

BURN TRAUMA INDUCES EARLY HMGB1 RELEASE IN PATIENTS. ITS CORRELATION WITH CYTOKINES

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Early HMGB1 release in burned patients

ABSTRACT - High mobility group box protein 1 (HMGB1) is a nuclear protein that may be released actively from monocytes and macrophages or passively from necrotic or damaged cells. Several experimental data suggest that burn injury is accompanied by elevated plasma HMGB, but there are only few data available about its changes in burned patients. The aim of this study was to follow the time course and the prognostic value of plasma HMGB1 and cytokine changes in patients with severe burn injury affecting more than 10 % of body surface area (n=26). Blood samples were taken on admission and on the following 5 days. Plasma HMGB1 concentration was measured by ELISA method, while IL-6, IL-8, IL-10 were assayed by the Cytometric Bead Array Kit. HMGB1 and IL-10 concentrations were elevated on admission and gradually decreased thereafter. Significant differences were observed between survivors and non-survivors in HMGB1 ($p<0.01$) and IL-10 ($p<0.001$) concentrations on admission with higher levels in non-survivors. IL-6 and IL-8 started to increase markedly from day 2. Positive correlation ($r=0.669$, $p<0.01$) was found between burned body surface and HMGB1 on admission. ROC analysis of data on admission showed that at a level of 16 ng/ml HMGB1 indicated lethality with 75.0 % sensitivity and 85.7 % specificity. Using the cutoff level of 14 pg/ml IL-10 predicted ICU mortality with 85.7 % sensitivity and 84.2 % specificity. Very early HMGB1 and IL-10 release may have an important impact on the immune function of patients following burn trauma.

KEYWORDS - burn injury, high mobility group box protein 1, interleukin-6, interleukin-8, interleukin-10, inflammation, necrosis

INTRODUCTION

Although the wound care and intensive therapy of burned patients improved in the last decades dramatically sepsis remained the leading cause of mortality and morbidity. Several studies suggest the important role of increased levels of circulating cytokines produced by T and B lymphocytes, monocytes, macrophages and keratinocytes in the pathophysiological processes after burn (1-3). Recently high mobility group box protein 1 (HMGB1) was also identified as a late mediator of systemic inflammation. HMGB1 is present in the nucleus of mammalian cells (4, 5). Pro-inflammatory activation signals caused by infections induce an active release of HMGB1 from activated monocytes and macrophages (6, 7) and it is also released passively by necrotic and damaged cells (8). Extracellular HMGB1 is a danger signal to responsive cells, and amplifies the signal by increasing production and secretion of other pro-inflammatory mediators (TNF- α , IL-1, IL-6, IL-8 etc.) and finally induces inflammation (5, 9). In apoptotic cells HMGB1 is firmly bound to hypo-acetylated chromatin, and does not transmit the inflammation signal (10). Serum HMGB1 level is low in healthy humans, and significantly elevated in different pathologic states. One study in septic patients showed higher plasma concentration in non-survivors than survivors (11), but the results were contradictory in another study (12). Burn injury is associated with severe hypovolemia due to excessive edema formation. HMGB1 may also be involved in the pathogenesis of haemorrhagic shock (13). Serum level of HMGB1 in patients with acute coronary syndrome has been suggested as a prognostic marker for the risk of developing an acute cardiac event within 1 month (14). Plasma HMGB1 is significantly increased within 1 h of mechanical trauma in humans with marked elevations occurring from 2 to 6 h postinjury (15).

IL-6 and IL-8 participate in trauma induced inflammatory processes. IL-6 plays a key role in B-lymphocyte regulation and acute-phase protein production (16). Serum levels of IL-

6 show association with the burned surface area (17), but it does not cause septic shock after administration in animal models in contrast to IL-8. Serum levels of IL-8 show quick increases after injury. The increase of IL-8 correlates with the development of ARDS and multiple organ failure (18). IL-10 is a key cytokine in the inhibition of inflammatory responses (19). In trauma patients higher levels of IL-10 showed a correlation with the development of sepsis (20). The survival chances can be improved by early antagonism of IL-10 in mice (21). The blocking of IL-10 in animal model could decrease the organ damage and increase survival. Elevated levels of the studied cytokines have been found in burned children on the third day after injury (1). After burn trauma a shift can be observed towards anti-inflammatory cytokine production (2) reflected in elevated levels of IL-10 leading to decreased resistance of infections (3, 22, 23).

