=8	NR group n=8	P-value	FR group n=8	NR group n=8	P-value	FR group n=8	NR group n=8	P-value
T 0 (Baseline - Start endotoxin infusion)	87.1 [78.7– 95.5]	84.5 [69.2– 99.8]	P = 0.68	2.78 [2.61, 3.18]	3.23 [2.78, 3.74]	P = 0.29	2270 [1918, 2815]	2000 [1757, 2111]	P = 0.21
T 3 (Start resuscitation)	44.6 [35.2– 54]	48.5 [38.8– 58.2]	P = 0.43	3.42 [2.52, 3.72]	2.91 [2.70, 3.04]	P = 0.41	1110 [950, 1230]	1155 [1029, 1340]	P = 0.53
T 4 (End resuscitation/endotoxin)	52.8 [48.4– 57.2]	60.4 [58.1– 62.7]	P < 0.001*	4.94 [3.91, 5.62]	3.08 [2.94, 3.70]	P = 0.006*	714 [630, 807]	1434 [1146, 1492]	P < 0.001*
T 6 (2 hours after resuscitation)				3.52 [3.08, 3.96]	2.53 [2.27, 3.28]	P = 0.046*	1236 [1043, 1379]	1858 [1425, 2176]	P = 0.02*
T 8 (4 hours after resuscitation)	60.1 [57.5– 62.7]	57.8 [53.6– 62]	P = 0.20	3.62 [2.92, 4.28]	2.98 [2.59, 3.66]	P = 0.40	1212 [982, 1372]	1518 [1248, 1722]	P = 0.09
T 12 (8 hours after resuscitation)				4.17 [3.62, 4.98]	3.10 [2.76, 4.71]	P = 0.21	1006 [871, 1149]	1412 [806, 1513]	P = 0.21
T 16 (12 hours after resuscitation)	56.9 [53.5– 60.3]	61.9 [59.5– 64.3]	P = 0.15	4.02 [3.14, 5.26]	3.85 [3.47, 5.53]	P = 0.99	910 [814, 1036]	1147 [827, 1269]	P = 0.25

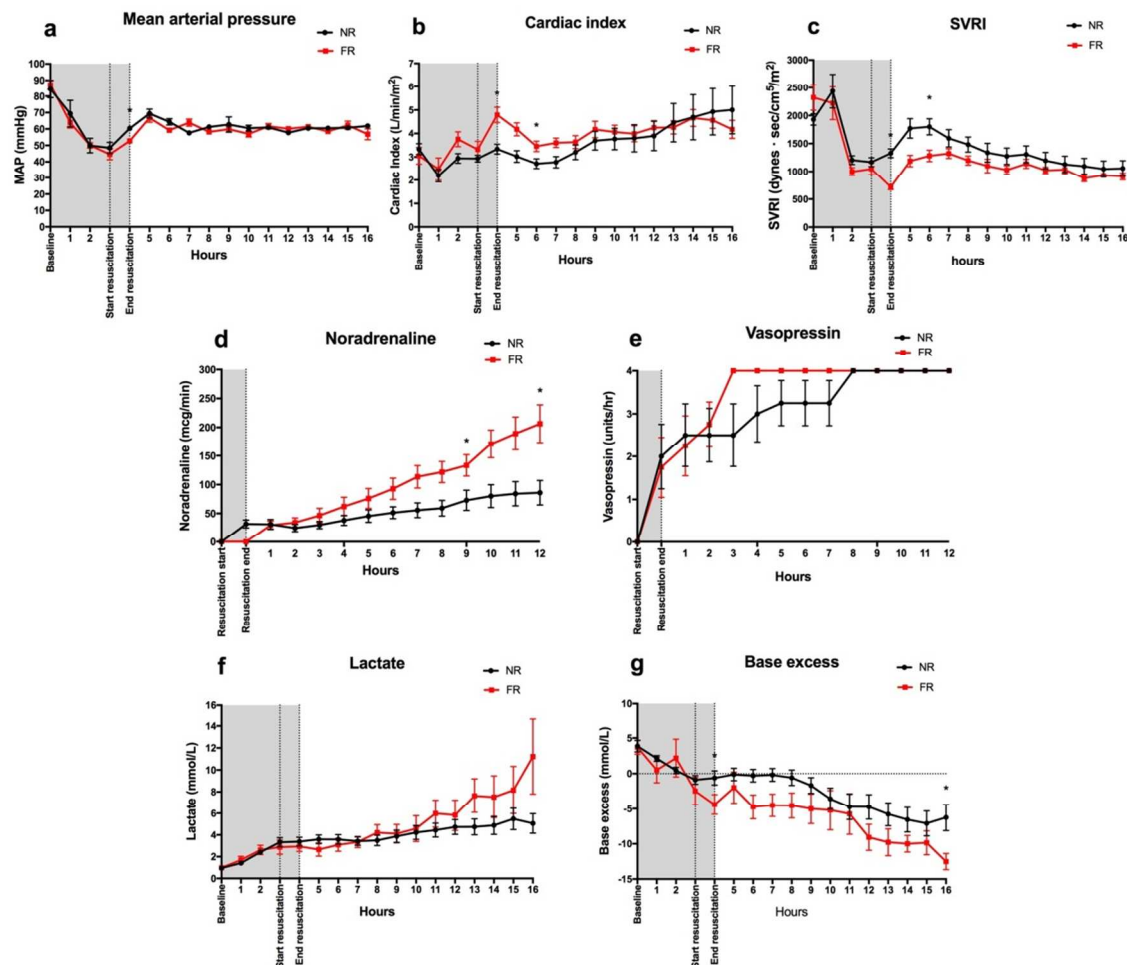
*Indicates statistically significant difference p value < 0.05.

