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Direktor: Prof. Dr. med. Hans-Christoph Pape

Arbeit unter wissenschaftlicher Betreuung von

PD Dr. sc. nat. Paolo Cinelli

The acute inflammatory response of adipose tissue in porcine as mono- and poly-trauma animal model

Inaugural-Dissertation

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vorgelegt von

Marko Canic

Tierarzt

aus Kroatien

genehmigt auf Antrag von

PD Dr. sc. nat. Paolo Cinelli, Referent

Prof. Dr. med. vet. Margarete Arras, Korreferentin

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Contents

Abstract

Severely injured patients are at risk to develop lethal complications including systemic inflammatory response syndrome (SIRS), or multi organ failure during their hospital stay. The underlying inflammatory mechanisms are not yet sufficiently known. Therefore, a better understanding of the inflammatory response in severely injured patients may improve the systemic complication rates in severely injured patients.

In the study utilizes a standardized porcine trauma model comparing groupt Polytrauma (PT) and Monotrauma (MT). The local inflammation was analysed in subcutaneous adipose tissue with qPCR and inluded: high mobility group protein 1 (HMGB1), heat shock protein 70 (HSP-70), and interleukins (IL-6, IL-8, IL-10).

The local adipose tissue revealed a different inflammation in MT and PT. Expression of HMGB1 at the fracture site of MT (3.75 ± 6.937) is higher than in PT (1.297 ± 1.98), Interleukin 6 (MT 407.57 ± 678.79, PT 119.47 ± 174.33), interleukin 8 (MT 612.906 ± 1219.17, PT 17.579 ± 340.07).

The different inflammatory response might base on the injury distribution or the cumulative injury severity. Further research is needed to understand better and to elucidate why differences between the local inflammation response in mono- and poly-trauma exist.

Zusammenfasung

Zu den potentiell letalen systemische Komplikationen von Schwerverletzten zählen das systemische Inflammations-response syndrome (SIRS), oder das Multiorgan-Versagen (MOV). Die inflammatorische Ursache für diese Komplikationen sind noch unzureichend geklärt. Diese Studie vergleicht die lokale Entzündungsreaktion in Polytrauma mit Monotrauma.

In dieser Studie wurde ein standardisiertes Polytrauma (PT) sowie ein standardisiertes Monotrauma (MT) an männlichen Edelschweinen durchgeführt. Die Überwachungszeit betrug sechs Stunden. Die lokale Inflammation wurde im subkutanen Fettgewebe an der Fraktur sowie an der nicht-frakturierten Seite mittels qPCR gemessen.

Lokal-inflammatorische Mediatoren waren an der Frakturierten Seite höher im Monotrauma verglichen zum Polytrauma. Zu diesen zählen Interleukin 6 (IL-6) (407.57 ± 678.79 vs 119.47 ± 174.33), oder IL-8 (612.906 ± 1219.17 vs. 17.579 ± 340.07).

Die Ursache der unterschiedlichen inflammatorischen Reaktion liegen möglicherweise in der Verletzungsverteilung und der summativen Verletzungsschwere und sollten in weiteren Studien untersucht werden.

Introduction

World Health Organization and the Global Burden of Disease study declare/disclose that injuries are responsible for five million deaths per year worldwide [1, 2]. The mortality related to trauma can be described as trimodal: immediate [3], early [4], and late [5]. Immediate and early mortalities are mainly due to devastating brain injuries, massive bleeding, and critical injuries to vital organs [3, 4, 6]. Unlike immediate and early mortality, late mortality is a result of an imbalanced systemic immune response to the multiple insults that develop after trauma [7]. The inflammatory response is recognized as a physiologic reaction to injury as a result of the interplay between various mediators produced at the site of injury. The severity of the inflammatory response correlates with the severity of injury: in severely injured patients, the local inflammatory response may be strong enough to trigger a systemic inflammatory response, which might lead to systemic inflammatory response syndrome (SIRS). Exposure of the organism to the systemic inflammatory response, as a consequence of uncontrolled production and release of pro- and anti-inflammatory mediators, might lead to multi-organ dysfunction syndrome (MODS) that is associated with an increased mortality rate [8]. Regulation and modulation of pro- and anti-inflammatory processes is especially important in the treatment of severely injured patients where it is seen as a promising new component of modern resuscitation protocols and future therapies [9, 10].

Post-traumatic inflammatory response

Trauma-related tissue injury leads to the activation of local immune cells and secretion of various kinds of mediators. The activation of this local inflammatory response is a physiological reaction that serves as a protection against infections and initiates tissue repair and wound healing [11].

Immediately after trauma or injury of the tissue, necrotic cells release intracellular molecules [(DNA, ATP, uric acid, heat shock proteins (HSPs), high mobility group box 1 (HMGB1)]. Those molecules are known as a damage-associated molecular pattern (DAMPs) or alarmins. Their release has the potential to trigger innate immune cells. Subsequently, innate immune cells (mainly dendritic cells (DCs) and neutrophils) recognize DAMP molecules and become active. After the activation, DCs and neutrophils, promote the further release of cytokines and recruitment of immune cells to infiltrate tissue to begin tissue repair (Figure 1).

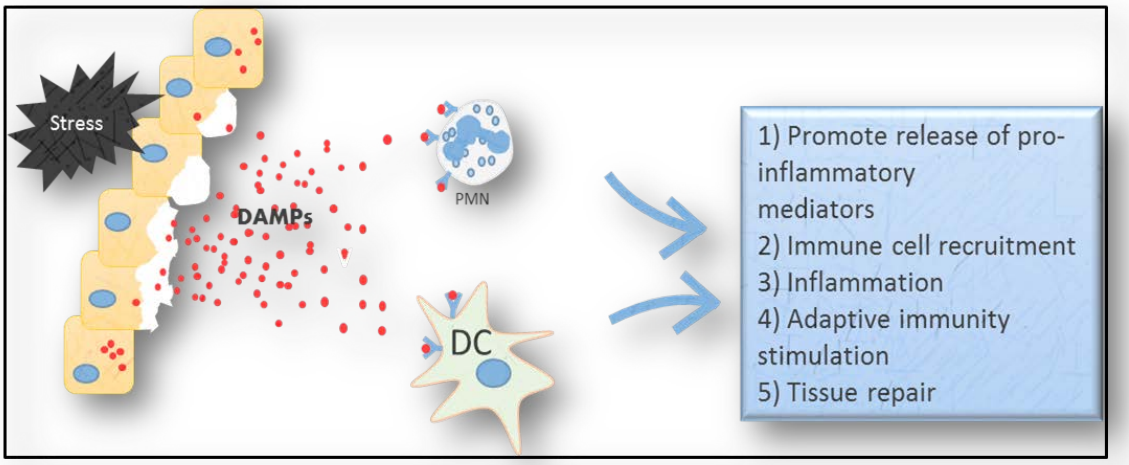


Figure 1: Danger model showing how DAMPs activate innate immune cells by binding to their receptors and what are results of the DAMPs binding to dendritic cells (DCs) and neutrophils (PMNs).

Alarmins

Alarmins are endogenous, chemotactic, and immune activating proteins that are released as a result of degranulation, cell injury, cell death, or in response to immune induction. They act as intercellular signals by interacting with chemotactic pattern recognition receptors to stimulate immune cells in host defense [12]. In response to cell injury, alarmins are released and initiate the innate immune responses.

Effects of alarmins on the innate immune system are:

1. activation of resident leukocytes (such as macrophages, and mast cells),
2. production of inflammatory mediators (cytokines, chemokines, and lipid metabolites),
3. recruitment of neutrophils and macrophages,
4. eliminate invading microorganisms and injured tissues

The list of the alarmins that are involved in immunological response is quite long and it continues to grow because there are still a number of alarmins that are still undiscovered [13, 14]. However, so far the most understood and the most researched alarmins are HMGB1 and HSP70 [15, 16].

High Mobility Group Box 1 (HMGB1)

The HMGB1 protein in human organism, primarily intracellular protein, is tightly involved in the regulation of DNA transcription. After a tissue injury, HMGB1 is either secreted by activated immune cells or passively released from necrotic cells [17]. In such an extracellular form, it has a significant role in inflammation, immunity, cell growth, cell proliferation, and cell death. Its main immune-stimulatory action is the promotion of the maturation of dendritic cells. Additionally, it poses a chemotactic stimulus to monocytes, macrophages, and neutrophils [18].

Heat Shock Protein 70 (HSP-70)

HSP-70 molecules are found in almost every cell type as intra- or extracellular protein. Under stress, intracellular HSP-70 protects cells against lethal damage and supports the folding and transport of newly synthesized, aberrant proteins [19]. Further, extracellular HSP-70 has many immunomodulatory functions, such as: facilitating the cross-presentation of antigens and acting as “chaperonins,” i.e., stimulators of innate immune responses [20]. Apart from its capacity to induce pro-inflammatory signaling, extracellular HSP-70 also possess some anti-inflammatory properties [21].

Cytokines

Cytokines are a large group of proteins that are secreted by specific cells of the immune system. They belong to the category of signaling molecules that mediate and regulate immunity and inflammation. Secreted cytokines have autocrine and paracrine functions. In some cases, they act as endocrine mediators as well [22]. Cytokines produced by lymphocytes are also referred to as lymphokines, and many of them are further known as interleukins (ILs) as they affect leukocytes. Some cytokines promote inflammation and therefore are called pro-inflammatory cytokines; others are suppressing pro-inflammatory mediators and are hence called anti-inflammatory cytokines.

Following injury, endogenous factors, such as ‘DAMPs’ or ‘alarmins,’ are secreted by activated immune or necrotic cells (e.g., neutrophils) [17, 23]. Alarmins directly activate several immune cell types as well as the complement system [24, 25]. The activation of complement and inflammatory cells triggers the production and release of the interleukins [26].

