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## Systemic release of cytokines and heat shock proteins in porcine models of polytrauma and hemorrhage

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### Abstract

**Objective**—To define systemic release kinetics of a panel of cytokines and heat shock proteins (HSP) in porcine polytrauma/hemorrhage models and to evaluate whether they could be useful as early trauma biomarkers.

**Design and Setting**—Prospective study in a research laboratory.

**Subjects**—Twenty-one Yorkshire pigs.

**Measurements and Main Results**—Pigs underwent polytrauma (femur fractures/lung contusion, P), hemorrhage (mean arterial pressure 25-30mmHg, H), polytrauma plus hemorrhage (P/H) or sham procedure (S). Plasma was obtained at baseline, in 5-15min intervals during a 60min shock period without intervention and in 60-120min intervals during fluid resuscitation for up to 300min. Plasma was assayed for IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/IL-23p40, IL-13, IL-17, IL-18, IFN $\gamma$ , TGF $\beta$ , TNF $\alpha$ , HSP40, HSP70 and HSP90 by ELISA. All animals after S, P and H survived (n=5/group). Three of six animals after P/H died. IL-10 increased during shock after P and this increase was attenuated after H. TNF $\alpha$  increased during the shock period after P, H and also after S. P/H abolished the systemic IL-10 and TNF $\alpha$  release and resulted in 20-30% increased levels of IL-6 during shock. As fluid resuscitation was initiated TNF $\alpha$  and IL-10 levels decreased after P, H and P/H, HSP 70 increased after P, IL-6 levels remained elevated after P/H and also increased after P and S.

**Conclusions**—Differential regulation of the systemic cytokine release after polytrauma and/or hemorrhage, in combination with the effects of resuscitation, can explain the variability and inconsistent association of systemic cytokine/HSP levels with clinical variables in trauma patients. Insults of major severity (P/H) partially suppress the systemic inflammatory response. The plasma concentrations of the measured cytokines/HSPs do not reflect injury severity or physiological

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changes in porcine trauma models and are unlikely to be able to serve as useful trauma biomarkers in patients.

### Keywords

biomarker; resuscitation; inflammation; injury severity; shock; mortality

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### Introduction

Severe trauma has been documented to induce dysfunction of various immune cells (1-6). Their production of mediators that induce and determine the magnitude of the systemic inflammatory response syndrome (SIRS) is regarded as a key contributing factor for patient morbidity and mortality (7-9). Accordingly, systemic levels of various markers of inflammation, such as cytokines or heat shock proteins (HSP), have been used to document inflammation and have been associated with disease severity and outcomes (10-21). However, robust correlations between systemic levels of inflammation markers with injury severity and clinically relevant outcome variables after trauma in patients are missing. Many reasons exist to explain the inability of systemic inflammation markers to serve as clinically useful trauma biomarkers. Trauma patient populations are inherently heterogeneous. Besides the diversity of the individual organ and tissue injuries, various degrees of injury-associated hemorrhage complicate the disease pathology. Furthermore, the mediator response to trauma shows large inter-individual variability, the systemic half-life of cytokines is usually short and their release kinetics in patients are poorly defined (22-26). Therefore, blood sampling intervals that have been used in previous observational studies in patients could have missed a useful diagnostic or prognostic window, and subsequent therapeutic interventions may have further confounded relevant associations (3, 27).

It is obvious that a detailed characterization of the early release kinetics of inflammation markers after trauma in patients is problematic. Thus, trauma models are required to define their release kinetics, to establish possible associations with injury severity, organ dysfunction and outcome, and to provide a rational framework for the design of future clinical observational studies.

In the majority of trauma models, the mechanical trauma component, such as isolated extremity fracture or laparotomy, is minor and hemorrhage is superimposed to produce shock and enhance the physiological and immunological responses to injury (28-31). Accordingly, it was suggested that hemorrhage-associated ischemia and reperfusion injury is probably the predominant pathophysiological effector in trauma models that use a combination of minor injury and hemorrhage (29). Thus, little information is currently available about the influence of major trauma, hemorrhage and their combination on the systemic release of inflammation markers. In order to understand their association with clinically relevant variables, a detailed characterization of their release kinetics after major trauma, hemorrhage, and major trauma plus hemorrhage is needed. To fill this gap, we sought to define the systemic release patterns of a broad panel of cytokines and heat shock proteins (HSPs) in a porcine model of polytrauma. In this model, we aimed to evaluate the effects of an additional hemorrhage component on those markers of inflammation, which increase in the systemic circulation after polytrauma alone. We hypothesized that the addition of a hemorrhagic component would increase the overall injury severity which will be manifested by a further increase in systemic concentrations of cytokines and/or HSPs.

## Material and Methods

### General instrumentation and data collection

All procedures were performed according to National Institutes of Health Guidelines for Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Twenty-one male and female Yorkshire pigs (30-40 kg of body weight) were fasted overnight. Anesthesia was induced with 10 mg/kg ketamine and 1 mg/kg xylazine intramuscularly. Peripheral intravenous (i.v.) access was obtained for continuous infusion of 10 mg/kg/h ketamine, 0.25 mg/kg/h xylazine and 50  $\mu$ g/kg/h fentanyl for maintenance of anesthesia throughout the experiment. Orotracheal intubation was performed using 5.0-6.0 mm internal diameter cuffed endotracheal tubes (Teleflex Medical) under direct laryngoscopy. Animals were mechanically ventilated (Evita XL, Draeger Medical) with intermittent mandatory ventilation adjusted to tidal volumes of 12 mL/kg at 12-18 breaths per minute to maintain a partial pressure of CO<sub>2</sub> (pCO<sub>2</sub>) between 35-45 mmHg. The fraction of inspired oxygen (FiO<sub>2</sub>) was 0.4 and positive end expiratory pressure (PEEP) 5 mmHg, except where otherwise noted. Core body temperature was maintained using conductive warming blankets (Gaymar T/Pump 500 T/Pad). A central venous catheter (Triple-Lumen 7Fr. AGB+ Arrow Catheter, Teleflex Medical) was placed in the external jugular vein for administration of fluids, anesthesia and continuous monitoring of central venous pressure (CVP). The ipsilateral common carotid artery was cannulated for continuous measurement of mean arterial pressure (MAP). Electrocardiography, pulse oximetry, capnography (Evita XL Capnography module, Draeger Medical) and body temperature were measured and monitored continuously. Arterial blood was sampled every 30 min for measurements of pH, pCO<sub>2</sub>, pO<sub>2</sub>, hemoglobin, sodium, potassium, chloride, glucose and lactate using a blood gas analyzer (Stat Profile pHox Plus L, Nova Biomedical). Venous blood was obtained at baseline (two samples per animal in 20 min intervals) and every five minutes for the first 30 minutes after injury, followed by increasing intervals (15 – 120 min) for the remaining 270 minutes. Venous blood was collected in lithium heparin tubes (APP Pharmaceuticals), centrifuged at 1000  $\times$  g for 30 min, plasma separated, aliquoted and stored at –80°C until further analyses of total protein content, cytokines, HSPs and creatine kinase (CK) levels. Plasma protein was measured using the NanoDrop micro-volume methodology (Thermo Scientific) and CK plasma levels were determined in the routine veterinary laboratory of the Loyola University Chicago comparative medicine facility.