Figure 1: experimental timeline



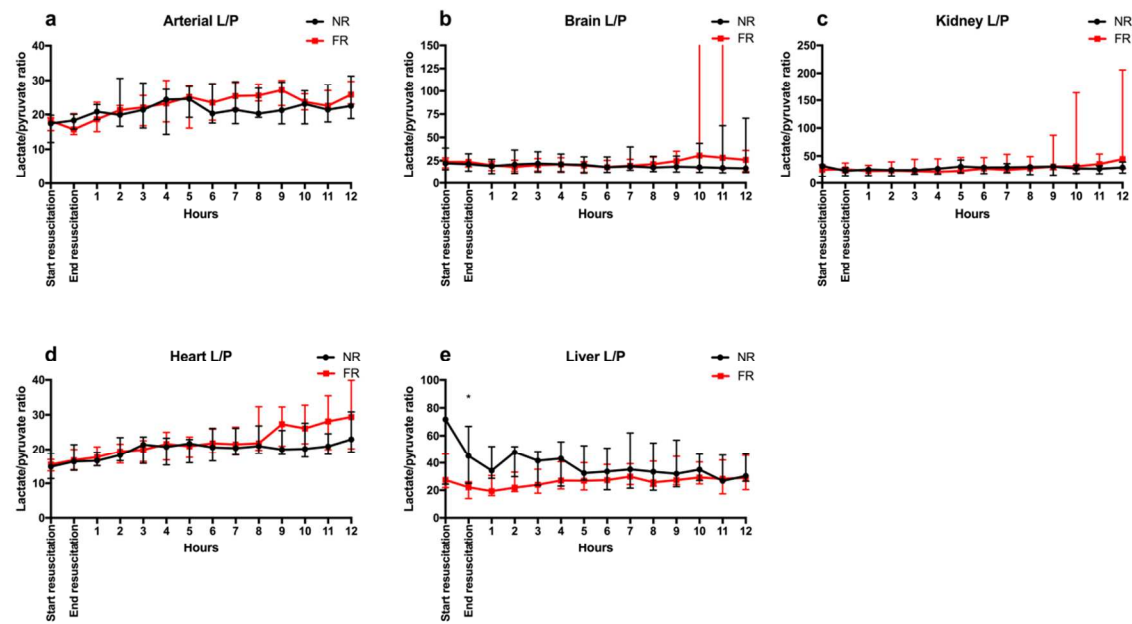
Schematic representation of the experimental protocol. Anesthesia and surgical instrumentation were followed by a 1 hour stabilization period during which no interventions were performed. Endotoxemic shock was induced with a 4 hour escalating dose endotoxin infusion. Resuscitation occurred in the last hour of the endotoxin infusion with animals either receiving a 40ml/kg bolus of 0.9% saline or commencing protocolized haemodynamic support. After resuscitation all animals were monitored for a further 12 hours during which both groups received protocolized haemodynamic and respiratory support. Blood gases were taken hourly during the 16 hours of the experiment and analyzed immediately on an ABL800 Flex (Radiometer, Copenhagen, Denmark). During the monitoring period hourly microdialysis samples were recovered and analyzed for lactate and pyruvate on an ISCUS clinical microdialysis analyzer (Hammarby Fabriksväg, Stockholm, Sweden). The bottom scale indicates the blood sampling timepoints throughout the experiment. * Indicates timepoints at which blood was taken for serum cytokines (IL-1 β , IL-6, IL-8, IL-10 and TNF α), troponin, ANP, BNP, hyaluronan and creatinine. † Indicates timepoints at which blood was taken to measure hyaluronan only. ** Indicates timepoints at which blood was taken to measure serum cytokines and hyaluronan. †† Indicates timepoints blood was taken to measure serum cytokines. Cytokine measurements were performed using an in house ELISA for interleukin 1-beta (IL-1 β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10) and tumour necrosis factor alpha (TNF α). The methodology of the cytokine analysis has been published previously¹⁵. Cardiac troponin I was measured using Beckman Coulter Unicel Dxl AccuTnI+3 immunoassay (Beckman Coulter, Brea, CA, USA). Hyaluronan was measured using a hyaluronan quantikine ELISA Kit (R&D systems, Minneapolis, USA). Serum ANP and BNP were measured using custom ovine radioimmunoassay (Endolab, Christchurch Heart Institute, Christchurch, New Zealand). Creatinine was measured using a COBAS Integra 400 blood chemistry analyzer (Roche Diagnostics, Australia).