Proinflammatory Cytokines

Interleukin 6 (IL-6)

Interleukin-6 (IL-6) is a multifunctional cytokine that is involved in many processes necessary to protect an organism from injury or pathogens. The engagement of IL-6 in acute phase response, such as immune cell activation, maturation, or hemostasis, makes it one of the most prominent cytokines in response to trauma. Regarding its function in host defense, IL-6 activates different cell populations with relevant functions in the acute phase response during inflammation. Furthermore, IL-6 has an essential role in immune cell maturation with induction of immunoglobulin production of B-cells and differentiation of T-cells [27]. After the trauma, IL-6 stimulates platelet production of megakaryocytes, but also it is involved in thrombin and fibrin accumulation [28].

Interleukin 8 (IL-8)

IL-8 is a cytokine that is released mainly by macrophages early after injury [29]. In addition to macrophages, epithelial cells, airway smooth muscle cells, and endothelial cells release IL-8. The leading role of IL-8 in inflammation is the recruitment of neutrophils [30]; it also is responsible for attraction and activation of monocytes, lymphocytes, basophils, and eosinophils at sites of inflammation. It was shown that IL-8 is involved in many cellular processes including cell proliferation, tissue remodeling, and angiogenesis.

Anti-inflammatory Cytokines

Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine that acts as a potent deactivator of monocyte/macrophage proinflammatory cytokine synthesis [31]. It plays a central role in limiting host immune response to pathogens, thereby preventing damages to the host. Impaired IL-10 expression or signaling can enhance the clearance of pathogens during an acute infection. However, an exaggerated inflammatory response may result in exacerbated activation with potential subsequent tissue damage [32].

The aim of this study

Previous studies focused on traumatic injuries and local inflammatory response, investigated early production of pro-inflammatory cytokines in adipose tissue[33]. Those studies described the increase of IL-6 and IL-8 as an early local activation of inflammation in adipose tissue. Up to now, the local immune response of adipose tissue was never compared in standardized mono- and poly-trauma groups. Therefore, in this study, we aimed to compare the expression of the local inflammatory response of adipose tissue in a standardized mono- and poly-trauma porcine model. Understanding the local inflammatory response would improve understanding of the relationship between the local and systemic inflammation and ultimately aid the prevention of multiple organ dysfunction syndrome or systemic inflammatory response syndrome (SIRS) as one of the critical complications in patients who suffered severe trauma.

Material and Methods

Animals

Male Edelschwein pigs were used for this study at the Department for Surgical Research, University Hospital of Zurich. The mean weight of the animals was 50.78 kg ± 4.46 kg, and a mean length was 124.42 cm ± 4.45 cm. The experiment was done under the license number 138/2017 which was approved by the Cantonal Veterinary Office Zürich, Switzerland. The animals were randomly allocated to sham, mono-trauma (MT) and poly-trauma group (PT). Animals of the sham group received no trauma, but they were anaesthetized, and samples were collected from them according to protocol.

Animals of group MT underwent a standardized femoral fracture. Animals group PT received a standardized femoral fracture, blunt chest trauma, liver laceration and mean-arterial pressure (MAP) controlled hemorrhagic shock. To simulate the transportation and initial assessment time (as often the case in humans) the injuries were treated 1 hour after induction of trauma. Femoral fractures were treated with retrograde nailing, liver laceration was treated by 'packing' (4 gauzes), and the hypovolemic shock was addressed with volume resuscitation (three times the volume of drawn blood). The blunt chest trauma was treated with a chest tube if the radiologic control showed pneumo- or haemothorax. If there were no obvious radiological pathologies to the chest, the thoracic trauma was continuously observed and regulated by ventilation.

Anesthesia and Preparation

After the transport from the breeder, all animals were acclimatized to the housing conditions of the experimental facility for at least seven days. Prior to induction of anesthesia, animals were pre-medicated in the stable with an intramuscular injection of ketamine 10-30mg/kg, midazolam 0.3-0.5 mg/kg and atropine 0.05 mg/kg. After confirmation of appropriate sedation, they were transported to the preparation room. A venous catheter was aseptically placed in the auricular vein cleaned with Kodan (Schulke & Mayr UK Ltd., Sheffield, United Kingdom). After the placement of the venous catheter, a bolus of 40 mg of 1% Propofol (Braun, Sempach, Switzerland) was given, and the animal was placed in sternal position for endotracheal intubation. Afterward, the animal was placed back in lateral recumbency in order to be shaved and prepare the place for the operation. During the preparation time, animals were kept anesthetized with propofol, which was administered as needed. In the operation theatre, the general anesthesia was maintained with a combination of Isoflurane (1-2%) (Provet AG, Lyssach, Switzerland), propofol (5-10mg/kg/h CRI) and sufentanil forte (0.01 mg/kg/h CRI) (Janssen-Cilag AG, Zug, Switzerland). Ventilation was performed using a volume-controlled ventilator (Draeger, Primus, Danvers, MA) to obtain normocapnia with an FIO2 of 30%, and tidal volume of 10 mL /kg and positive end-expiratory pressure (PEEP) of 5 mm Hg was kept. The primary goal was to maintain a partial pressure of CO2 (pCO2) approximately about 35 ± 5 mm Hg. Fluid management was performed following the experimental protocol with Ringerfundin B. Braun (Medical AG, Sempach, Switzerland) with 21 mL/kg per hour.

For hemorrhagic shock induction, resuscitation (fluid substitution) and drug administration as well as for measuring hemodynamic parameters intra-vascular sheets (French size 10 and long 23 cm, AVANTI® +,Cordis, California, USA) were placed in femoral vein and artery, as well in jugular vein by the Seldinger technique and ultrasound guidance. The urinary output was monitored via a urinary bag (Cystofix, Braun, Melsungen, Germany) that was connected to a suprapubic urinary catheter (Marflow AG, Adliswil, Switzerland) inserted thru the incision in the abdomen and urinary bladder. Sham animals were only anesthetized and arterial, venous, and urinary lines were placed.

Induction of Trauma, Haemorrhagic Shock, Resuscitation, and Timeline

Mono-trauma

Animals in group MT underwent a standardized femur fracture. Animals were placed in right-sided recumbence, and the left hind limb was shaved and aseptically prepared. Through a small incision (2-4 cm) a specially manufactured led plate was placed in direct contact with the femur bone and firmly fixed in place. The

femur fracture was induced by shooting the captive bolt gun (9 X 17, Dynamit Nobel AG, Troisdorf, Germany) onto the lead plate that was placed above the middle-third of the femoral bone. Femur fracture was confirmed using fluoroscopy (Figure 2). During 1 hour post-fracture period FIO2 was decreased to 21%, and infusions were decreased to 10ml/kg/h in order to simulate the environment at the potential accident site.

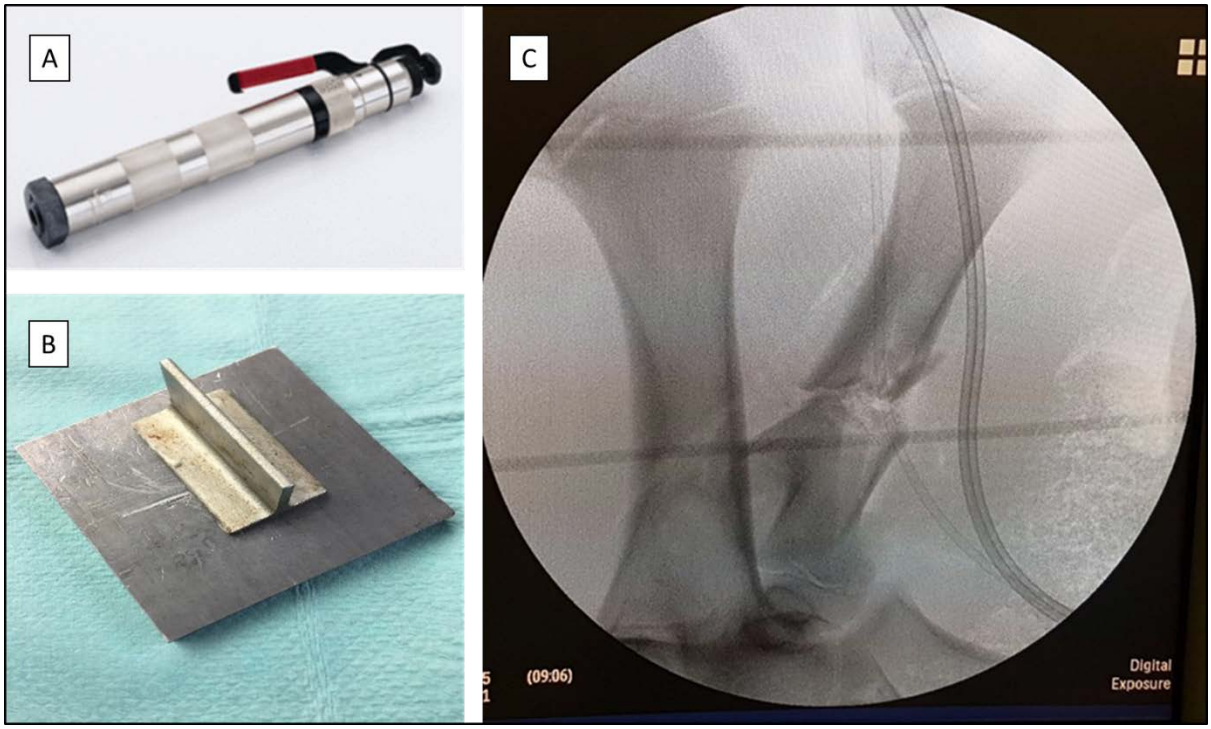


Figure 2: A captive bolt gun (A) which together with a lead plate (B) was used to perform femoral fracture in the diaphysis (C) and afterward confirmation of the fracture with the fluoroscope.