### Polytrauma and hemorrhage

Polytrauma consisted of bilateral open femur fractures and lung contusion, modified as described previously (32, 33). Injuries were produced with a captive bolt gun (Karl Schermer), modified with exchangeable mushroom shaped metal heads (1 and 2.5 inches in diameter). In brief, the bolt gun with the small metal head was placed vertically against the femur and fired while the animal was in supine position and the leg extended. The metal head perforated the skin and produced a 2<sup>nd</sup> degree complex open femur fracture without injury of major vessels. After both femurs were fractured, the small metal head was exchanged for the large metal head and the bolt gun was fired against the right chest wall in the midaxillary line at the level of the fourth intercostal space with a 45° cephalad trajectory. As confirmed by necropsy, this resulted in lung contusion involving approximately 20-30% of the right lung without producing hemo/pneumothoraces or rib fractures. All injuries were produced within 5 min. Based on an Abbreviated Injury Score (AIS) of 3 for the extremity trauma and 3 - 4 for the chest trauma, the estimated Injury Severity Score was 18 – 25 (34). In the experimental groups with hemorrhage, blood was rapidly withdrawn from the arterial catheter to maintain a MAP of 25-30 mmHg until t=60 min, as described previously (32, 35).

## Experimental groups and treatment protocol

After achieving stable baseline conditions (at least 30 min after instrumentation) animals were randomized to one of the following four experimental groups: 1. *Polytrauma only* (n = 5): Animals were subjected to bilateral femur fractures plus lung contusion. 2. *Hemorrhage only* (n = 5): Animals were hemorrhaged to a MAP of 25 - 30 mmHg for 60 min. 3. *Polytrauma plus hemorrhage* (n = 6): Animals were subjected to bilateral femur fractures plus lung contusion followed by hemorrhage to a MAP of 25 - 30 mmHg for 60 min. 4. *Sham control* (n = 5): No injury or hemorrhage.

To simulate a 60 min period of shock after trauma, animals were ventilated with FiO<sub>2</sub> 0.21, PEEP 0 mmHg and no resuscitation was allowed other than the minimum amount of lactated Ringer's solution required for delivery of anesthesia. At t=60 min, ventilation was adjusted to FIO<sub>2</sub> 0.4, PEEP 5 mmHg and resuscitation to a MAP of 70 mmHg was performed with warmed lactated Ringer's solution. Between t=60 - 120 min, lactated Ringer's solution was administered in i.v. boluses of 500 mL until the MAP reached 70 mmHg to simulate typical human resuscitation regimens during the pre-hospital phase. From 120 min until the end of the experiment, resuscitation was performed continuously to maintain a MAP of at least 70 mmHg to simulate in-hospital resuscitation. At the conclusion of the experiment (t=300 min), a mixture of saturated potassium chloride was infused via the central venous catheter for euthanasia while the animal was under general anesthesia.

## Cytokines and HSPs

Plasma levels of cytokines and HSPs were measured utilizing the following commercially available porcine enzyme linked immunosorbent assays (ELISAs): Interleukin (IL)-1 $\beta$  (R&D Systems; lower detection limit (LDL): 10 pg/mL), IL-4 (Invitrogen; LDL: 2 pg/mL); IL-5 (USCN Life Science; LDL: 3.9 pg/mL), IL-6 (R&D Systems; LDL: 9 pg/mL), IL-8 (R&D Systems; LDL: 4.6 pg/mL), IL-10 (Alpco Diagnostics; LDL: 3 pg/mL), IL-12/IL-23 p40 (R&D Systems; LDL: 9 pg/mL), IL-13 (USCN Life Science; LDL: 7.8 pg/mL), IL-17 (USCN Life Science; LDL: 7.8 pg/mL), IL-18 (Invitrogen; LDL: 21.3 pg/mL), Interferon (IFN) $\gamma$  (R&D Systems; LDL: 6.1 pg/mL), Transforming Growth Factor (TGF) $\beta$ 1 (R&D Systems; LDL: 4.6 pg/mL), Tumor Necrosis Factor (TNF) $\alpha$  (R&D Systems; LDL: 3.7 pg/mL), HSP40 (USCN Life Science; LDL: 78 pg/mL), HSP70 (USCN Life Science; LDL: 78 pg/mL), HSP90 (USCN Life Science; LDL: 78 pg/mL). All assays were performed according to the manufacturers' protocols using plasma that had not been thawed previously. Measurements were performed after all animal experiments had been completed.

## Algorithm for cytokine/HSP measurements, data analysis and statistics

The following algorithm was used to limit the number of cytokine/HSP measurements: All cytokine/HSPs were measured in plasma from animals after polytrauma only (group 1). This was done because hemorrhage without significant tissue damage is unlikely to occur after severe blunt trauma in patients. Only cytokines/HSPs which were consistently detectable (50% of all specimens after polytrauma with plasma levels above the detection limit of the ELISA) and which showed significant increases of their plasma concentrations from baseline after polytrauma alone were then measured in the specimens obtained from groups 2-4. Data are described as mean  $\pm$  standard deviation (SD) or median with range (in parenthesis), as appropriate. Normal distribution was assessed with the Komolgorov-Smirnov test. Most of the cytokine/HSP plasma concentrations did not pass the normality test ( $\alpha = 0.05$ ). Therefore, Kruskal-Wallis H test with Dunn's post hoc correction to control for multiple testing were used to compare differences between the experimental phases (baseline, shock, resuscitation) and groups. The average plasma level during each of the experimental phases was calculated for each animal and used for statistical analyses. Because physiological and clinical parameters were normally distributed, these data were

analyzed with Student's t-test or one-way analysis of variance with Tukey post-hoc test to correct for multiple testing. Chi-square test was used for dichotomous categorical variables. Data analyses were calculated with the GraphPad Prism program (GraphPad Software). A two-tailed  $p < 0.05$  was considered significant.