Figure 2: Hemodynamic, vasopressors and acid/base variables during the experiment in both fluid resuscitated (FR) and non-fluid resuscitated animals (NR)



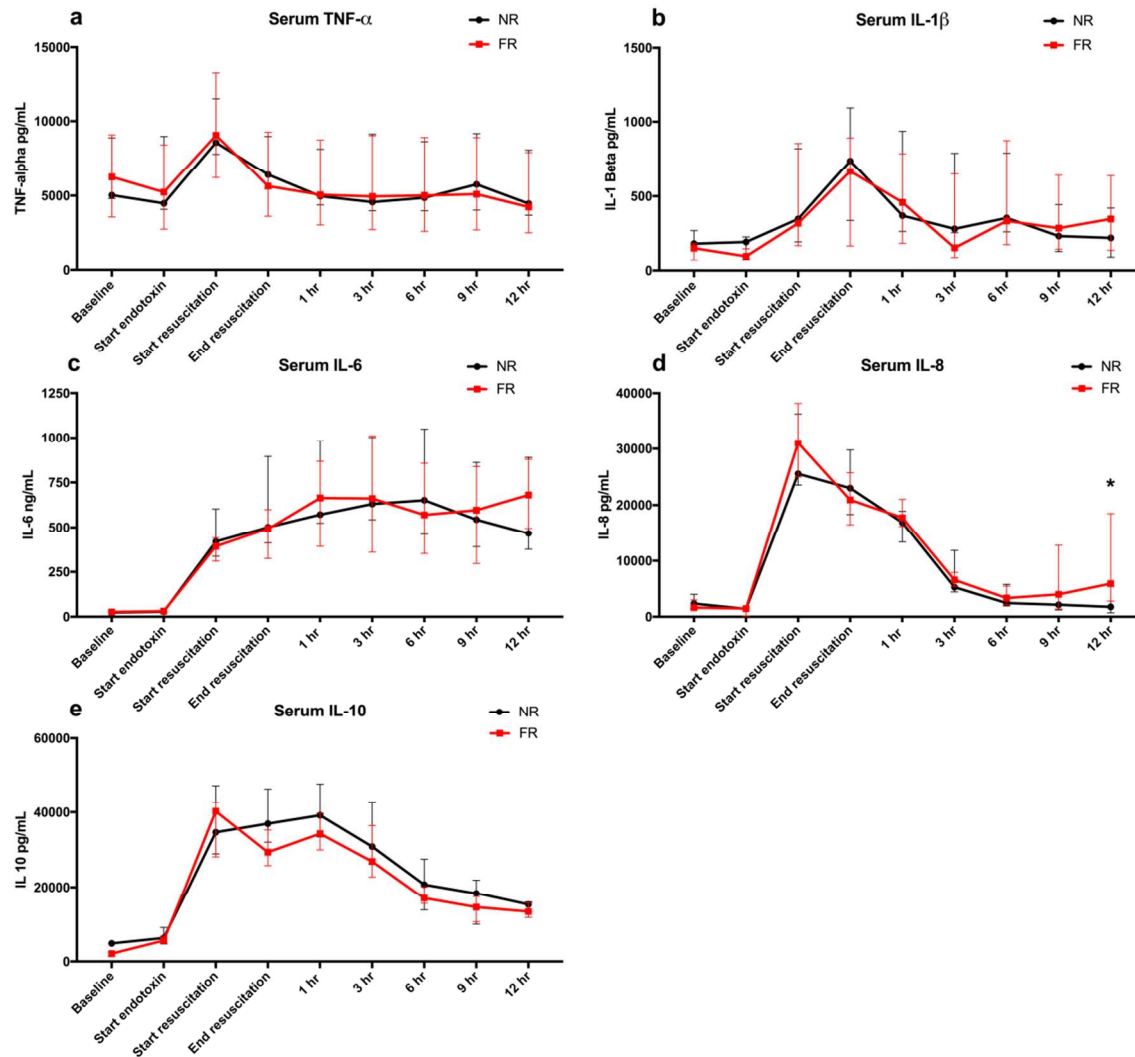
(A) Mean arterial pressure during endotoxemia, resuscitation and post-resuscitation monitoring period. Grey shading corresponds to time period of endotoxin infusion. Results are expressed as mean $\pm$ SD. (B & C) Cardiac index and systemic vascular resistance during the experiment. Results are expressed as median values with IQR. (D) Noradrenaline infusion rate during the experiment, median and IQR. Linear regression analysis demonstrated the rate of increase to be significantly greater in the FR group. (E) Vasopressin infusion rates during the experiment, median and IQR. There was no significant difference in vasopressin infusion rates. All animals required the maximal dose of vasopressin by the end of the study. (F & G) Lactate and base excess over the experiment, data represented as mean $\pm$ SD. * indicates timepoints with a statistically significant difference ($p < 0.05$)