Polytrauma

Animals in group PT received a femoral fracture as described in MT group (Figure 2). Additionally, a liver laceration, blunt chest trauma, and a MAP-controlled hemorrhagic shock was performed. Hemorrhage was induced by withdrawing blood continuously until a MAP of 25 ± 5 mm Hg was reached. During this time, the animal was turned into a supine position and prepared for the induction of the blunt thoracic trauma. The blunt thoracic trauma was performed using bolt shot on a led panel at the right dorsal lower chest using a captive bolt gun (9 X 17, Dynamit Nobel AG, Troisdorf, Germany) (Table 3). A radiograph controlled the status of the chest and the lung, in case of pneumothorax or haemothorax a chest tube was inserted in the usual manner. Afterward, mini-laparotomy access at the upper third of the abdominal midline was used in order to expose the left lobe of the liver and induce the liver laceration. One puncturing incisions was done to the right upper liver lobe using a sharp, custom-made three-edged blade (Figure 3). After 30 seconds of uncontrolled bleeding, the liver was packed with four sterile gauzes (Figure 3).

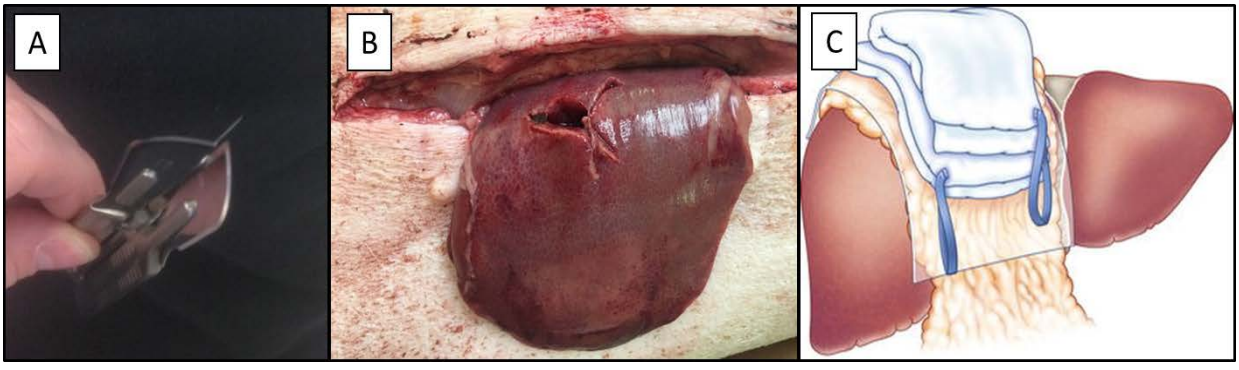


Figure 3: Custom made three edge blade (A) was used to perform a standardized liver laceration (B). A schematic image of "packing" of the liver after the laceration (C).

Shock parameters

Body temperature, mean arterial pressure, heart rate, lactate, hematocrit, potassium and partial pressure of oxygen are parameters that were measured and recorded regularly throughout the experiment. Mentioned parameters were recorded to get better perception and insight of the hemorrhagic shock, its depth and severity, and the response of the animals on all injuries.

Resuscitation

The resuscitation started one hour after induction of the femoral fracture. The fluids resuscitation of hypovolemic animals (group PT) was initiated with an infusion of crystalloids (Ringerfundin B. Braun, Medical AG, Sempach, Switzerland) with three times the volume of the amount of blood that was drawn for one hour. Simultaneous the femoral fracture was treated with retrograde nailing (Closed Reduction, Internal Fixation, CRIF). The reduction was achieved with X-ray guidance. A suprapatellar incision served as the approach to the intercondylar plane. The femoral cavity was opened with an awl. In order to ream the medullary canal, a drill guide wire was used as a guidance for the reaming device (Synthes). Afterward, the femoral fracture was stabilized with an intramedullary nail and interlocking screws (Synthes). The reduction was confirmed under the fluoroscope. Simultaneously FIO2 was increased to 30% to maintain PaCO2 between 35 and 45 mmHg and the infusions were increased to 100ml/h in both mono and multiple trauma animals.

Timeline

After anesthesia and preparation, the baseline values were taken. With the femoral fracture, the Shock-phase started (1h). During this phase, vital parameters and blood gas analysis (BGA) were taken every 15minutes. One hour after a trauma, the resuscitation phase began. The total observation time was 6 hours after trauma. From the Resuscitation timepoint to the termination time point (6 hours after trauma) BGAs and vital parameters were documented every hour (Figure 4).

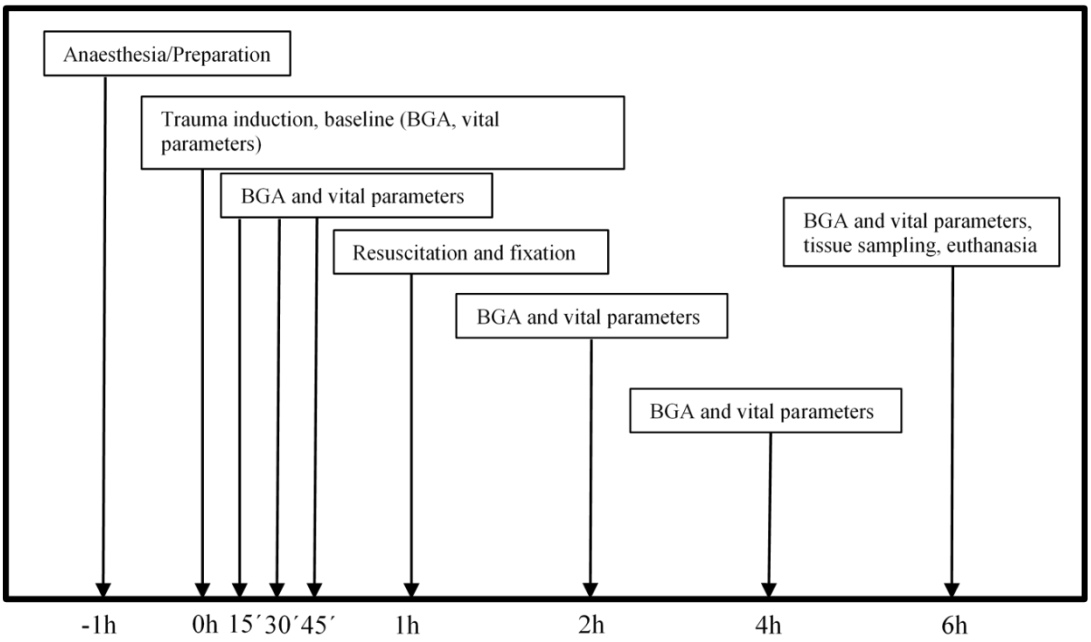


Figure 4: The timeline of this study

Tissue sampling and analysis

Samples of adipose tissue were collected before the induction of trauma and at the end of 6 hours follow-up period (before animals were euthanized). Before trauma, samples were harvested from the site where the fracture was planned. After 6 hours samples were collected from the injured and non-injured site. Collected samples were immediately stored in RNA stabilization reagent, snap-frozen in liquid nitrogen, and stored at -80 °C until analysis.

Prior to the analysis, samples were defrosted, and approximately 90 mg of fatty tissue per sample was used. For RNA extraction samples were disrupted and then homogenized in 2ml microcentrifuge tubes. From the lysate, RNA was extracted using RNeasy Lipid Tissue Mini Kit according to the manufacturer's instructions (RNeasy Mini Handbook). The next step was to measure the amount and purity of the purified RNA. This step was crucial for determining the amount of each sample to use in downstream applications; reverse transcription and RT-PCR. The amount of 500µg of total RNA was transcribed to complementary DNA using an Invitrogen SuperScript III Reverse Transcriptase (Thermo Fischer, Switzerland) according to the manufacturer's protocol. Gene expression was analyzed by real-time polymerase chain reaction on Rotor-gene Q (Qiagen AG, Hombrechtikon, Switzerland) and normalized to the expression of porcine Glyceraldehyde-3-Phosphate-Dehydrogenase (GAPDH). The list of primers used in the study is listed in Table 1.

	Sense	Antisense
GAPDH	5`TCT CAT GGT TCA CGC CCA TC	5`AGT GAA CGG ATT TGG CC GC
Interleukin 6	5`GTC CCC CAG CTA CAT TAT CC	5`GAA TCC AGA CAA AGC CAC CA
Interleukin 8	5`GTT GTT GTT GCT TCT CAG TTC	5` CTT CCA AAC TGG CTG TTG CC
Interleukin 10	5`CGGGAA CCT TGG AGC AGA TT	5`CGG CGC TGT GAT CAA TTT CT
Heat shock protein 70	5`CAG GCT TCC CTT TAG CTC GG	5`GGC CTC CTT CGG CCI TTT TC
High mobility group box 1	5`TCC CCA CGG TAG GAA ACG	5`GCC CTG AAT CCG CAG AAT A

Table 1: Porcine primer for quantitative PCR (qPCR)

Statistics

All data were tested for normal distribution using the Kolmogorov-Smirnoff test and the Shapiro test. Nominally called variables were tested by Chi-square analysis. Proportions were evaluated using the Yates-corrected statistics. Logarithmic distributed variables were logarithmized to result in a normal distribution. For parameters without normal distribution, continuous variables were compared using the Mann-Whitney U-test. Categorical variables were compared using the Chi-square test. All calculation and graph plotting were performed with R (R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/), SPSS (Version 25, IBM Inc., Amork, NY, USA) and GraphPad Prism (Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Statistical significance was assumed at $p < 0.05$.