## Results

### Physiological responses to trauma and hemorrhage

There were no statistically significant differences between physiological variables among the four experimental groups at baseline ( $p > 0.05$  for all measurements). CK plasma levels increased from  $449 \pm 154$  U/mL at baseline to  $2000 \pm 674$  U/mL at 300 min after polytrauma alone ( $p < 0.05$ ). The MAP (Fig. 1A) decreased to 50-55 mmHg during the shock period after polytrauma alone. The hemorrhage volume to achieve the MAP target value of 25-30 mmHg during the shock period in the group with hemorrhage only was  $37 \pm 7$  mL/kg and  $24 \pm 8$  mL/kg in the group with polytrauma plus hemorrhage ( $p < 0.05$  vs. hemorrhage only). Animals in the control group required  $34 \pm 20$  mL/kg of i.v. fluids to maintain MAP of 70 mmHg during the observation period (Fig. 1B). The fluid requirements to maintain the target MAP increased to  $103 \pm 60$  mL/kg after polytrauma alone ( $p < 0.05$  vs. control) and to  $257 \pm 63$  mL and  $271 \pm 50$  mL/kg after hemorrhage alone and hemorrhage plus polytrauma (both groups:  $p < 0.05$  vs. sham control and polytrauma alone), respectively. Hematocrit values (Fig. 1C) remained constant in the sham control group (30%), decreased in all other groups and remained constant during the resuscitation period at 18-20% after polytrauma alone and at 13-15% in the groups with hemorrhage. Plasma protein concentrations paralleled changes in hematocrit values (Fig. 1D). Lactate levels increased in all trauma and/or hemorrhage groups ( $p < 0.05$  vs. control) and reached peak levels of  $5.5 \pm 2.2$  mmol/L,  $6.5 \pm 0.5$  mmol/L and  $9.2 \pm 3.2$  mmol/L between 60-120 min after polytrauma only, hemorrhage only and polytrauma plus hemorrhage ( $p > 0.05$  between groups), respectively (Fig. 1E). In all trauma/hemorrhage groups the ratio of arterial oxygen concentration to the fraction of inspired oxygen (P/F) decreased during the shock period below 200 and recovered during the resuscitation period when ventilated with PEEP of 5 mmHg,  $FiO_2$  0.4 (Fig. 1F). Mortality was 50% after polytrauma plus hemorrhage and 0% in all other groups ( $p < 0.05$ ; Fig. 1G).

### Plasma levels of cytokines and HSPs

The plasma concentrations of the various cytokines and HSPs during experimental baseline conditions, shock and resuscitation are summarized in Tab. 1. IL-1 $\beta$ , IL-18 and IFN $\gamma$  were not detectable. IL-4, IL-8, IL-17 and HSP40 were detectable in only few plasma specimens and did not show significant changes between the experimental periods. IL-5, IL-12/IL-23 p40, IL-13, TGF $\beta$  and HSP90 were detectable in all plasma specimens. However, their plasma concentrations did not show significant changes. In contrast, IL-6, IL-10, TNF $\alpha$  and HSP70 were detectable in the majority of plasma specimens and their concentrations increased after polytrauma.

The time course of the plasma levels of the individual cytokines and HSPs that were consistently detectable after polytrauma are shown in Fig. 2-6. Due to the large differences among the individual molecules (Tab. 1), plasma levels are shown as percent of baseline to permit direct comparison of relative changes after polytrauma. In agreement with their average concentrations during the experimental periods (Tab. 1), plasma levels of IL-5, IL-12, IL-13, TGF $\beta$  and HSP90 were unchanged throughout the entire observation period (Fig. 2).



IL-10 (Fig. 3A/B) plasma concentrations increased significantly during the shock period after polytrauma and remained unchanged in sham control animals. When compared with polytrauma alone, the increase in IL-10 plasma levels during the shock phase was attenuated in magnitude and duration after hemorrhage. The combined insult of polytrauma plus hemorrhage did not result in increased IL-10 plasma levels during the shock phase (Fig. 3A/B).

In contrast to IL-10, TNF $\alpha$  plasma levels increased during the shock period after polytrauma alone, hemorrhage alone and also after sham procedure (Fig. 4A/B). Similar to IL-10, TNF $\alpha$  plasma levels did not increase during the shock period after polytrauma with superimposed hemorrhage. As resuscitation was initiated, IL-10 and TNF $\alpha$  plasma concentrations decreased in the trauma/hemorrhage groups (Fig. 3B, Fig. 4B). These effects were also detectable when IL-10 and TNF $\alpha$  plasma levels were normalized to plasma protein content (not shown).

After polytrauma alone, IL-6 plasma levels were unchanged during the shock phase and increased during resuscitation (Fig. 5A/B). However, this effect was also detectable in sham control animals. Although hemorrhage alone was not associated with increases in IL-6 plasma levels, IL-6 levels increased during the shock period and remained constant during resuscitation after polytrauma plus hemorrhage.

HSP70 plasma levels did not show significant changes during the shock period (Fig. 6A/B). During resuscitation, HSP70 levels increased after polytrauma alone, but did not show significant changes in any other group.

## Discussion

In the present study, we provide the first detailed characterization of the immediate systemic release kinetics of a broad panel of inflammation markers in porcine models of polytrauma and hemorrhage. The injuries in our model created a pathophysiological condition that closely resembled the typical clinical characteristics of severely injured blunt trauma patients. The lactate levels after the shock period underscored the injury severity (36-39) and the increase in CK plasma concentrations after polytrauma documented significant soft tissue damage. Hemorrhagic shock alone produced physiological responses comparable with polytrauma alone. As expected, the combination of polytrauma plus hemorrhage resulted in further increases in fluid dependency and lactate levels, and produced mortality during the early resuscitation period. This demonstrates that the overall severity of the insult was highest after polytrauma plus hemorrhage.