Although the role of the different cytokines has been well studied, the dynamism and the prognostic role of their changes are not fully examined in humans following burn injury. Otherwise, several experimental data suggest that burn injury induces significant elevation of plasma HMGB1 concentration (24, 25), but there are only few data available about the changes in plasma HMGB1 concentration in patients (26). The aim of our study was therefore to follow up the time course of plasma HMGB1 and pro- and anti-inflammatory cytokine levels in the immediate postinjury-period to investigate their prognostic value in patients with severe burn injury.

MATERIALS AND METHODS

Patients

Twenty-six patients admitted with burn injury to Intensive Care Unit of Department of Anaesthesia and Intensive Therapy, University of Pécs Medical School between 2005 and

2008 were included in the study. The investigation was based on the permission of the local ethics committee in accordance with current recommendations. Informed consent was obtained from the patients or their nearest relative. Inclusion criteria were the presence of burn injury affecting more than 10% of body surface and in-hospital fluid resuscitation started within 3 h following burn injury. Burned body surface was estimated by the Lund–Browder chart. Exclusion criteria were electrical injury, chronic left heart or renal insufficiency and age less than 18 years. If inhalation injury was suspected (facial burn, soot in the throat, chest X-ray) bronchoscopy was performed for verification. All patients were mechanically ventilated during the study period and survived the first week. Tracheostomy was performed before the first grafting in order to avoid coagulopathy complications. The patients were examined regularly (twice a day) for the presence of sepsis and cases of suspected infection, cultures were taken. The diagnosis of sepsis was based on the American Consensus Conference criteria. Based on the clinical outcome, patients were divided into survivors and non-survivors.

Blood sampling and analysis

Blood samples were collected at the time of hospital admission (day 1) and on the 5 consecutive mornings (days 2 to 6) thereafter. White blood cell count and the relative number of granulocytes, lymphocytes and monocytes were determined. Plasma was isolated from EDTA anticoagulated blood samples by low speed centrifugation at 4 °C, and stored at -80 °C until analyzed in a single batch at the end of the study. Patient management was independent of these results, patients were treated according to our treatment protocol. Excision and grafting were started within 72 hours and enteral feeding was commenced on the first day after injury once hemodynamic stability was reached. Plasma concentration of HMGB1 was measured by a commercially available HMGB1 ELISA Kits (Shino-Test Corporation,

Kanagawa, Japan). Concentrations of IL-6, IL-8, IL-10, were measured by the Cytometric Bead Array Human Inflammation Kit (BD Biosciences, USA). Both assays were carried out according to the manufacturer's instructions.

Statistical analysis

Data were expressed as median and interquartile range (IQR (standard 25th-75th percentile)). Mann-Whitney test was used to compare the values of the survivors and non-survivors. Difference between survivors and non-survivors regarding the occurrence of sepsis was analysed with chi square test. Correlations between variables were analyzed with Spearman test. Values of $p < 0.05$ were considered significantly. Receiver Operating Characteristic (ROC) analysis was used for assessment of specificity and sensitivity of the HMGB1 and interleukin levels regarding mortality.

RESULTS

The study population consisted of 26 patients. Demographic data are presented in table 1. The age of survivors ($n=14$) was significantly lower than non-survivors ($n=12$) (58 %, IQR 46-73; 34 %, IQR 28-51, respectively ($p < 0.05$)), while burned body surface area was significantly higher in non-survivors compared to survivors (40 %, IQR 30-46; 19 %, IQR 15-25, respectively ($p < 0.001$)). Sepsis developed in 5 out of 14 patients in the survivors whereas in 11 out of 12 patients in the non-survivors ($p < 0.05$, respectively) after the observation period.