Results

For this study, 58 animals were randomly divided as following: 27 male animals in group PT, 25 male animals in group MT and six male animals in the sham group. Three animals in the PT group (11.11%) did not reach the end time-point. The data of these animals were included until the time of death.

Vital parameters

Body temperature was continuously measured and recorded. Measurements of body temperature show significant differences towards the end of the observational period when comparing groups PT, MT and Sham. Findings are summarized in Figure 5.

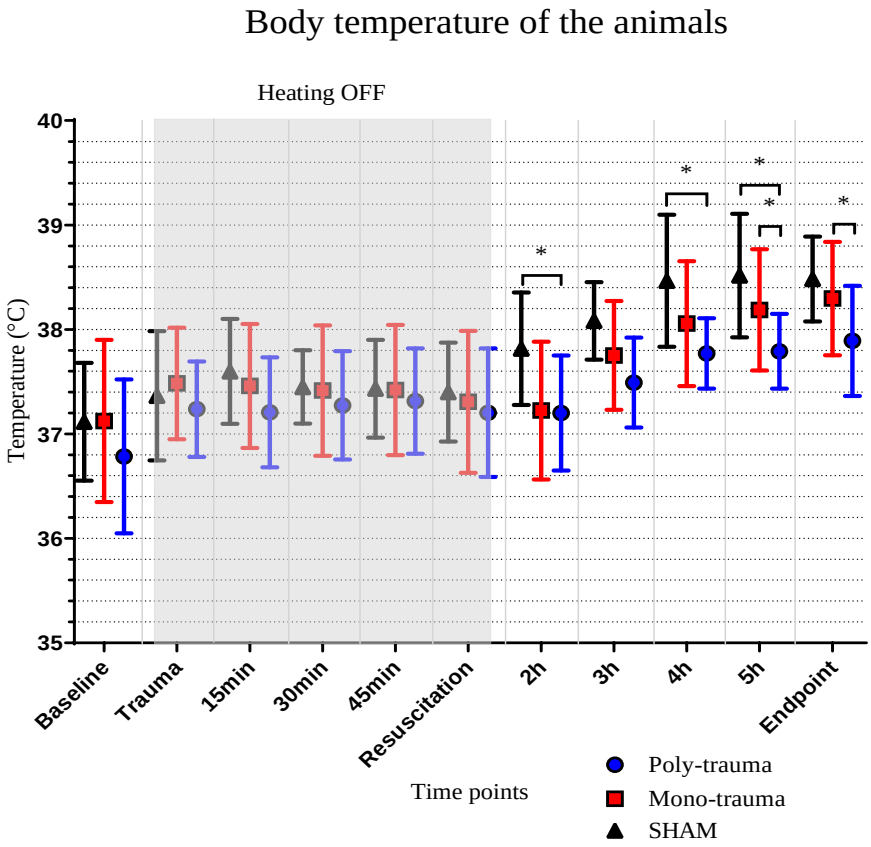


Figure 5: The temperature in all three groups have a similar course. Sham and MT group have a significantly higher temperature compared to PT at some time points. The significant increase of the body temperature in the sham group is noticed at the time point “two hours after the trauma” and then at four hours, and five hours after the induction of injury. Further significant differences are noticed between MT and PT group at five hours after the injury and the endpoint (*p<0.05).

Throughout the whole experiment, mean arterial pressure measurements show no differences between sham and MT group. However, the PT group shows significant lower values of MAP during the whole time of the experiment. Even baseline measurements showed significantly lower MAP in the PT group when compared to the MT group. Time points and significantly different values are marked in Figure 6 (* $p < 0.05$ and ** $p < 0.01$).

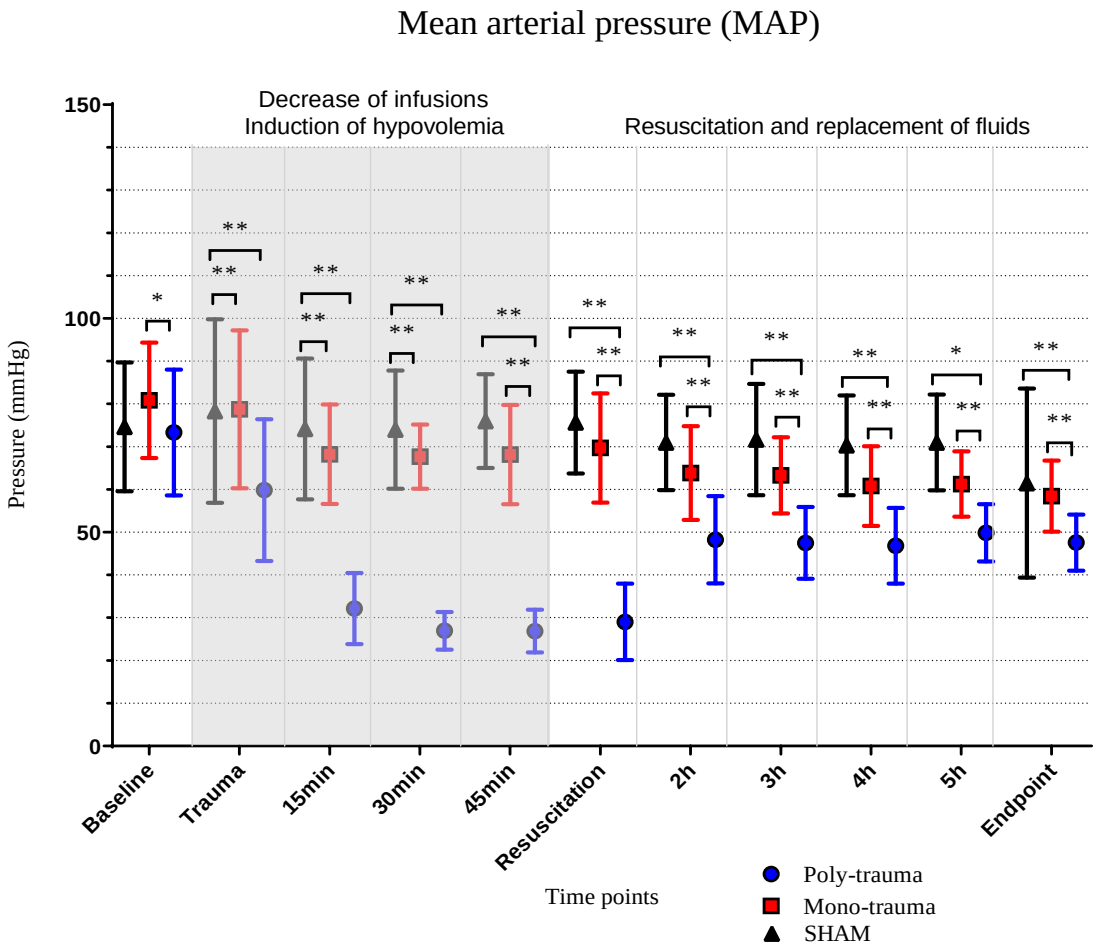


Figure 6: Mean arterial pressure values in all three animal groups. From the beginning of the experiment (baseline), a significant difference in MAP between mono and poly-trauma animals can be observed. Later, with the induction of hypovolemic shock in the PT group, a significant decrease of mean arterial pressure is spotted in comparison to sham and MT group. The values of MAP between sham and MT group are almost the same during the whole experiment (* $p < 0.05$ and ** $p < 0.01$).

Heart rate was monitored, and the values were recorded according to the timeline. The data shows that animals developed physiologically response to the hemorrhage and the drop of the mean arterial pressure. A first significant increase of the heart frequency, in PT group, is observed 30 minutes after the induction of hemorrhagic shock. The increased heart frequency of PT group lasts up to one our post-resuscitation and afterward was again recorded one hour before the endpoint and at the endpoint (Figure 7) (* $p < 0.05$ and ** $p < 0.01$).

Heart rate (HR)

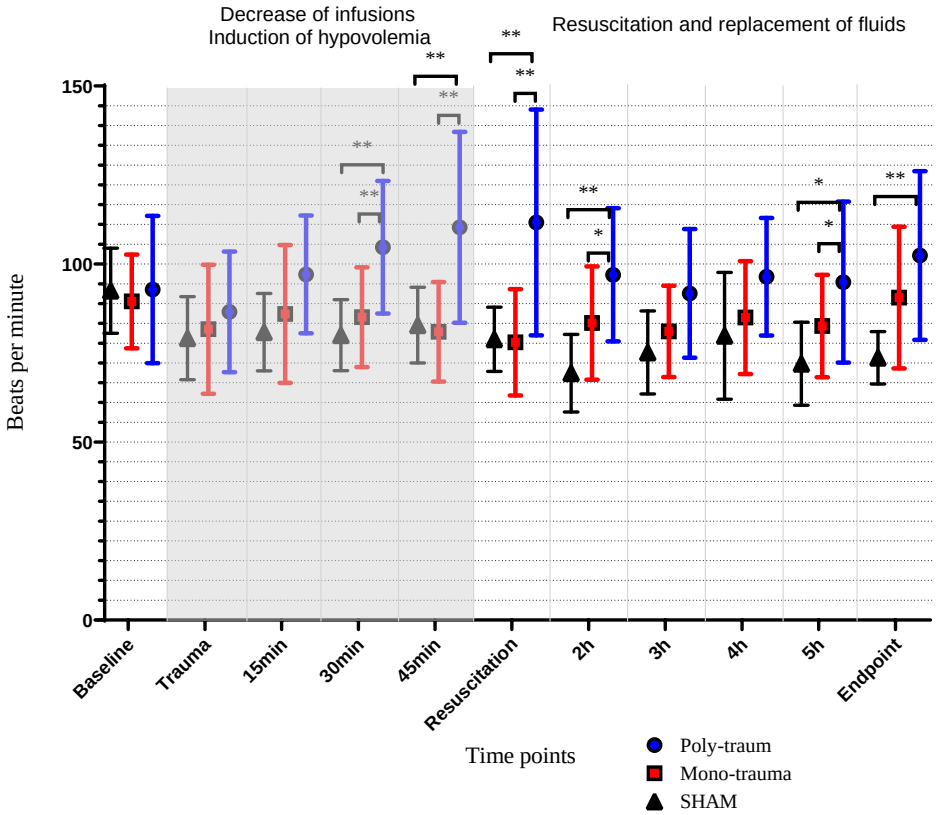


Figure 7: Pattern of heart rate during the observational period. The first significant increase of heart frequency match the time point 30 minutes after the induction of hemorrhage and last until two hours after the induction. A further significant increase of the heart rate in the PT group is observed at five hours post induction and the endpoint (* $p<0.05$; ** $p< 0.01$).