Figure 3: Individual organ lactate/pyruvate ratios recovered by microdialysis



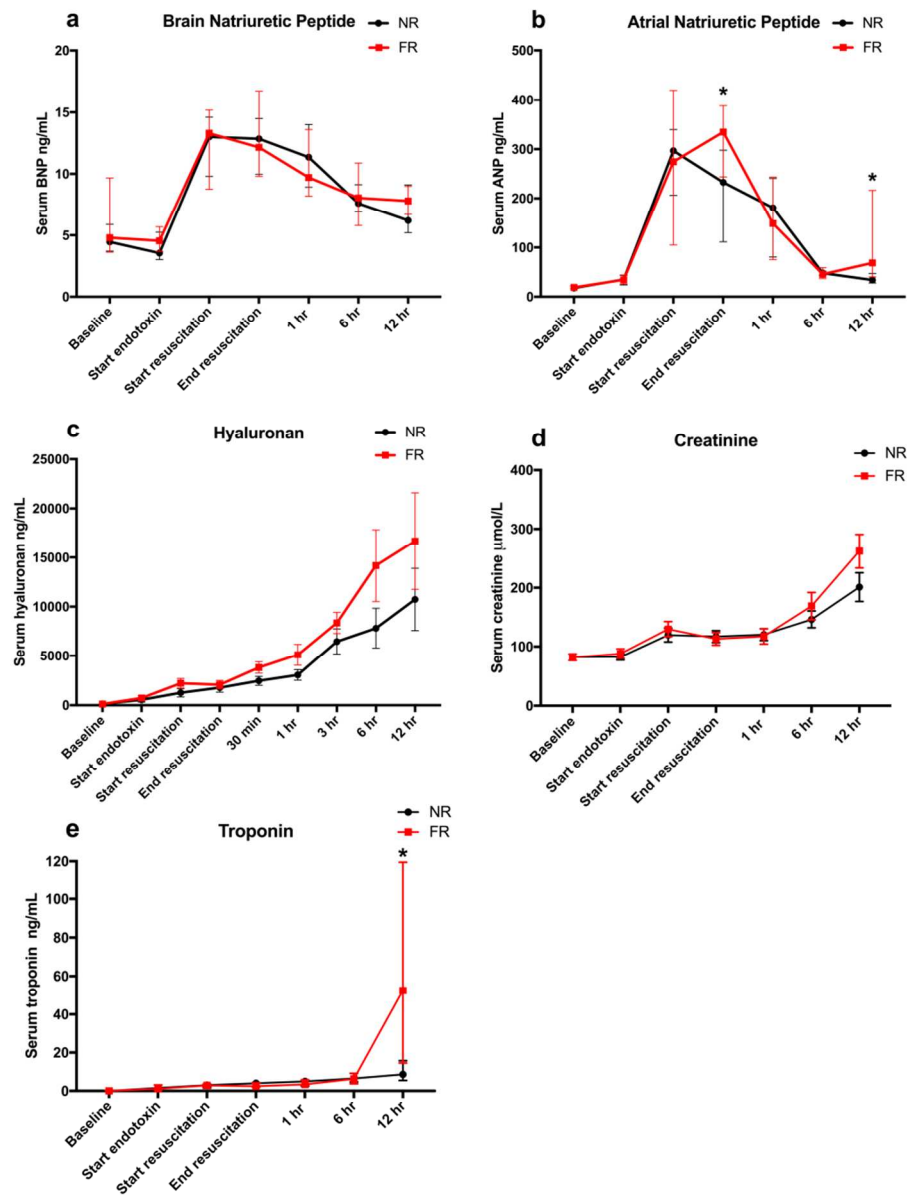
Individual organ lactate/pyruvate ratios for the period after resuscitation for fluid resuscitated (FR) and non-fluid resuscitated animals (NR). (A) Arterial L/P ratios during the post resuscitation period, values between groups were similar throughout. (B) Brain L/P ratios during the post resuscitation period, variance seen in IQR after hour 10 was due to the rapid increase in L/P ratio in a single animal in the final 3 hours of the experiment (>3400). (C) Kidney L/P ratio during the same period, there were no significant difference between groups at any of the timepoints. (D) Heart L/P ratio during the same period, there were no significant difference between groups at any of the timepoints. (E) Liver L/P ratios during the period after resuscitation. Values for the NR group started at a higher baseline than the FR group. After resuscitation values in the NR were significantly greater than those in the FR. All data represented as median values with IQR. * Indicates $P < 0.05$.

Figure 4: Plasma cytokines during the experiment in both fluid resuscitated (FR) and non-fluid resuscitated animals (NR)



Plasma cytokine levels for both fluid resuscitated and non-fluid resuscitated animals throughout the experiment. (A) Serum TNF- α increased during endotoxemia reaching peak values in both groups at the beginning of resuscitation before returning to near baseline levels. (B) Serum IL-1 β increased during endotoxemia in both groups reaching maximal values at the end of resuscitation. (C) Endotoxemia