On hospital admission white blood cell count was markedly elevated and differed significantly ($p < 0.01$) in survivors 13.01 G/l (IQR 8.77-15.38) and non-survivors 23.30 G/l (IQR 17.13-24.93) (Fig. 1a). Burn trauma induced acute severe granulocytosis (87.2 %, IQR 81.8-89.8; 87.3 %, IQR 80.0-88.4), and lymphocytopenia (9.2 %, IQR 7.3-16.8; 9.7 %, IQR

5.8-18.1) both in survivor, and non-survivor patients respectively(Fig. 1b,c).. The relative number of granulocytes decreased, the relative number of lymphocytes increased significantly from day 2 in survivors, while the ratio stagnated in non-survivors.

The plasma HMGB1 concentration was markedly elevated on hospital admission both in survivors 5.01 ng/ml (IQR 3.64-11.44) and non-survivors 21.19 ng/ml (IQR 14.81-34.36) (Fig. 2a). The difference between survivors and non-survivors was significant ($p<0.001$) only on the day 1. HMGB1 level significantly declined thereafter in both groups during the observation period.

IL-6, IL-8, IL-10 concentrations were higher in both groups than the normal values in human plasma throughout observation. IL-10 concentrations were markedly elevated at the time of hospital admission in non-survivors and gradually decreased thereafter, whereas in survivors it showed only a moderate elevation (Fig. 2b). Mean levels of IL-10 in non-survivors were significantly higher compared to survivors during the whole study period ((day 1 ($p<0.001$), day 2 ($p<0.05$), day 3 ($p<0.05$), day 4 ($p<0.01$), day 5 ($p<0.001$), day 6 ($p<0.05$)). Pro-inflammatory cytokines (IL-6 and IL-8) were only moderately elevated on admission and started to increase markedly from day 2. IL-6 reached the peak value in non-survivors on day 4 and a moderate decrease could be found later on (Fig. 2c). In survivors it peaked on day 2, and remained on a lower, but elevated level thereafter. Significant differences were found between survivors and non-survivors on day 4 ($p<0.05$) and 5 ($p<0.001$). IL-8 showed a marked elevation after admission reaching the peak value in survivors on day 3, in non-survivors on day 4 (Fig. 2d). After peaking IL-8 level started to normalise in survivors, whereas in non-survivors a plateau phase could be observed thereafter. Significant differences were found between survivors and non-survivors on day 4 ($p<0.05$), day 5 ($p<0.01$) and day 6 ($p<0.05$).

Positive correlations ($r=0.669$, $p<0.01$) were found between burned body surface and HMGB1 concentrations on admission (Fig. 3). ROC analysis of data on admission showed that at a level of 16 ng/mL HMGB1 indicated lethality with 75.0 % sensitivity and 85.7 % specificity (Fig. 4). Using the cutoff level of 14 pg/ml IL-10 predicted ICU mortality with 85.7 % sensitivity and 84.2 % specificity. IL-6 and IL-8 levels on admission did not show any predictive value. Comparing HMGB1 values to cytokine results a positive correlation was found between HMGB1 and IL-10 levels on admission ($r=0.746$, $p<0.01$), but close correlation could not be found between HMGB1 level on admission and IL-6 and IL-8 levels on admission and later on.

DISCUSSION

The results of the present study demonstrate an early increase in plasma HMGB1 and IL-10 within 3 hours of burn injury in humans. Additionally, we have found that shortly after burn injury HMGB1 and IL-10 levels were significantly higher in non-surviving patients and have good predictive value regarding mortality.

