Am J Transl Res 2021;13(9):10617-10624
www.ajtr.org /ISSN:1943-8141/AJTR0126471

Original Article

Analysis of polymorphonuclear cell apoptosis and inflammatory cytokines in trauma patients in the emergency department

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Received November 17, 2020; Accepted April 15, 2021; Epub September 15, 2021; Published September 30, 2021

Abstract: Introduction: Durring the immune-inflammation cascade in trauma patients, the roles of polymorphonuclear cells (PMNs) and inflammatory cytokines are very important; however, there is little research in this area, especially for patients with multiple traumas. This study aimed to determine the effects of inflammatory cytokines and apoptosis of PMNs on the prognosis of patients with multiple traumas in tertiary medical centers. Materials and methods: The study subjects were patients with multiple severe traumas who had visited the emergency department. More specifically, patients with multiple traumas included those who had visited the emergency department because of trauma and presented with trauma in more than two body regions. The severity of the traumas was evaluated using the Glasgow coma scale (GCS) and abbreviated injury scale (AIS). In addition, prognostic factors including the length of the hospital stay in the intensive care unit (ICU), the condition upon discharge from the emergency department (discharge, hospitalization in a general ward, hospitalization in the intensive care unit, transfer to a different hospital, surgical operation, death, etc.), outcome of the surgical operation, and presence of infection were examined. To examine the inflammatory response factors, blood samples were obtained. Flow cytometry was performed to analyze PMN cell apoptosis. For comparative analysis, the patients were categorized according to their admission type and the presence of hemorrhagic shock. Results: Ninety-six patients were enrolled in the study (mean age 51.4 ± 16.7 years). When inpatients that had been admitted to the ICU were compared with general-ward inpatients, apoptosis, ROS, MIF, TNF-α, and IL-6 levels were found to be higher, with levels of TNF-α showing a statistically significant difference (726.7 ± 1524.2 vs. 37.5 ± 83.0, P = 0.037). PMN cell apoptosis was rarely observed in shock patients compared with non-shock patients (5.1 ± 5.8 vs. 15.0 ± 26.1, P = 0.004). When subjects were classified based on AIS (11 points or more, no more than 11 points), no significant differences were found between groups. Conclusion: Findings of laboratory tests targeting trauma patients who required hospitalization showed that levels of inflammatory cytokines such as TNF-α were increased in ICU-hospitalized patients. PMN cell apoptosis was reduced according to the initial laboratory data of patients with hemorrhagic shock in the emergency department.

Keywords: Trauma, inflammation, apoptosis, PMN, cytokines

Introduction

Trauma patients account for a large proportion of patients admitted to the emergency department. Trauma is a major cause of acquired disability and is strongly associated with death and disability in those aged < 40 years [1]. As trauma lowers the patient's quality of life and influences social and economic aspects, it is widely dealt with in public medical services [2].

Current therapeutic strategies for trauma patients focus on hemostatic resuscitation, with permissive hypovolemia and blood product-based resuscitation, followed by definitive hemorrhage control before definitive restoration of circulating volume and tissue perfusion [3-5]. The low blood flow induced by trauma and hypovolemic shock triggers hypoxia and systemic inflammatory response syndrome (SIRS). Reduced immunity leads to compensatory anti-inflammatory response syndrome (CARS). Thereafter, failure to maintain homeostasis due to immune dysregulation-induced acute respiratory distress syndrome (ARDS), multiple organ failure (MOF), immune function reduction, and

inflammation overexpression, and other causes leads to life-threatening damage in patients [6-8].

In the immune-inflammation cascade in trauma patients, the roles of polymorphonuclear cells (PMNs) and inflammatory cytokines are very important [9-11]; however, there is little research on these factors, especially for patients with multiple-traumas. This study prospectively investigated multiple-trauma patients in tertiary medical centers to study the effects of inflammatory cytokines and PMN apoptosis on patient prognosis.

Materials and methods

The study subjects were patients with multiple severe traumas who had visited the emergency department. More specifically, patients with multiple traumas included those who had visited the emergency department because of trauma and presented with trauma in more than two body regions. To examine the inflammatory response factors, blood samples were obtained from patients who had agreed to participate in this research. The severity of their traumas was evaluated using the Glasgow coma scale (GCS) and abbreviated injury scale (AIS). In addition, prognostic factors, including the length of hospital stay in the intensive care unit, the condition upon discharge from the emergency department (discharge, hospitalization in a general ward, hospitalization in the intensive care unit, transfer to a different hospital, surgical operation, death, etc.), outcome of the surgical operation, and presence of infection were examined. For comparative analysis, the patients were categorized according to their admission type and presence of hemorrhagic shock.