Level of lactate was assessed as a clinical standard for shock assessment. Therefore, a significant increase was noticed only in the PT group. An initial significant increase was observed already 15 minutes after the induction of hemorrhage and lasted until the end of the experiment. At the termination point level of lactate is only significantly different between PT and MT group (Figure 8).

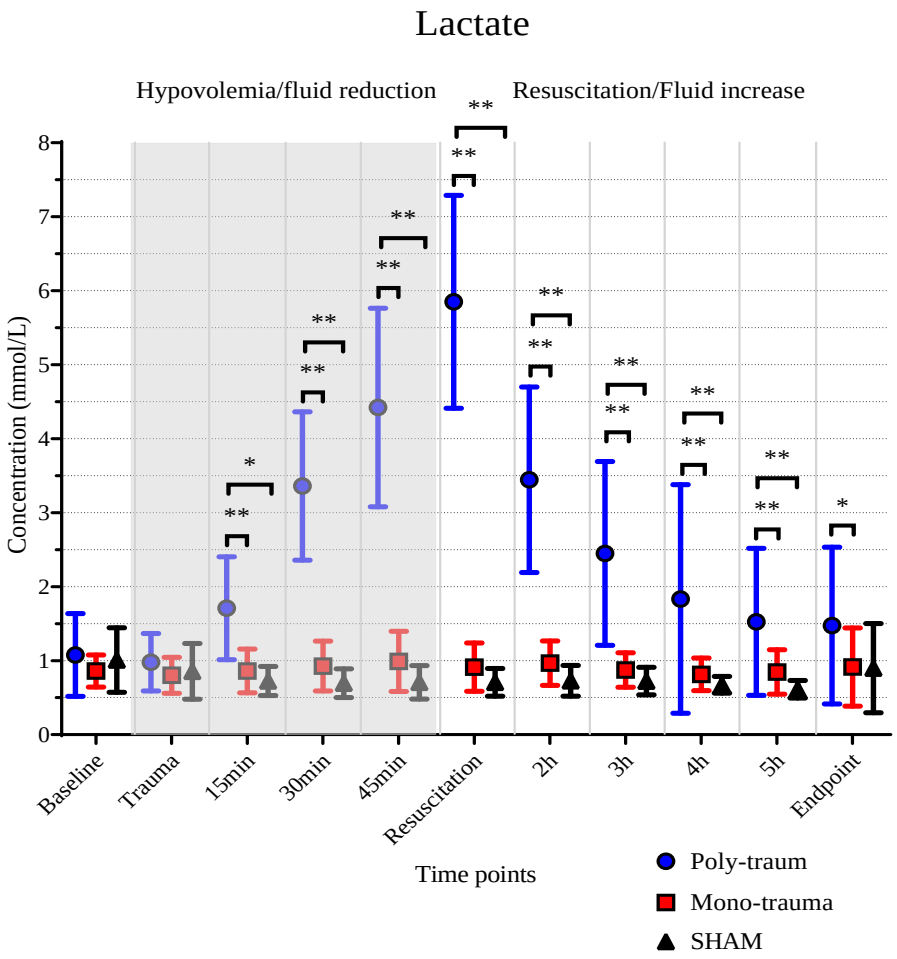


Figure 8: Comparison of lactate level in PT, MT and Sham groups. Significant increase of lactate level can be observed in PT goup, with onset as early as 15 minutes after haemorrhagic shock and persisted until the end of the experiment (*p< 0.05 and ** p< 0.01).

Potassium concentration in blood during the hypovolemia confirm the hypovolemic shock and hypoperfusion of the tissues. A significant increase in the potassium in the PT group is documented 15 minutes after the induction of hypovolemic shock. The significant increase in potassium lasts then until the resuscitation. Afterward, the next significant increase in potassium is noticed at four hours after the induction of traumas and preserved until the termination point (Figure 9).

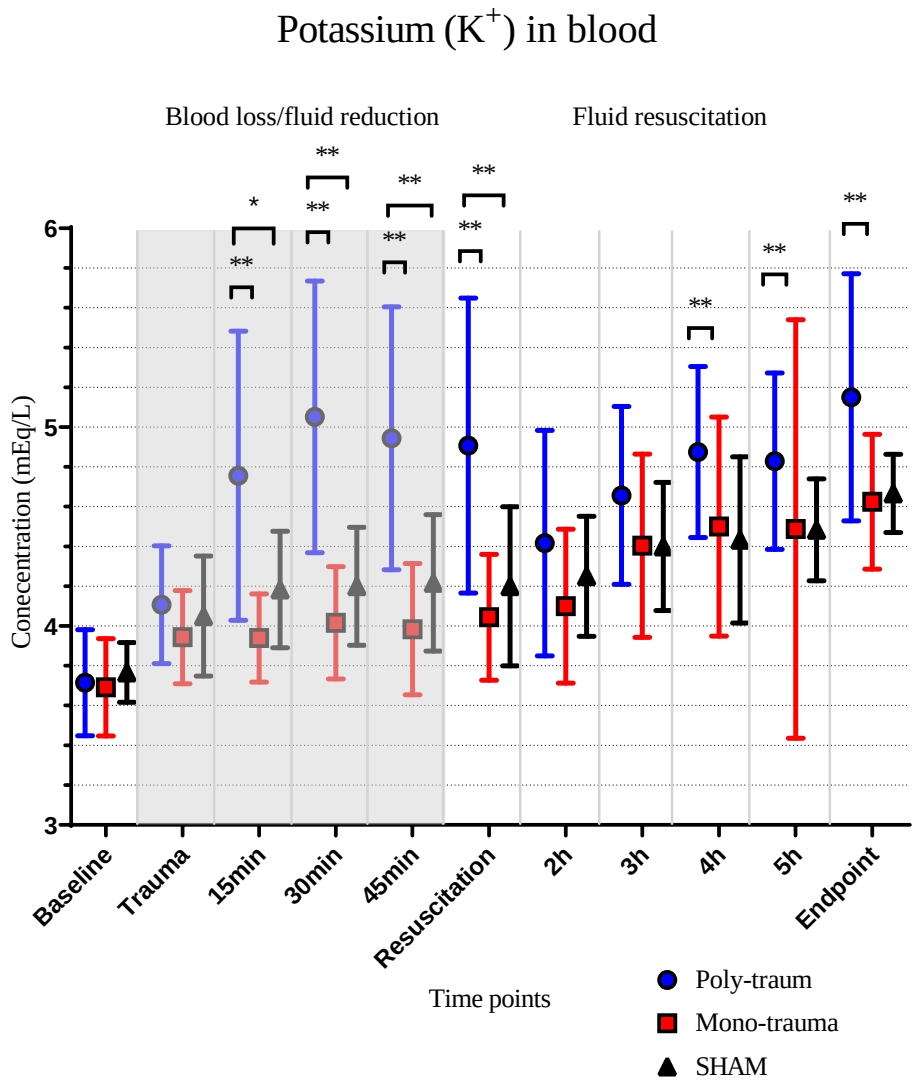


Figure 9: Significant increase in potassium concentration in group PT compared to group MT and Sham. Comparison of the course of lactate level in groups PT, MT and Sham. The significant increase of potassium concentration indicates tissue hypo perfusion (*p< 0.05 and ** p< 0.01).

Hematocrit values after the hemorrhage and during the hypovolemic shock remain unchanged. However, one hour after the resuscitation, compared to sham and MT group, PT group demonstrates significantly decreased hematocrit, which remained so until the endpoint (Figure 10).