Burn injury induces systemic inflammatory response in the early phase and it is often associated with sepsis in the late phase of the trauma, which can lead to multiple organ failure contributing to high mortality. HMGB1 was recently identified as a potent pro-inflammatory mediator playing an important role in the pathogenesis of human diseases including sepsis, hemorrhagic shock, mechanical trauma, surgical stress, cerebral and myocardial ischemia and pancreatitis (12, 13, 15, 27-29). Several experimental data confirm that burn injury induce an elevation in plasma HMGB1 concentration (24, 25). Using the same, commercially available enzyme-linked immunosorbent assay system, plasma median HMGB1 concentrations were below 2 ng/ml in healthy humans (15, 29). Our results confirmed a marked elevation in HMGB1 following burn injury, as reported recently by Dong and associates (26). In our study

plasma HMGB1 levels were markedly elevated in the very early hours after burn trauma. Using the same assay system Peltz and associates have published recently that plasma HMGB1 was elevated more than 30-fold above healthy controls within 1 hours of trauma injury, peaked from 2 to 6 hours postinjury (15). These results suggest that, in contrast to sepsis, HMGB1 release is an early event after traumatic or burn injury in humans. Moreover, HMGB1 level was significantly higher in our study in non-survivors than in survivors on admission, and despite the small study population it has predictive value indicating lethality with 75.0 % sensitivity and 85.7 % specificity.

Beside HMGB1 elevation following burn injury our results confirmed an overwhelming anti-inflammatory response reflected in marked elevation of IL-10 levels. The anti-inflammatory response was associated with more frequent occurrence of sepsis and higher mortality rate. Higher levels of pro- and anti-inflammatory cytokines following burn injury have been found in other studies (30, 31). IL-6, IL-8, IL-10 produced mainly by macrophages, play an important role in the initial phase of the post-burn pathophysiological processes. Higher levels of IL-6 and IL-10 were found in the early post-burn period (32) supporting our results however, contrary to our study significant differences were not found between survivors and non-survivors in the mean serum levels of IL-6 and IL-10 measured on admission. IL-10 levels showed significantly higher peak value just before the death of the patients (32) compared to survivors. Similarly to our study Ozbalkan and associates found significant differences between survivors and non-survivors regarding serum IL-10 and IL-8 levels measured on admission (33). In our study the levels of IL-10 on admission were not only significantly higher in the non-survivors but IL-10 levels on admission had prognostic value. Our results are in concordance with that of others (3, 22) and confirm an early shift towards anti-inflammatory cytokine production making the patient susceptible to infections due to suppression of T helper type 1 (TH1) cells (34). Our data showed that almost every

patient who died suffered from sepsis and levels of IL-10 were significantly higher compared to survivors on admission and on the following two days. It has been proven in several studies that elevated levels of IL-10 are associated with susceptibility to infection and this way these results support our findings. The importance of early elevation of IL-10 was underlined by the study of Lyons and associates (21). Immediate anti-IL-10 antibody treatment after injury could improve the survival of burned mice suffering from sepsis caused by cecum ligation and puncture. Although administration of anti-IL-10 antibody on the third day after injury could decrease the level of circulating IL-10 and restore the function of TH1 cells it could not increase the survival. These data underline the prognostic value of IL-10 level measured on admission.

HMGB1 could be released actively from monocytes and macrophages or passively from necrotic or damaged cells (6-8). HMGB1 levels on admission correlated well with the burned body surface. Although thermal injury has been shown experimentally to markedly enhance HMGB1 gene expression in various organs, it required 24 hours, or more (35). Burn injury was accompanied by an acute anti-inflammatory excess in our non-surviving patients, with significantly elevated IL-10 concentration on admission in non-survivors. Pro-inflammatory cytokines IL-6 and IL-8 increased significantly only from day 2. These findings support the hypotheses that HMGB1 measured on admission has been released from damaged cells and not due to inflammation. Hypovolemia and tissue hypoxia following burn trauma may additionally contribute to the elevation of plasma HMGB1 concentration.

Our results may indirectly support the experimental findings of Zhang et al. (24) that HMGB1 released after major burns is associated with the development of immune suppression, and HMGB1 also acts as an immunosuppressor cytokine complementary to its role in the pathogenesis of septic response. Elevated level of HMGB1 could inhibit the production of pro-inflammatory cytokines in T helper 1 cells and in macrophages, and could

lead to T cellular immune dysfunction (24, 26). In addition burn injury in our patients induced a marked decrease in the relative number of lymphocytes that gradually increased in survivors, and stagnated in non-survivors, significantly differing from the day 2 of trauma. Septic episodes in our patients occurred in the later phase of the treatment, so the slight elevation in plasma HMGB1 concentration at the end of the observation period might be a sign of impending sepsis, but a close correlation could not be verified.