Whole blood samples were collected from the patients and stored in tubes with ethylenediaminetetraacetic acid (EDTA). A modification of Boyum's technique was applied to separate PMNs. The separated PMNs were collected for the evaluation of apoptosis. Then, in the separated plasma, the levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured.

Approval was obtained from the Institutional Review Board (IRB) of the Korea University Guro Hospital (IRB No. KUGH17229-001).

Subject inclusion and exclusion criteria

The study subjects were patients with multiple severe traumas who had visited the emergency department within a one-year period. This study involved comparative observational research without treatment interventions.

The inclusion criteria for this study were: (i) adults aged > 19 years; (ii) written informed consent; (iii) patients who were diagnosed with multiple traumas and underwent blood tests in the emergency department; and (iv) those with trauma in > 2 regions.

The exclusion criteria were: (i) cases in which blood sample collection and oxygen saturation analysis affected patient treatment and those with other contraindications for blood sampling and oxygen supply; (ii) patients already registered in other studies; (iii) pregnant women and young children aged < 18 years (**Figure 1**).

Separation of PMNs and measurements for apoptosis evaluation

PMNs were separated using a modification of Boyum's technique, as described earlier. First, whole blood samples were collected and placed in tubes with EDTA treatment under sterile conditions. Each 3-mL whole blood sample was placed over 3 mL of PolymorphPrep (Nycomed Pharma AS, Oslo, Norway) in a 15-mL test tube without mixing. Then, the samples were centrifuged at 500× g for approximately 35 min. Layers formed after centrifugation, and the layer of PMNs between the monocyte and erythrocyte layers was collected using a pipette. To disintegrate the red cells remaining in the collected PMNs, a 0.2% saline solution was applied for 30 s. To normalize the osmotic pressure, a 1.8% saline solution was added and then centrifuged at 450× g for 10 min. Repeated washing was performed 1-2 times with phosphate-buffered saline. The separated PMNs were stained with annexin V-fluorescein isothiocyanate (AnV-FITC) and propidium iodide (PI) (ApoScan Annexin V FITC Apoptosis Detection Kit; Genzyme, Cambridge, MA, USA). Flow cytometry (FACSCalibur flow cytometer, Becton Dickinson, San Jose, CA, USA) was performed to examine programmed cell death. If the PI uptake was high, the cells were considered dead. If no uptake of AnV was observed, the cells were considered necrotic. If uptake of

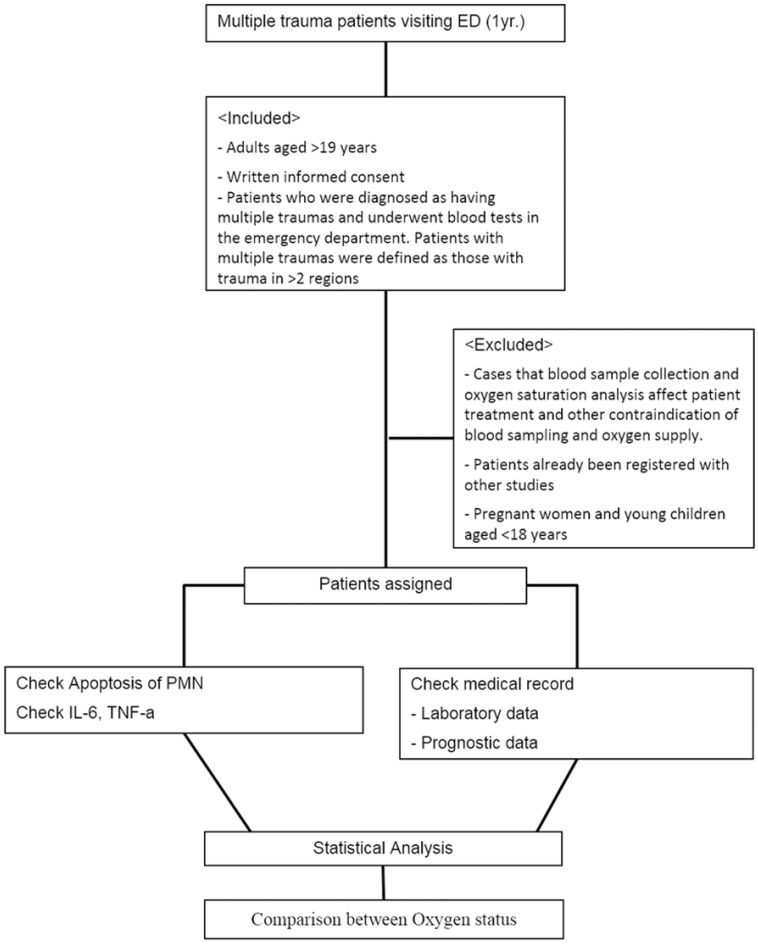

Figure 1. Flowchart of patient enrollment data.