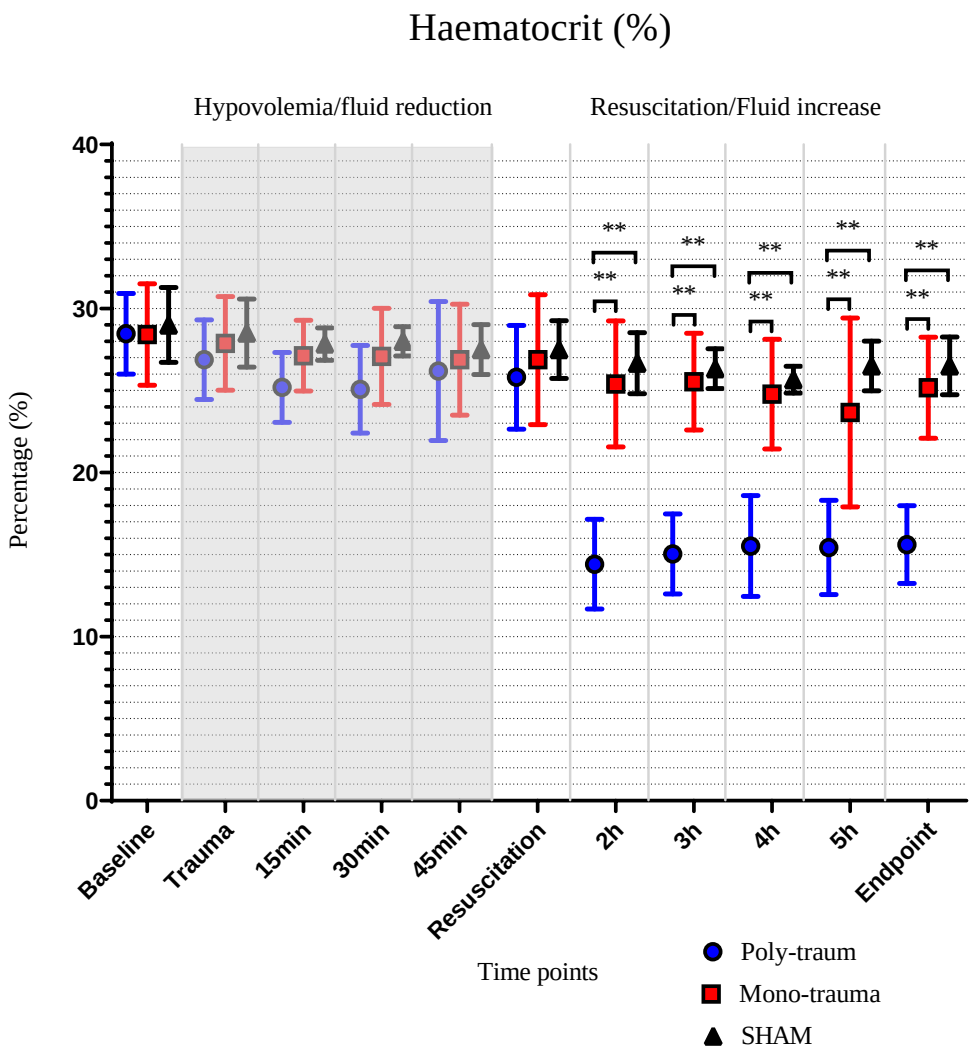


Figure 10: Hematocrit values during the hypovolemic phase remained the same in all three groups of animals. However, after the resuscitation hematocrit values of the PT group significantly declined and the values remained significantly lower than in sham and MT group for the duration of the experiment (*p< 0.05 and ** p< 0.01).

The partial pressure of oxygen (pO_2) followed the same trend in all three groups. The first drop of pO_2 in all three groups is seen immediately at the time point of trauma induction when the oxygen was decreased to 21%. The significant difference in partial pressure between animals was noticed after 15 minutes after the trauma and lasted until resuscitation (Figure 11).

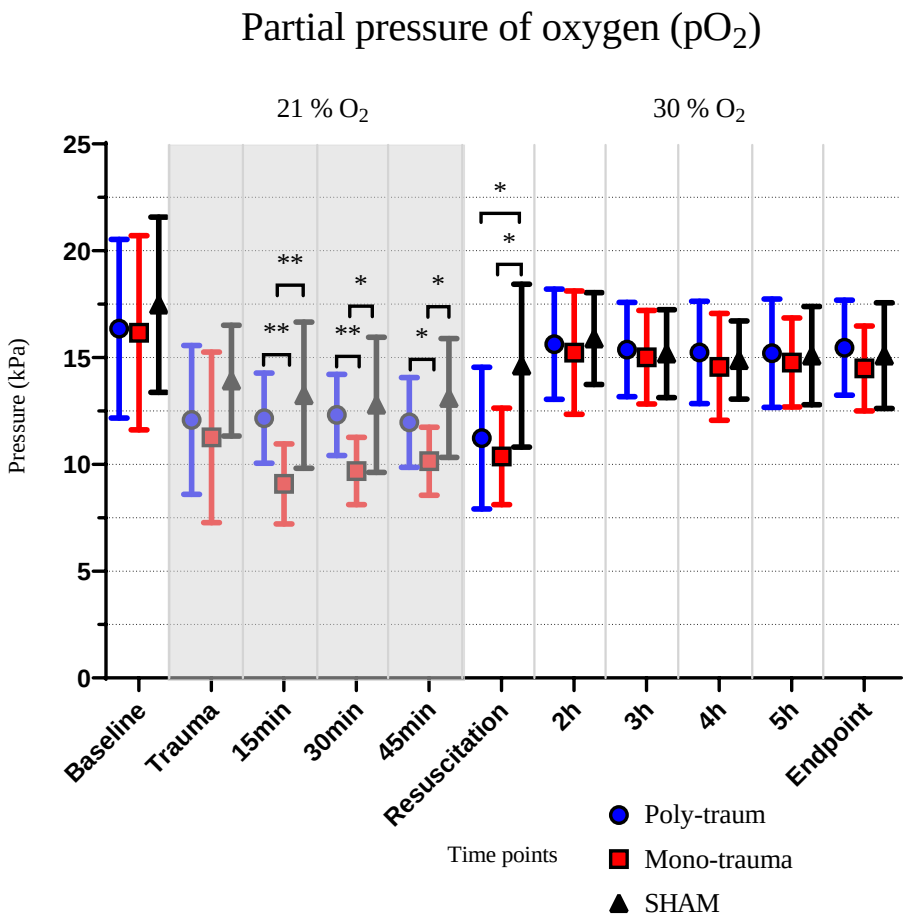
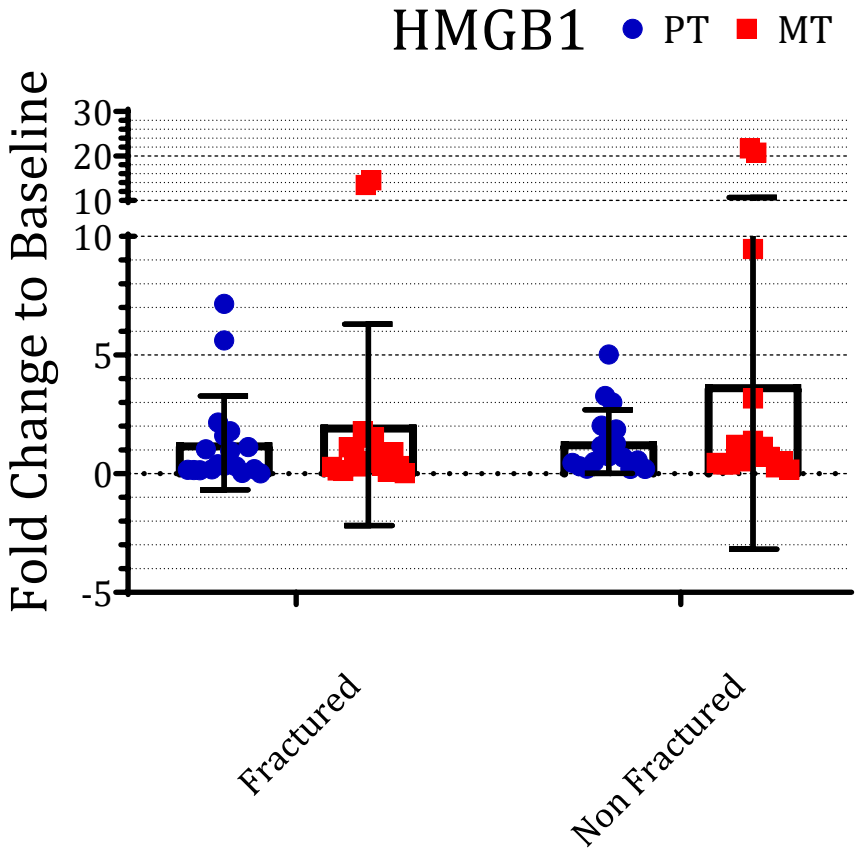


Figure 11: Partial pressure values in all three groups tend to stay grouped. When the oxygen saturation was decreased (during the period of one hour after the trauma) values of pO_2 dropped. However, despite similar values and a decrease of the values, the group of sham animals has significantly higher pO_2 in comparison to a group of PT and MT (*$p< 0.05$ and ** $p< 0.01$).

Local inflammatory response of adipose tissue

Quantitative PCR analysis of HMGB 1 indicates higher expression of the inflammatory protein in the MT group in comparison to the PT group. It can be observed that at fracture site MT group (3.75 ± 6.937) developed a stronger response when compared to the PT group (1.297 ± 1.98) from the same site. Animals from MT group at the non-fractured site (3.75 ± 6.937) developed as a well stronger inflammatory response than PT group at the same site (1.35 ± 1.34) (Figure 12). The statistical analysis did not reveal any statistically significant differences.

Figure 12: Expression of HMGB1 in local adipose tissue. The expression is shown as fold changes compared to



the baseline measurement. A trend towards higher expression in group MT compared to group PT could be observed, but the statistical analysis did not reveal any significant difference between groups.

Analysis and comparison of HSP 70 proteins from fractured and non-fractured site revealed the following result; the expression of HSP 70 proteins from MT group (fractured: 12.551 ± 29.17; non-fractured: 8.27 ± 26.98) is higher than at PT group (fracture: 4.9 ± 11.33; non-fractured: 1.603 ± 2.278). However, statistical analysis failed to demonstrate significant differences between the groups. The summary of the findings can be seen in Figure 13.