Although we expected that plasma levels of several pro- and anti-inflammatory cytokines would increase after polytrauma alone, and that the combined insult of polytrauma plus hemorrhage would further exaggerate their systemic concentrations, only TNF $\alpha$  and IL-10 levels increased during the shock period after polytrauma alone, and the combined insult abolished their release into the systemic circulation. Despite the similarity of the physiological responses to polytrauma alone and hemorrhage alone, comparison of the systemic release kinetics of TNF $\alpha$  and IL-10 documented distinct plasma profiles. This implies differential regulation of the systemic cytokine release after major tissue injury and after hemorrhage, and advises caution in the interpretation of findings from trauma models that rely on a hemorrhage component to evoke physiological changes.

Only IL-6 plasma levels showed a small (25-30%) but statistically significant increase during the shock period after the combination of polytrauma plus hemorrhage, when compared with hemorrhage or polytrauma alone. Our measurements in short intervals during the shock phase excluded transient peak levels of the other molecules during a simulated

pre-hospital phase. These findings make it unlikely that transient peak levels of these molecules during the pre-hospital phase have been overlooked in previous clinical observational studies, and also that measurements of cytokines or HSPs at intervals closer to the traumatic insult may not provide a diagnostic or prognostic advantage.

Although the time dependent and monophasic increase in IL-10 plasma levels during the shock period could be attributed to the inflammatory response induced by blunt trauma, measurements of TNF $\alpha$  after polytrauma alone, hemorrhage alone and sham procedure revealed that the plasma concentrations during the shock phase were identical in regards to magnitude and time progression. This suggests that minor interventions, such as adjustment of PEEP and FiO<sub>2</sub>, modulate the systemic TNF $\alpha$  release to the same degree as severe tissue injury or blood loss and demonstrates that TNF $\alpha$  levels are unable to discriminate the degree of tissue injury. The TNF $\alpha$  and IL-10 plasma levels measured after polytrauma plus hemorrhage also show that their systemic release kinetics do not reflect the magnitude of the physiological response or the severity of the overall insult. Our results indicate that the combined effects of polytrauma and hemorrhage on systemic IL-10 and TNF $\alpha$  plasma concentrations are neither synergistic nor additive. On the contrary, polytrauma plus hemorrhage resulted in significant mortality but did not induce the systemic release of these cytokines. These findings are in agreement with previous observations, which indicated that mortality from trauma in patients and from thoracotomy plus hemorrhage in pigs is associated with a blunted systemic TNF $\alpha$  release (40). Thus, our findings support the concept that major trauma in patients or the combination of polytrauma plus hemorrhage in the experimental setting is associated with the inability to mount an appropriate cytokine response in the systemic circulation (3, 40). As an additional hemorrhage component appears to attenuate systemic release of TNF $\alpha$  and IL-10 after polytrauma, these observations may further reflect that the combined insult leads to the induction of a state of immune paralysis (41).

The unchanged plasma levels of IL-6 during the shock period after polytrauma and hemorrhage alone and the slightly increased levels after a combined insult are consistent with plasma concentrations of IL-6 in trauma patients when blood was collected on the scene of the accident (42, 43). Although IL-6 was the only molecule that showed increased plasma levels with higher injury severity during the shock period, this increase was small and variable.

The changes in plasma levels of cytokines that we detected during the resuscitation period after polytrauma alone are consistent with changes that have been observed in severely injured trauma patients on hospital admission (4, 43-50), which confirms the clinical relevance of our model. However, standard fluid resuscitation resulted in distinct and differential effects on plasma cytokine/HSP concentrations in each experimental scenario. Our findings that TNF $\alpha$  and IL-10 levels also decreased when plasma concentrations were normalized to total plasma protein content, and that all other detectable cytokines and HSPs showed increased or unchanged plasma levels during resuscitation, document that this decrease cannot be attributed to hemodilution. Resuscitation fluids have been shown to influence immune responses after trauma (51-53). Thus, it appears more likely that the multiple changes of TNF $\alpha$ , IL-10, IL-6 and HSP70 levels that were observed during resuscitation in sham control and injured animals are related to the immune modulatory effects of the resuscitation fluid, which further hampers interpretation of findings on systemic cytokine levels from observational studies in trauma models or in patients.

An obvious limitation of the present study is that we did not measure all cytokines/HSPs in all experimental groups. Although such data may provide valuable information from an experimental standpoint, the resources required to perform these analyses would be

extensive. As a controlled hemorrhage only model is of limited clinical relevance and the expected gain in clinically relevant information would be minimal, we refrained from these measurements.

The present study was designed as an observational study to assess the association of numerous inflammation markers with physiological variables and injury severity. Therefore, our data do not permit conclusions on the molecular mechanisms regulating the systemic release of cytokines or on their possible biological roles in the systemic circulation or at local sites after trauma. Nevertheless, it might be speculated that the differentially regulated systemic release kinetics of TNF $\alpha$ , IL-10 and IL-6 in our models are due to distinct patterns of alarmins (damage associated molecular pattern molecules, DAMPs) that are released in response to the specific modes of injury (54, 55). Irrespective of the underlying mechanisms, our findings make it unlikely that these molecules could be useful as a prognostic or diagnostic tool in the clinical setting. The differential effects of polytrauma alone, hemorrhage alone and polytrauma plus hemorrhage, in combination with the influence of fluid resuscitation, are able to explain the large variability and inconsistent association of systemic cytokine levels with clinical variables and outcomes in severely injured patients (13, 14, 45, 56). Because an injury severity that is associated with significant mortality appears to suppress the systemic inflammatory response, at least for TNF $\alpha$  and IL-10, proteins or other molecules that are actively secreted from immunological competent cells in response to trauma are unlikely to be able to serve as useful trauma biomarkers early after injury.