AnV was observed, the cells were considered apoptotic. If no uptake of PI and AnV was found, the cells were considered viable.

Measurement of cytokine levels in plasma and culture supernatants using enzyme-linked immunosorbent assay

The first blood sample (2 mL) collected after each patient's visit to the emergency department was stored in a sodium citrate tube and centrifuged at 1500× *g* at 4°C for 10 min. The plasma was kept at -80°C until analysis. IL-6 and TNF-α levels were measured using an enzyme-linked immunosorbent assay kit.

Clinical data

Information on the general characteristics of the patients, including age, sex, past history, mental status, presence of hemorrhagic shock, and progress, was collected. For the assessment of the physiological trauma score, the Glasgow coma scale was applied. The correlations between apoptosis, prognosis, PMNs, and IL-6 and TNF-α levels were analyzed. With respect to post-blood-collection experiments and prognosis, the treatment period was investigated on the basis of the time to and condition upon discharge from the emergency department, hospitalization duration, and time to and condition upon discharge from the hospital.

Data and statistical analyses

Frequency and comparative analyses were conducted using SPSS 21.0. Each measured value is described as the mean ± standard deviation. As a statistical method, a t-test was conducted for simple comparison between the two groups. For the comparison of AIS scores and oxygen concentrations between > 3 groups, one-way analysis of variance was conducted. Variables with p values < 0.05 were considered statistically significant.

Results

General characteristics

Ninety-six patients were enrolled in the study. The mean age was 51.4 ± 16.7 years. Of the total patients, 71 (74.0%) were male.

Regarding past disease history, 67 (69.7%) patients had none, 14 had diabetes (14.6%), 22 had hypertension (22.9%), one had hepatitis, and one had cancer.

Regarding mental status at the time of emergency room visit, 86 patients were in an alert state (89.6%), 2 were in a verbal state (2.1%), 7 were in a painful state (7.3%), and 1 was in an unresponsive state.

Table 1. Clinical variables of enrolled patients

Patients with multiple traumas (n = 96)	
Age (yr)	51.4 ± 16.7
Male	71 (74.0%)
Past history	
None	67 (69.7%)
Diabetes	14 (14.6%)
Hypertension	22 (22.9%)
Tuberculosis	0
Hepatitis	1 (1.0%)
Cancer	1 (1.0%)
Mental status	
Alert	86 (89.6%)
Verbal	2 (2.1%)
Painful	7 (7.3%)
Unresponsive	1 (1.0%)
Hemorrhagic shock (SBP < 90)	8 (8.3%)
Glasgow coma scale score	14.2 ± 2.3
AIS score	5.80 ± 3.77
Head	1.26 ± 1.28
Face	0.98 ± 1.25
Chest	0.94 ± 1.07
Abdomen	0.63 ± 1.20
Extremity	1.23 ± 1.24
External	0.78 ± 1.11
KTAS	
1	1 (1.0%)
2	11 (11.5%)
3	72 (75.0%)
4	10 (10.4%)
5	2 (2.1%)
Progress (length of stay)	
ICU admission	14 (21.8 ± 13.4 days)
GW admission	24 (10.0 ± 6.3 days)
Discharge	58
Death	1 (1.0%)
Apoptosis (%)	5.9 ± 9.3 (0-78.6)
ROS	29.1 ± 33.4 (0.7-249.0)
MIF (pg/mL)	2500.4 ± 3001.6 (127.33-20648.66)
TNF-α (pg/mL)	855.7 ± 2134.2 (0.04-11937.83)
IL-6 (pg/mL)	587.8 ± 3782.6 (0-36938.62)
iNOS (uM)	124.0 ± 62.0 (0-253.29)

SBP: systolic blood pressure; AIS: abbreviated injury scale; KTAS: Korean triage and acuity scale; ICU: intensive care unit; GW: general ward; ROS: reactive oxygen species; MIF: macrophage migration inhibitory factor; TNF: tumor necrosis factor; IL: interleukin; iNOS: inducible nitric oxide synthase.

The mean Glasgow coma scale score was 14.2 ± 2.3, and the average AIS score was 5.80 ± 3.77.