In the present study we have followed the changes in HMGB1 and pro, and anti-inflammatory cytokines in patients with severe burn injury on admission and for 5 consecutive days. This study is limited by a small study population of burned trauma patients, but the results clearly reveal that burn injury induces a very early HMGB1 and IL-10 release in humans, and it may have an important impact on the immune function of patients following burn trauma. Future research with larger number of patients might further elucidate these potential relationships with HMGB1 and interleukin levels. The role of HMGB1 and IL-10 in posttrauma inflammation and organ dysfunction makes it a potential target for therapy directed at reducing postinjury morbidity and mortality.

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Tables

Table 1.: Demographic data of patients (Median, and interquartile range (25th-75th percentile)).

	Survivors (n=14)	Non-survivors (n=12)	p value
Age (years)	34 (28-51)	58 (46-73)	p< 0.05
Male/female	11/3	10/2	NS
Body mass index (kg m ⁻²)	27 (22-33)	25 (19-31)	NS
Burned surface area (%)	19 (15-25)	40 (30-46)	p< 0.01
Deep burn injury (%)	15 (12-21)	23 (18-30)	p< 0.01
Inhalation injury (n)	10	9	NS
Flame burn injury (n)	8	6	NS
Blast burn injury (n)	2	3	NS
Scald burn injury (n)	4	3	NS
Occurrence of sepsis (n)	5	11	p< 0.05
ICU length of stay (days)	39 (25-52)	23 (14-35)	p<0.01

NS = non significant

Figure legends

Fig. 1. Changes in white blood cell count (A), relative number of granulocytes (B) and lymphocytes (C) in survivor (open box), and non-survivor (patterned box) patients (Median, and interquartile range (25th-75th percentile, and 5th-95th percentile)). # = p<0.05, ## = p<0.01 ### = p<0.001 Survivors vs. Non-survivors.

Fig. 2. Changes in plasma high mobility group box protein 1 (A), IL-10 (B), IL-6 (C), IL-8 (D) concentration in survivor (open box), and non-survivor (patterned box) patients. Data are expressed as median and interquartile range (standard 25th-75th percentile, and 5th-95th percentile)). # = p<0.05, ## = p<0.01 ### = p<0.001 Survivors vs. Non-survivors.

Fig. 3. Correlation between burned body surface and plasma high mobility group box protein 1 concentration in patients on admission. (r=0.669, p<0.01)

Fig. 4. Receiver Operating Characteristic curve of burned plasma high mobility group box protein 1 and interleukin concentrations on admission.