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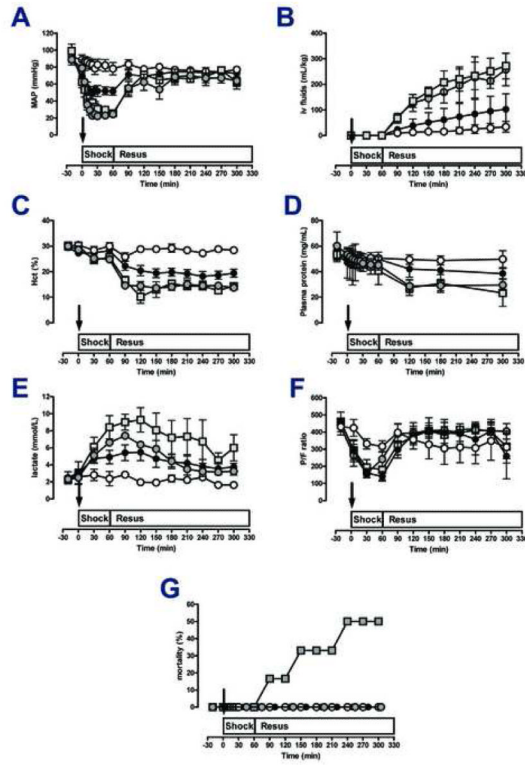
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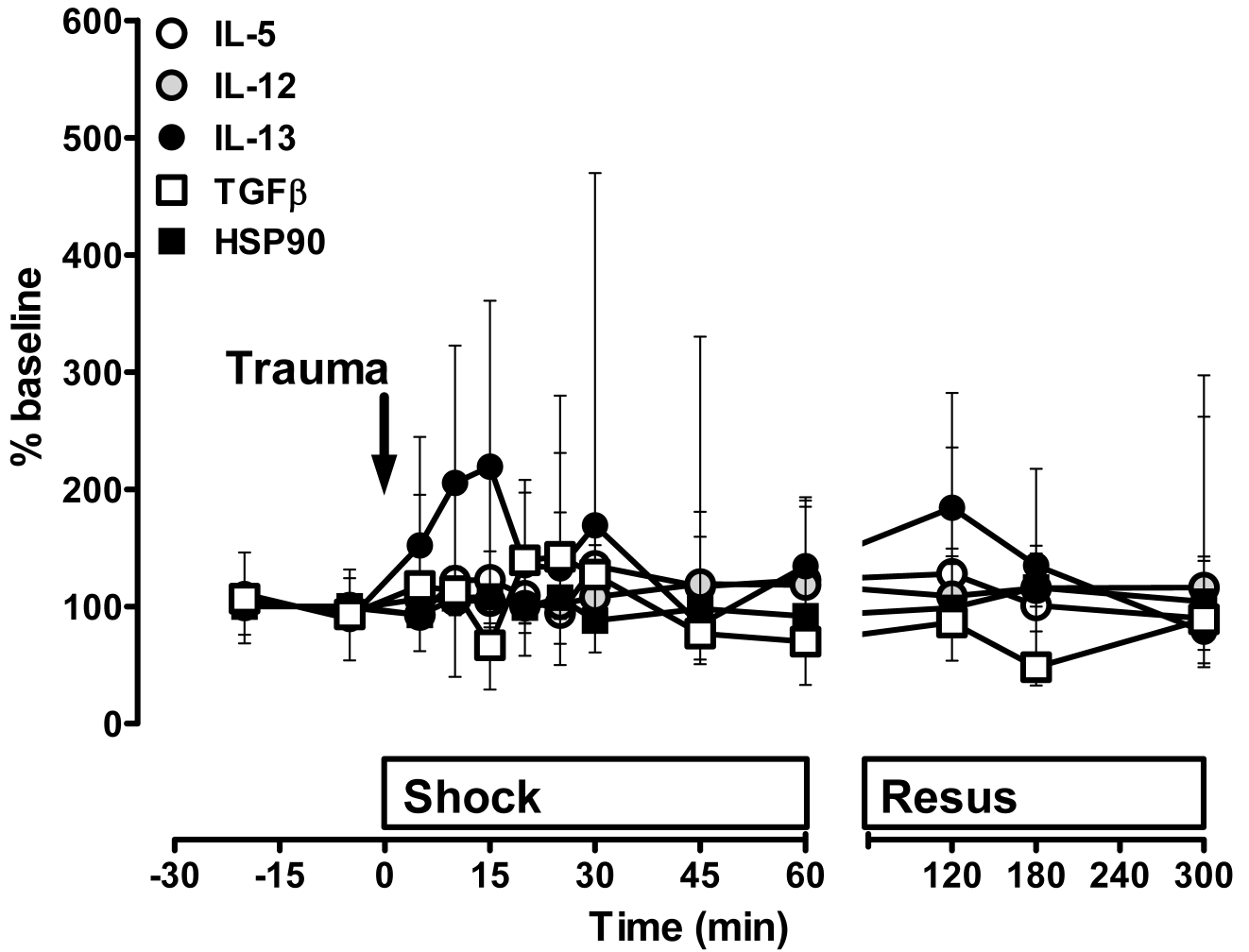
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**Figure 1. Physiological responses to trauma and hemorrhage**

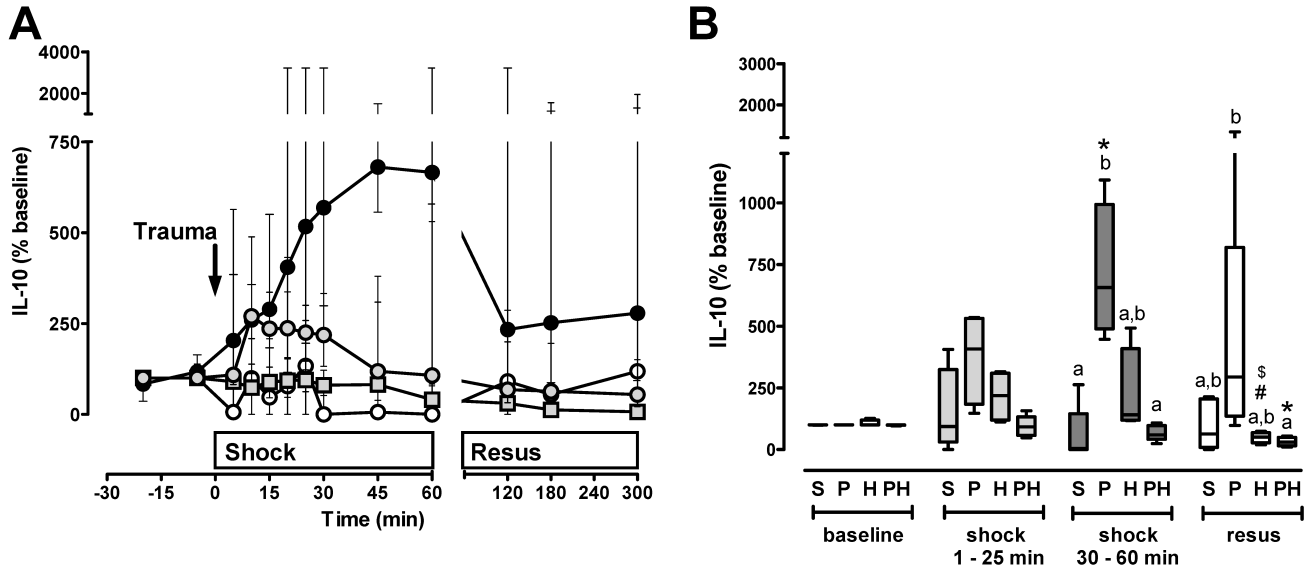
Arrows indicate the time point of injury/hemorrhage. Resus: Resuscitation period. Data are mean ± SD. Open circles: sham control, n=5. Black circles: Polytrauma only, n=5. Grey circles: Hemorrhage only, n=5. Grey squares: Polytrauma plus hemorrhage, n=3-6. **A.** Mean arterial pressure (MAP, mmHg). **B.** I.v. fluids: Intravenous fluid requirements (mL/kg body weight). **C.** Hematocrit (%). **D.** Plasma protein concentrations (mg/mL). **E.** Plasma lactate concentrations (mmol/L). **F.** P/F ratio: ratio of arterial oxygen concentration to the fraction of inspired oxygen. **G.** Mortality (%).