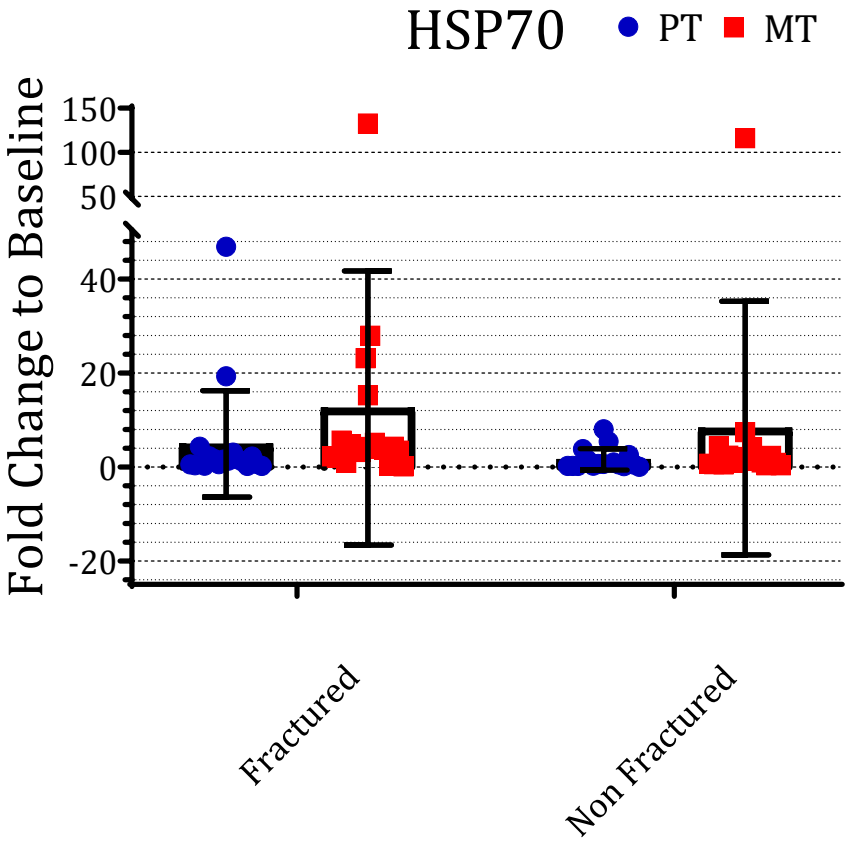


Figure 13: Fold change differences of HSP70 in fatty tissue in MT and PT group at the fractured and non-fractured site. The increase in standard deviation may indicate a different distribution expression of this inflammatory mediator.

Analysis of IL-6 shows higher expression of mentioned interleukin in the MT group when compared to the PT group. Fractured and non-fractured sites of MT group (fracture: 407.57 ± 678.79; non-fractured: 23.58 ± 66.87) revealed higher expression of IL-6 within MT group than in PT group (fracture: 119.47 ± 174.33; non-fractured: 20.35 ± 45.62) (Figure 14). Mentioned comparisons did not reveal statistically significant differences.

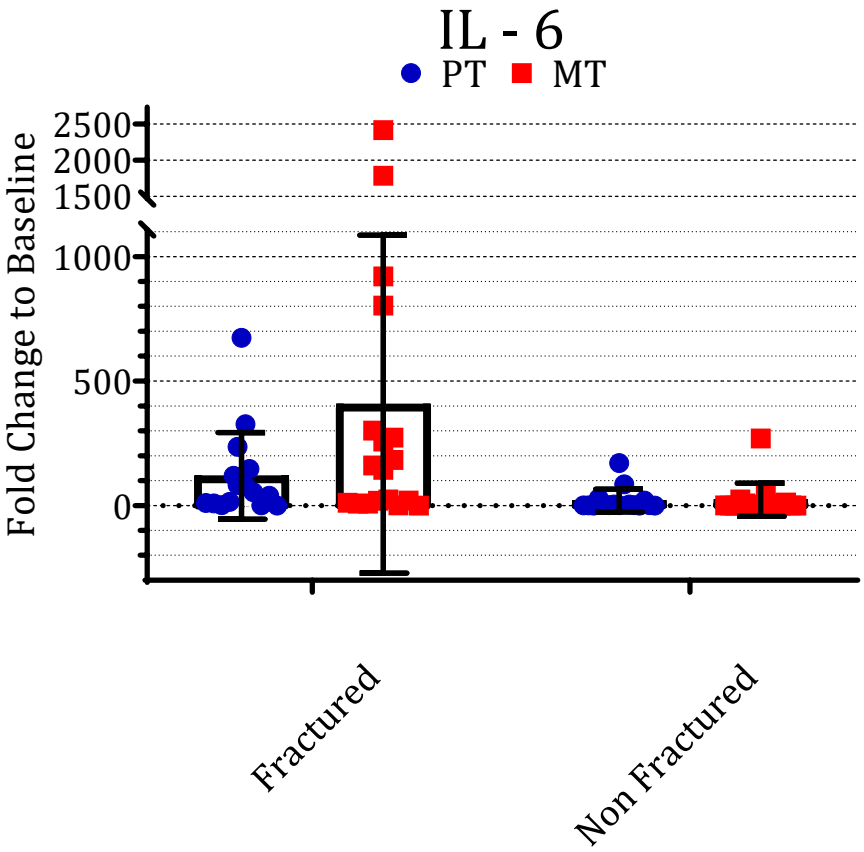


Figure 14: Analysis of IL-6 cytokine presents the higher expression of the inflammatory protein in the MT group than in PT groups at the fracture site. The higher expression of the mentioned protein is also higher at the non-fractured site in MT group when compared to the PT group. However, the expression is not that extreme like at fracture site. Differences are not statistically different.

Further analysis of local inflammatory mediators in adipose tissue once again exposed higher expression of IL-8 in the fractured site (MT: 612.906 ± 1219.17; PT: 17.579 ± 340.07) when compared to the non-fractured site (MT 35.92 ± 125.88; PT: 9.95 ± 26.52). From the gathered data it can be noticed that at the fractured site MT group (612.906 ± 1219.17) developed stronger local inflammatory response than PT 173.579 ± 340.07) (Figure 15). However, data did not reveal any statistically significant difference.

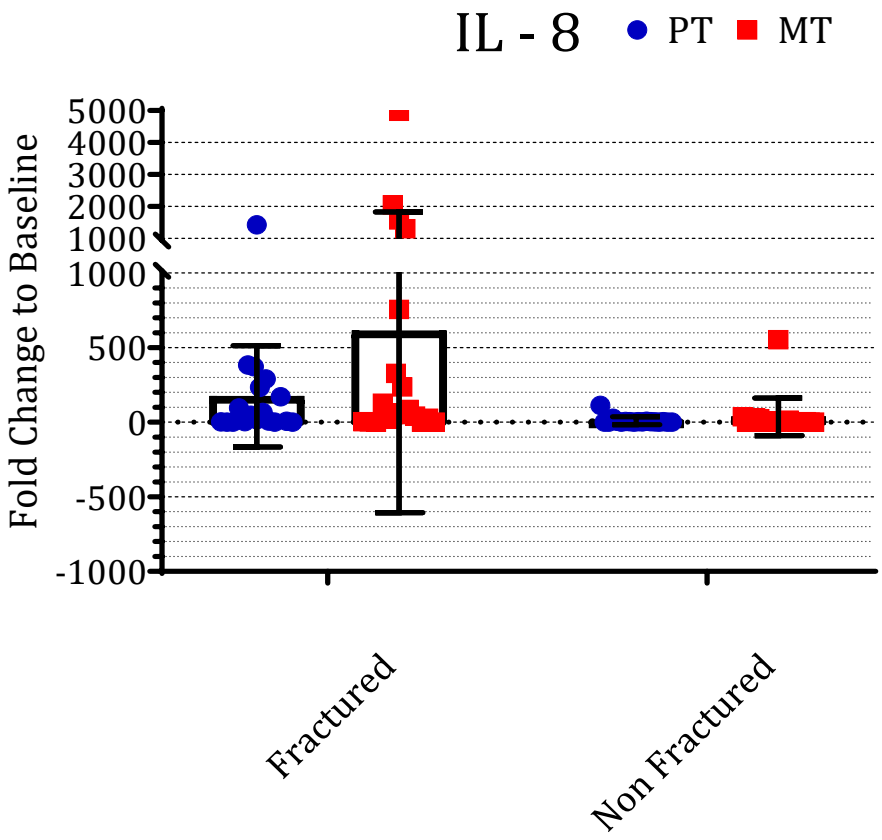


Figure 15: Expression of IL-8 at the fractured and non-fractured site in MT and PT group of animals. MT group of animal developed higher local inflammatory response both in the fractured and non-fractured site. At the fracture site in the MT group, IL-8 is more expressed than at the non-fractured site. Statistical analysis did not reveal a significant difference.

Analysis of IL-10 showed higher expression of mentioned cytokine in the MT group at the fractured site (10.458 ± 16.168) than in the PT group (4.54 ± 3.98). At the non-fractured site, the MT group showed again higher expression (3.051 ± 3.22) when compared to the PT group (2.39 ± 2.12) (Figure 16). Statistical analysis did not expose any statistically significant differences.