Figures

Fig. 1

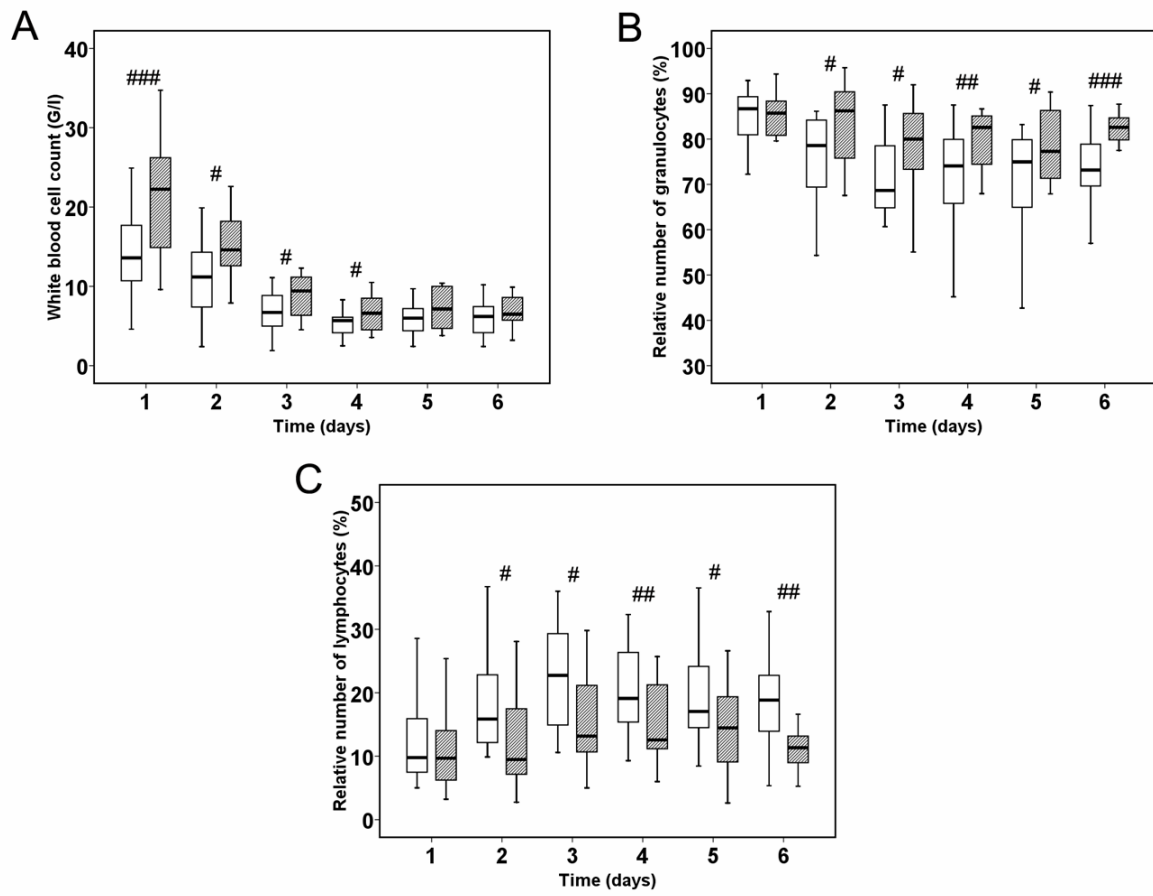


Fig. 2