**Figure 2. Plasma concentrations of cytokines/heat shock proteins without significant changes after polytrauma alone**

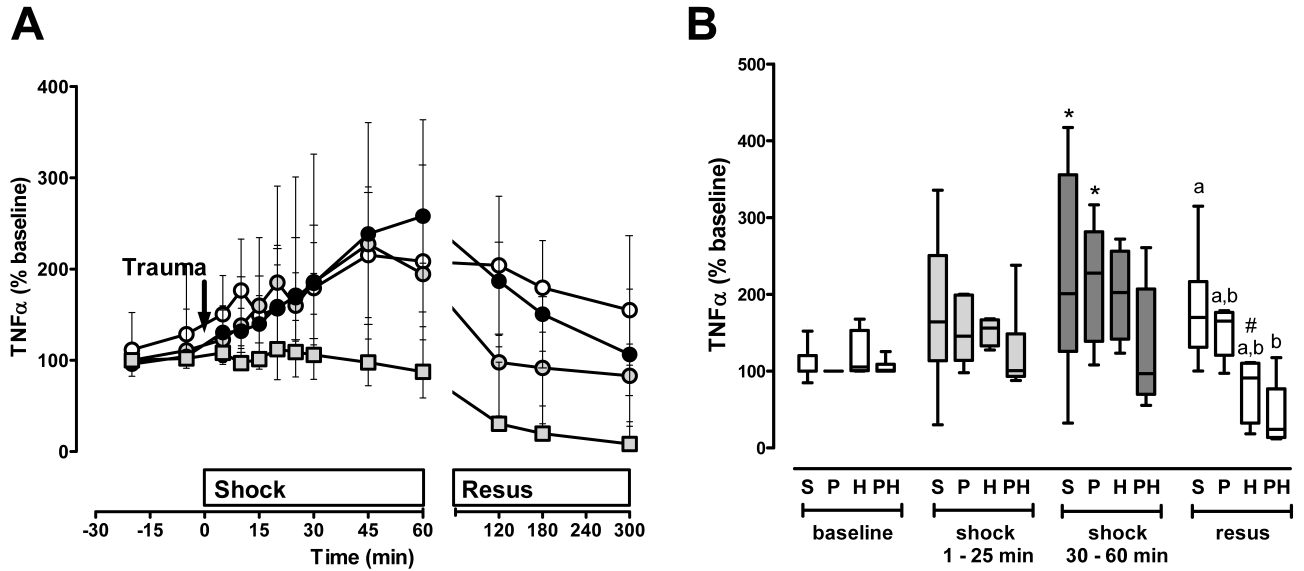
Data are expressed as % of the individual baseline concentrations and plotted as median with interquartile range. n=5 per time point. Open circles: IL-5. Grey circles: IL-12. Black circles: IL-13. Open squares: TGFβ. Black squares: HSP90. The arrow indicates the time point of injury. Resus: Resuscitation period.





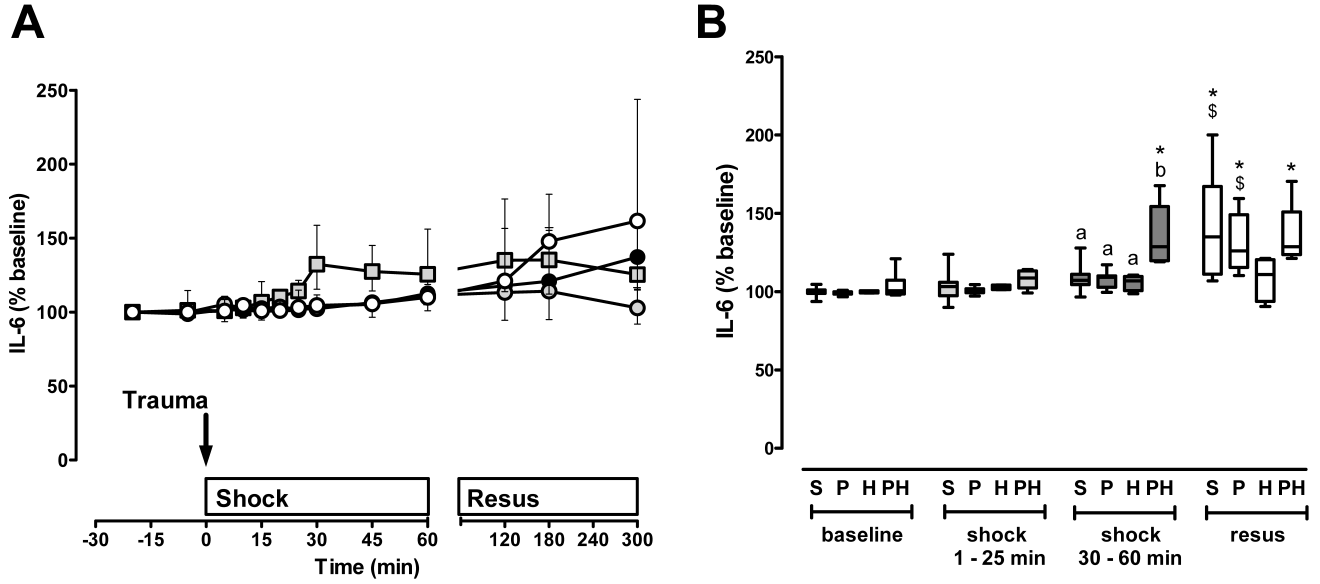
**Figure 3. IL-10 plasma concentrations after polytrauma and hemorrhage**