produced large increases in IL-6 in both groups that were sustained throughout the study. (D) Serum IL-8 reached maximal values in both groups at the beginning of resuscitation. IL-8 decreased in both groups after resuscitation, however there was a late increase in IL-8 in the FR animals with final IL-8 values statically significantly different between groups ($p=0.04$). (E) Endotoxin produced a large increase in IL-10 in both groups that slowly decreased throughout the monitoring period. Results are expressed as median values $\pm$ IQR.

Figure 5: Cardiovascular and renal biomarkers during the experiment in both fluid resuscitated (FR) and non-fluid resuscitated animals (NR).



(A) Endotoxemia produced a large increase in plasma BNP in both groups that then declined throughout the observation period in both groups (median + IQR). (B) Serum ANP rose in response to endotoxemia in both groups. Plasma ANP peaked at the initiation of resuscitation in the non-fluid resuscitation group. In the fluid resuscitation group plasma ANP rose with endotoxemia and increased further after resuscitation. There was a statistically significant difference in plasma ANP between groups after resuscitation ($p=0.02$) (median + IQR). (C) Hyaluronan increased in both groups during the study (mean + s.d.). (D) Serum creatinine was not significantly different at any timepoint and increased in both groups throughout the study (median + IQR). (E) Serum troponin values were similar between groups until the end of the experiment where there was a large increase in the fluid resuscitated animals (median and IQR) ($P=0.03$).