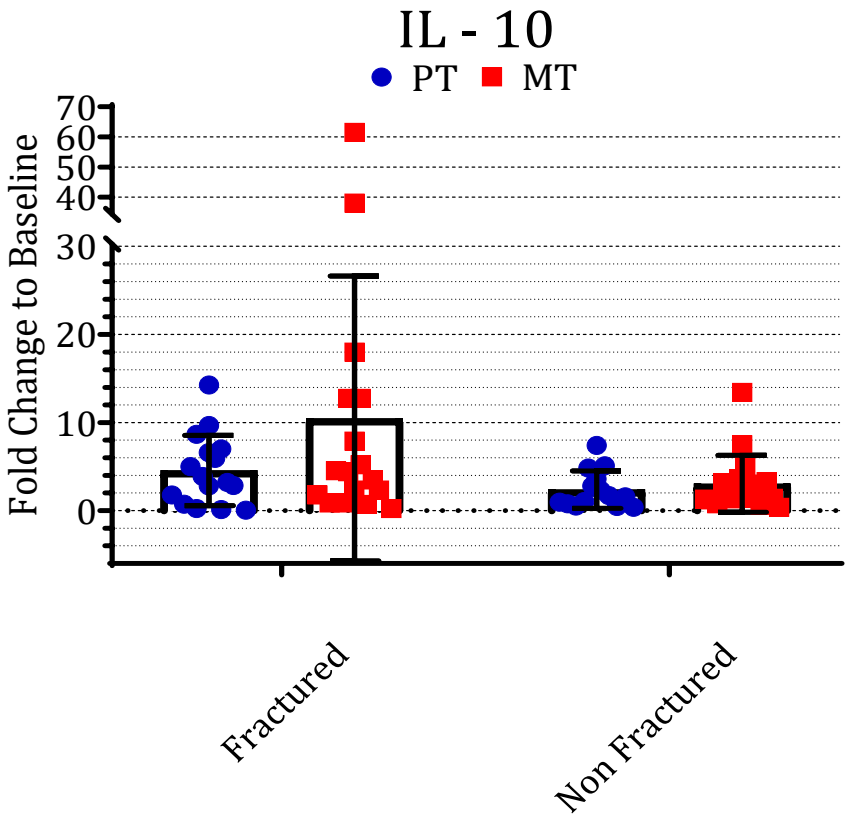


Figure 16: Interleukin 10 expression is more pronounced at the fractured site in both MT and PT group when compared to the non-fractured site. However, when two animal groups are compared within the same site, the MT group has higher expression of IL-10 than PT group. In addition, at the non-fractured site, it can be noted that MT group developed higher local inflammatory response than the group of PT. Differences between groups and sites did not reveal any statistical significance.

Discussion

Injuries are still one of the leading causes of death among the population worldwide [2]. Early mortality occurs immediately or shortly after the accident due to severe bleeding, head trauma or massive organ injury. Numerous improvements in emergency care resulted in a decrease in early mortality in the past decade [3, 4, 6]. However, disorders of the immune system in severely injured patients remain a significant cause of late mortality, which can sometimes appear even weeks after the initial trauma. Previous studies have shown that early local inflammatory response plays a vital role in the initial systemic reaction in order to preserve immune integrity, reestablish homeostasis, and initiate tissue repair and wound healing [34, 35]. However, in severely injured patients local immune response might initiate a stronger immune reaction, which eventually may lead to uncontrolled systemic immune over-activation and consequently to organ failure and death[7]. The exact trigger and connection between local and systemic inflammatory response in severely injured patients is weakly understand. Hence, the primary goal of this study was to investigate the influence and relationship between local and systemic inflammatory response. The better understanding of the interplay between local and systemic inflammation and pro- and anti-inflammatory immune response may be fundamental in the development of new immunomodulatory therapies for severely injured patients [9, 10].

Considering ethical and social aspects, the first choice of animal model for hemorrhagic shock and poly-trauma would be rodents. However, porcine physiological, immunological and some anatomical properties replicate better human-like response to hemorrhagic shock and trauma [36, 37]. Human-like response together with highly reproducible hemorrhagic shock, long bone fracture, and blunt chest trauma makes porcine as a pivotal large animal model in hemorrhagic shock resuscitation and traumatic injury studies [38, 39].

In this study, just like in previous studies, feasibility and reproducibility of hemorrhagic shock [40-42], long bone fracture [43-45], and blunt chest trauma [46] were demonstrated in the porcine animal model. Unlike in previous studies, in this study several injuries were combined: long bone fracture, blunt chest trauma, and pressure controlled severe hemorrhagic shock. In order to compare the local inflammatory response in poly-trauma (PT) and mono-trauma (MT) group, in the PT group, multiple injuries were combined all together on an animal. In the MT group, only femoral fracture was done. Feasibility, reproducibility, and standardization of the injuries, simultaneously with low mortality (5% of the whole number of animals) verified establishment of valuable mono- and poly-trauma animal model.

Mentioned injuries were successfully replicated on all animals. Each femoral fracture and blunt chest trauma was confirmed, assessed and recorded immediately after the injury. Performance of standardized haemorrhagic shock was possible due to pressure-controlled haemorrhage with targeted mean arterial pressure of 25-30 mmHg. Targeted value of MAP in the study reflects hemorrhagic shock and organ hypoperfusion seen in humans during shock and intensive care period [47]. The severity of the hemorrhagic shock and hypoperfusion of tissue is authenticated from the arterial blood gas analysis. The indicators for hypovolemic shock and tissue hypo-perfusion like decrease partial pressure of oxygen, lactate level, and the rise of potassium were significantly changed in all poly-trauma animals when compared to mono-trauma and sham group. Apart from the significant changes in arterial blood gas analysis, poly-trauma animals developed, just like human patients with severe bleeding in clinics, physiological response to hemorrhagic shock. Respond to the hemorrhage manifested itself as increased heart rate, drop of the body temperature and drop of the hematocrit [36, 37].

Investigating local inflammatory response in our study revealed intriguing results. In both animal groups, inflammatory mediators in adipose tissue were elevated 6h after injury when compared to the baseline, which suggests the development of early inflammatory response in adipose tissue. It is noticed that inflammatory mediators were expressed and elevated not only at the fracture site but also at the non-injured site (i.e., contra-lateral limb). The analysis of HMGB 1 expression did not assist our premise that local inflammation mediators would be more expressed at the injured site. Indeed, HMGB 1 was more expressed at the non-fractured site what was not expected. However, our presumption for HSP 70, and interleukins -6, -8, and -10 expressions were confirmed. Their expression did comply with our premise. Mentioned mediators were more expressed at the fractured site in both animal groups. In the end, a comparison of local inflammation in adipose tissue at the fractured site between mono- and poly-trauma animals showed differences. In general, it is noticed that mono-trauma animals have higher expression of all inflammatory mediators in the adipose tissue. However, relations in differences vary. Although, expression pattern exists, and numbers show different expression of inflammatory mediators statistically significant differences were not found when the same sites between two animal groups were compared.

One of the first issue that could affect statistical non-significance from fractured sites in mono- and poly-trauma models are outliers. Each inflammatory mediator that was analyzed has few outliers that are very high, but each outlier is different. It means that outliers do not belong to the same animal or sample; they are not mutually connected. The second issue that can be a potential problem for the non-significant difference of our

data could be the length of the follow-up period. Possibly, six hours of follow up were not enough to develop a significant inflammatory response in adipose tissue in respective animal models. Some studies, which have analyzed alarmins and cytokines, have followed up of forty-eight hours [48, 49]. However, on the contrary, for some other inflammatory mediators maybe harvesting time point after six hours was already a time when those mediators started to decrease, and the tissue was harvested in anti-inflammatory phase. For example, Horst et al. in 2015 published a study about the HSP 70 proteins in which was noticed a decrease of HSP 70 after two and a half hours after the trauma. Last but not the least essential presumption could be the wrong choice of the inflammatory mediators. Maybe some other inflammatory mediators are better expressed in adipose tissue after local injury or some other inflammatory mediators are more specific for porcine trauma model. A similar situation has been seen in a study mentioned before in a publication from Horst et al. from 2015[48].

Gathered data and experience proves that this animal model presents a valuable animal model, which may be used in further research of local inflammatory response in mono- and poly-trauma. However, some modification of the protocol for adipose tissue inflammatory response analysis may be considered.

Conclusion

From what is known, this is the first study focused on analyzing and comparing local inflammatory response in adipose tissue of two separate porcine trauma models. Both groups of animals demonstrated them self as realistic and potential animal models for an upcoming study of trauma. Molecular analysis of local inflammatory response did not reveal clear answers if inflammatory response varies or not in mono- and poly-trauma animal models. Although significant differences do not exist, some evidences suggests that they exist. Accordingly, further research in this area will be required to understand local inflammation response. Furthermore, analyzing other soft tissues and its local inflammatory response may be useful to provide valuable data that would possibly explain the relationship between local and systemic inflammation and their influence on multi-organ failure.

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Curriculum Vitae

Name	Marko Canic
Geburtsdatum	24. Juli 1988
Geburtsort	Zagreb
Nationalität	Kroate

Sep 1995 – Juli 2003	Grundschule Tar, Kroatien
Sep 2003 – Juni 2007	Juraj Dobrila Gymnasium, Pazin, Kroatien
Mai 2007	Bundesweit Abiturprüfung
Sep 2008 – Mai 2015	Studium der Veterinärmedizin an der Universität Zagreb, Kroatien
27. Mai 2015	Abschlussprüfung vet. med., Universität Zagreb, Kroatien

Januar 2016 – November 2016	Doktorand, Institute für Regenerative Medizin, Universität Zürich, Schweiz
April 2018 – Juni 2019	Anfertigung der Dissertation unter Leitung von PD. Dr.sc. nat. Paolo Cinelli am Klinik für Traumatologie, Universitäts Spital Zürich Klinikdirektor: Prof. Dr. med. Hans-Christoph Pape
Ab Februar 2017	Wissenschaftlicher Mitarbeiter, Zentrum Forschung Chirurgie, Universitäts Spital Zürich, Schweiz

April 2018 – Juni 2019	Doktorand, Abteilung Forschung Chirurgie, Universität Zürich, Schweiz

Zürich, 17. Juni 2019