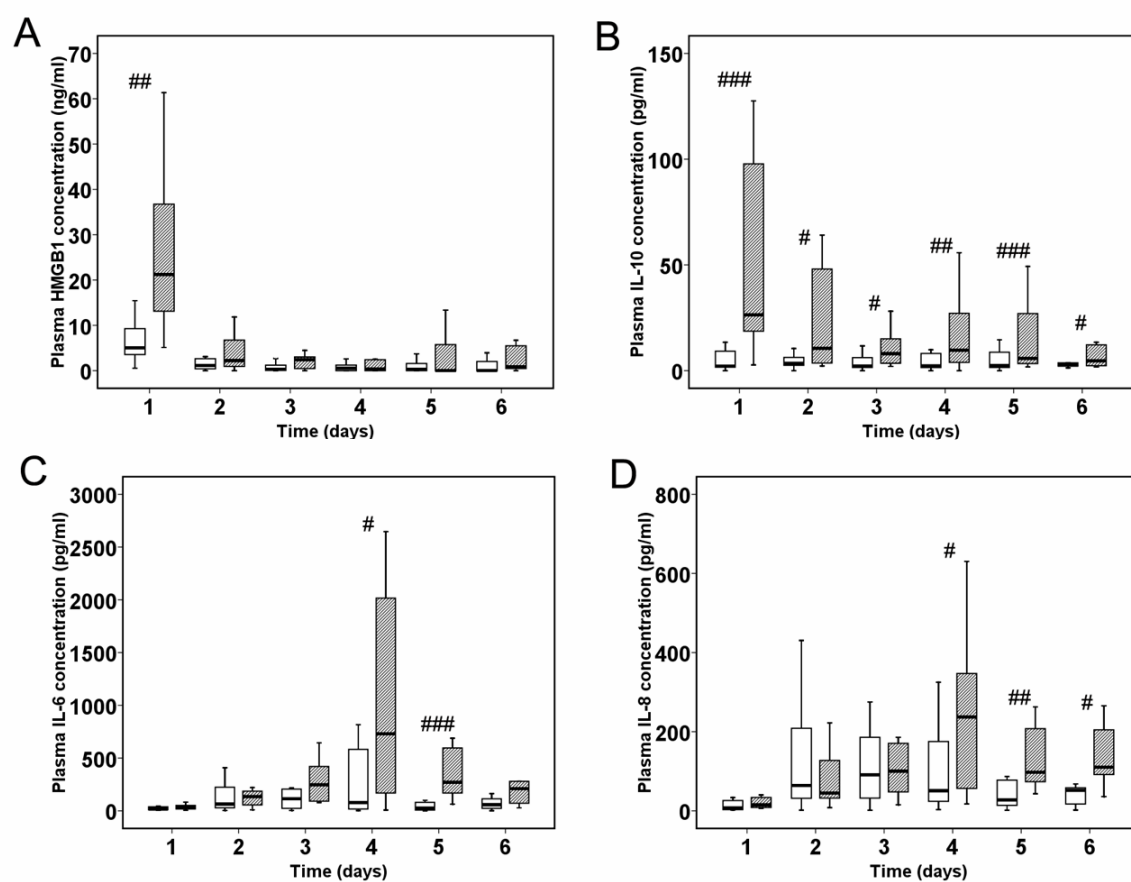


Fig. 3

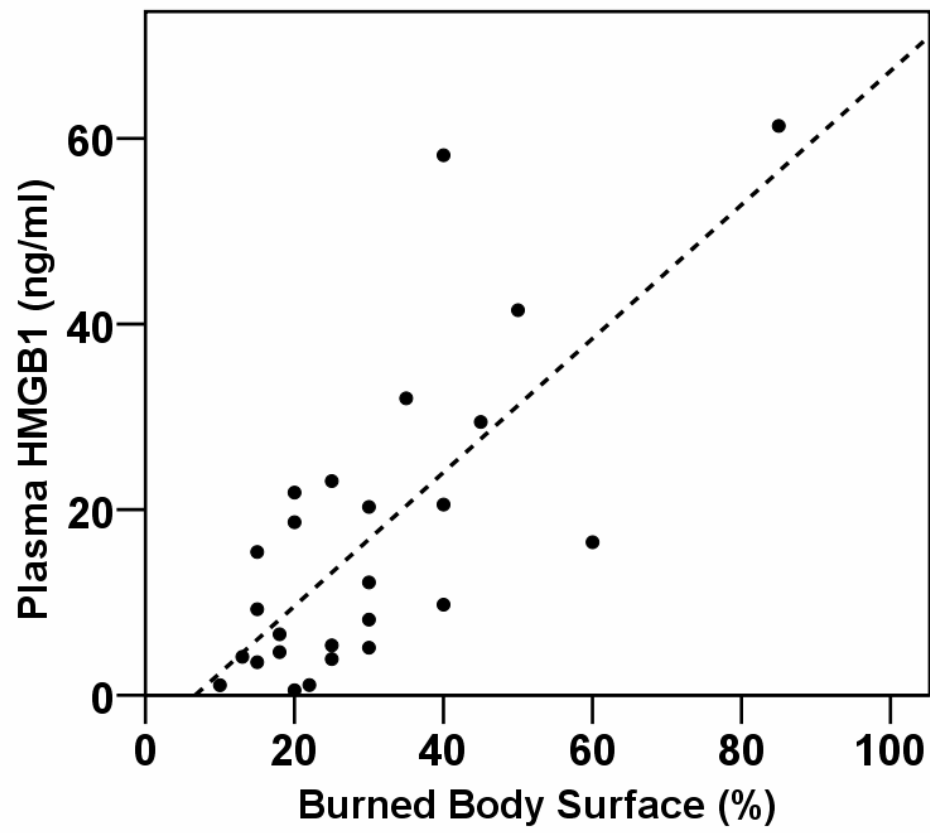


Fig. 4