**A.** Time course. Data are expressed as % of the individual baseline concentrations and plotted as median with interquartile range. Open circles: Sham control, n=5. Black circles: Polytrauma only, n=5. Grey circles: Hemorrhage only, n=5. Grey squares: Polytrauma plus hemorrhage, n=3-6. The arrow indicates the time point of injury/hemorrhage. Resus: Resuscitation period. **B.** Average IL-10 plasma levels during the individual experimental phases (baseline, early shock phase (t=1-25 min), late shock phase (30-60 min), resuscitation phase (resus)). S: Sham control. Average plasma levels for each animal were calculated as the mean of all plasma levels which were determined during each time period; data from A. P: Polytrauma only. H: Hemorrhage only. PH: Polytrauma plus hemorrhage. Boxes extend from the 25<sup>th</sup> to 75<sup>th</sup> percentile; the horizontal line shows the median. Error bars show the 10<sup>th</sup> and 90<sup>th</sup> percentile. Groups not sharing the same letter are significantly different (p<0.05) during each individual experimental phase. \*: p<0.05 vs. baseline. \$: p<0.05 vs. early shock period (1-25 min). #: p<0.05 vs. late shock period (30-60 min).



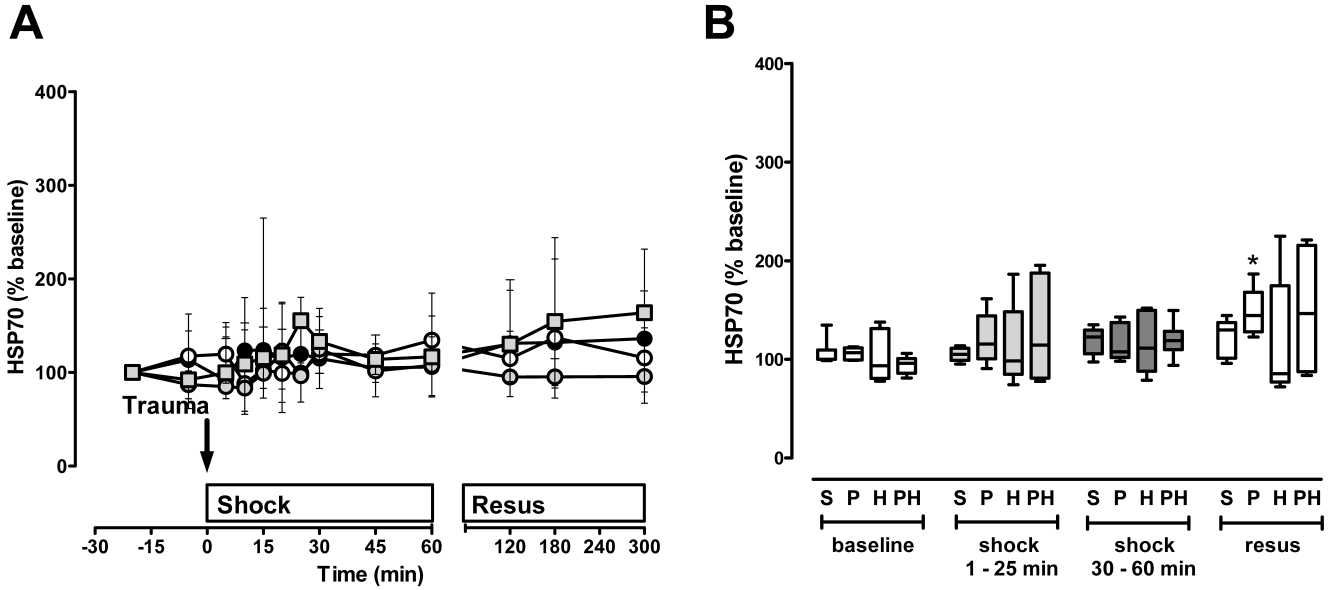
**Figure 4. TNF $\alpha$  plasma concentrations after polytrauma and hemorrhage**

**A.** Time course. Data are expressed as % of the individual baseline concentrations and plotted as median with interquartile range. Open circles: Sham control, n=5. Black circles: Polytrauma only, n=5. Grey circles: Hemorrhage only, n=5. Grey squares: Polytrauma plus hemorrhage, n=3-6. The arrow indicates the time point of injury/hemorrhage. Resus: Resuscitation period. **B.** Average TNF $\alpha$  plasma levels during the individual experimental phases (baseline, early shock phase (t=1-25 min), late shock phase (30-60 min), resuscitation phase (resus)). S: Sham control. Average plasma levels for each animal were calculated as the mean of all plasma levels which were determined during each time period; data from A. P: Polytrauma only. H: Hemorrhage only. PH: Polytrauma plus hemorrhage. Boxes extend from the 25<sup>th</sup> to 75<sup>th</sup> percentile; the horizontal line shows the median. Error bars show the 10<sup>th</sup> and 90<sup>th</sup> percentile. Groups not sharing the same letter are significantly different (p<0.05) during each individual experimental phase. \*: p<0.05 vs. baseline. \$: p<0.05 vs. early shock period (1-25 min). #: p<0.05 vs. late shock period (30-60 min).



**Figure 5. IL-6 plasma concentrations after polytrauma and hemorrhage**

**A.** Time course. Data are expressed as % of the individual baseline concentrations and plotted as median with interquartile range. Open circles: Sham control, n=5. Black circles: Polytrauma only, n=5. Grey circles: Hemorrhage only, n=5. Grey squares: Polytrauma plus hemorrhage, n=3-6. The arrow indicates the time point of injury/hemorrhage. Resus: Resuscitation period. **B.** Average IL-6 plasma levels during the individual experimental phases (baseline, early shock phase (t=1-25 min), late shock phase (30-60 min), resuscitation phase (resus)). S: Sham control. Average plasma levels for each animal were calculated as the mean of all plasma levels which were determined during each time period; data from A. P: Polytrauma only. H: Hemorrhage only. PH: Polytrauma plus hemorrhage. Boxes extend from the 25<sup>th</sup> to 75<sup>th</sup> percentile; the horizontal line shows the median. Error bars show the 10<sup>th</sup> and 90<sup>th</sup> percentile. Groups not sharing the same letter are significantly different (p<0.05) during each individual experimental phase. \*: p<0.05 vs. baseline. \$: p<0.05 vs. early shock period (1-25 min). #: p<0.05 vs. late shock period (30-60 min).



**Figure 6. HSP70 plasma concentrations after polytrauma and hemorrhage**

**A.** Time course. Data are expressed as % of the individual baseline concentrations and plotted as median with interquartile range. Open circles: Sham control, n=5. Black circles: Polytrauma only, n=5. Grey circles: Hemorrhage only, n=5. Grey squares: Polytrauma plus hemorrhage, n=3-6. The arrow indicates the time point of injury/hemorrhage. Resus: Resuscitation period. **B.** Average HSP70 plasma levels during the individual experimental phases (baseline, early shock phase (t=1-25 min), late shock phase (30-60 min), resuscitation phase (resus)). S: Sham control. Average plasma levels for each animal were calculated as the mean of all plasma levels which were determined during each time period; data from A. P: Polytrauma only. H: Hemorrhage only. PH: Polytrauma plus hemorrhage. Boxes extend from the 25<sup>th</sup> to 75<sup>th</sup> percentile; the horizontal line shows the median. Error bars show the 10<sup>th</sup> and 90<sup>th</sup> percentile. Groups not sharing the same letter are significantly different (p<0.05) during each individual experimental phase. \*: p<0.05 vs. baseline. \$: p<0.05 vs. early shock period (1-25 min). #: p<0.05 vs. late shock period (30-60 min).

**Table 1**

Plasma levels of cytokines/heat shock proteins after polytrauma.

Marker (pg/mL)	Baseline	Shock (t=1-60 min)	Resuscitation (t=61-300 min)
<b>IL-1<math>\beta</math></b>			
Mean $\pm$ SD	0	0	0
Median (Min – Max)	0 (0)	0 (0)	0 (0)
<b>IL-4</b>			
Mean $\pm$ SD	0.1 $\pm$ 0.13	0.08 $\pm$ 0.05	0.05 $\pm$ 0.09
Median (Min – Max)	0.05 (0 – 0.31)	0.11 (0.02 – 0.13)	0 (0 – 0.24)
<b>IL-5</b>			
Mean $\pm$ SD	39.3 $\pm$ 12.5	49.2 $\pm$ 9.7	41.6 $\pm$ 13.3
Median (Min – Max)	37.8 (24 – 58)	48.7 (35 – 60)	50.1 (23 – 52)
<b>IL-6</b>			
Mean $\pm$ SD	134 $\pm$ 7	141 $\pm$ 8	188 $\pm$ 15 <sup>a</sup>
Median (Min – Max)	131 (128 – 145)	142 (131 – 152)	184 (170 – 206)
<b>IL-8</b>			
Mean $\pm$ SD	0	12 $\pm$ 29	2.2 $\pm$ 4.2
Median (Min – Max)	0 (0)	0 (0 – 71)	0 (0 – 10.5)
<b>IL-10</b>			
Mean $\pm$ SD	15.3 $\pm$ 14.2	80.9 $\pm$ 63.6	90.7 $\pm$ 56.4 <sup>a</sup>
Median (Min – Max)	8.9 (0 – 31)	76.3 (15 – 174)	92.9 (39 – 175)
<b>IL-12/IL-23 p40</b>			
Mean $\pm$ SD	739 $\pm$ 268	791 $\pm$ 257	900 $\pm$ 294
Median (Min – Max)	654 (582 – 1213)	681 (628 – 1245)	857 (621 – 1389)
<b>IL-13</b>			
Mean $\pm$ SD	6.5 $\pm$ 3.4	9.6 $\pm$ 3.1	7.2 $\pm$ 2.3
Median (Min – Max)	6.2 (2.5 – 11.1)	10 (5.4 – 13.6)	66.5 (5.7 – 11.2)
<b>IL-17</b>			
Mean $\pm$ SD	0	0.5 $\pm$ 1.1	0.4 $\pm$ 0.9
Median (Min – Max)	0 (0)	0 (0 – 2.5)	0 (0 – 1.9)
<b>IL-18</b>			
Mean $\pm$ SD	0	0	0
Median (Min – Max)	0 (0)	0 (0)	0 (0)
<b>IFN<math>\gamma</math></b>			
Mean $\pm$ SD	0	0	0
Median (Min – Max)	0 (0)	0 (0)	0 (0)



Marker (pg/mL)	Baseline	Shock (t=1-60 min)	Resuscitation (t=61-300 min)
<b>TNF<math>\alpha</math></b>			
Mean $\pm$ SD	133 $\pm$ 69	261 $\pm$ 186	249 $\pm$ 60 <sup>a</sup>
Median (Min – Max)	122 (44 – 265)	199 (54 – 574)	249 (153 – 327)
<b>TGF<math>\beta</math></b>			
Mean $\pm$ SD	926 $\pm$ 407	1006 $\pm$ 508	1084 $\pm$ 861
Median (Min – Max)	1019 (173 – 1289)	1033 (395 – 1731)	866 (422 – 2770)
<b>HSP40</b>			
Mean $\pm$ SD	0.41 $\pm$ 0.5	0.04 $\pm$ 0.06	0
Median (Min – Max)	0.25 (0 – 1.2)	0 (0 – 0.16)	0 (0)
<b>HSP70</b>			
Mean $\pm$ SD	144 $\pm$ 48	155 $\pm$ 29	193 $\pm$ 43 <sup>b</sup>
Median (Min – Max)	169 (79 – 189)	168 (122 – 184)	216 (145 – 234)
<b>HSP90</b>			
Mean $\pm$ SD	3.4 $\pm$ 1.0	3.4 $\pm$ 0.6	3.8 $\pm$ 0.9
Median (Min – Max)	3.4 (1.9 – 4.8)	3.5 (2.6 – 4.2)	3.3 (3.3 – 5.4)

Average plasma concentrations at baseline, during shock and resuscitation (n = 5 per time period).

<sup>a</sup> p<0.05 vs. baseline;

<sup>b</sup> p<0.05 vs. shock. The average plasma concentration for each animal was calculated as the mean of two, eight and three plasma levels that were determined at baseline, during shock and resuscitation, respectively.