Eight patients (8.3%) had hemorrhagic shock.

When classified based on the Korean triage and acuity scale (KTAS), the number of patients classified as KTAS 1, 2, 3, 4, and 5 were 1, 11, 72, 10, and 2, respectively.

According to the laboratory test results, apoptosis was found in 5.9 ± 9.3% of patients, ROS was 29.1 ± 33.4, MIF was 2500.4 ± 3001.6 pg/mL, TNF-α was 855.7 ± 2134.2 pg/mL, IL-6 was 587.8 ± 3782.6 pg/mL, and iNOS was 124.0 ± 62.0 uM.

Judging by the course of the patients, 14 patients were admitted to the ICU, with an average funding period of 21.8 ± 13.4 days. Twenty-four patients were admitted to the general ward, with an average funding period of 10.0 ± 6.3 days.

Fifty-eight patients were discharged, and one died (**Table 1**).

Analysis

When inpatients that had been admitted to the ICU were compared with general-ward inpatients, apoptosis, ROS, MIF, TNF-α, and IL-6 levels were higher, and levels of TNF-α showed a statistically significant difference. (726.7 ± 1524.2 vs. 37.5 ± 83.0, P = 0.037) (**Table 2**; **Figure 2**).

When the target patients were classified based on the presence or absence of shock, apoptosis was rarely observed in the shock patients.

Table 2. Comparison between two groups (ICU admission)

	GW admission (n = 24)	ICU admission (n = 14)	*p*-value
Apoptosis (%)	4.2 ± 3.5	4.9 ± 4.2	0.578
ROS	27.7 ± 22.0	30.1 ± 23.7	0.754
MIF (pg/mL)	1314.4 ± 626.8	1978.4 ± 1864.8	0.207
TNF-α (pg/mL)	37.5 ± 83.0	726.7 ± 1524.2	0.037*
IL-6 (pg/mL)	152.5 ± 210.2	265.2 ± 837.4	0.626
iNOS (uM)	143.9 ± 63.7	122.0 ± 51.1	0.251

ICU: intensive care unit; GW: general ward; ROS: reactive oxygen species; MIF: macrophage migration inhibitory factor; TNF: tumor necrosis factor; IL: interleukin; iNOS: inducible nitric oxide synthase. *p<0.05.

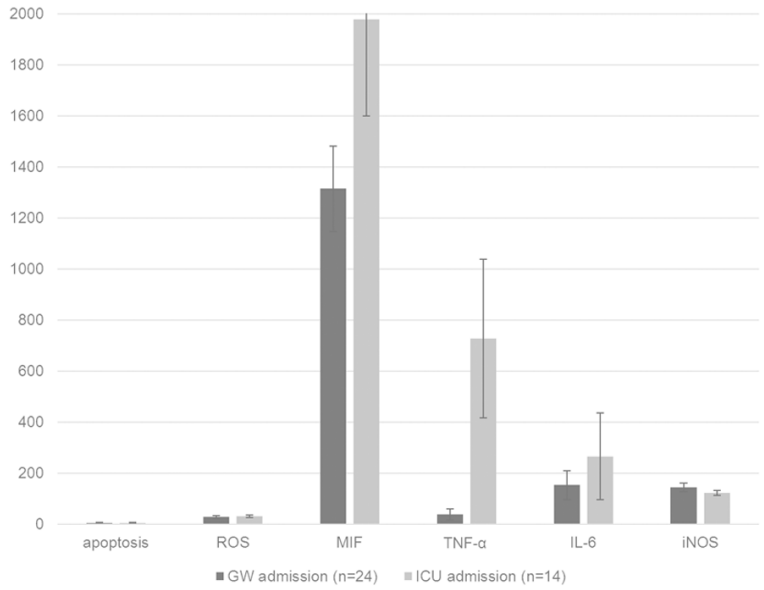

Figure 2. Comparison between ICU-admission group and GW-admission group.

Table 3. Comparison between two groups (hemorrhagic shock)

	Shock (-) (n = 88)	Shock (+) (n = 8)	*p*-value
Apoptosis (%)	15.0 ± 26.1	5.1 ± 5.8	0.004*
ROS	47.8 ± 82.1	27.4 ± 25.2	0.098
MIF (pg/mL)	1972.3 ± 2216.0	2548.4 ± 3068.4	0.606
TNF-α (pg/mL)	897.1 ± 2489.6	852.0 ± 2115.4	0.955
IL-6 (pg/mL)	362.0 ± 557.1	608.3 ± 3948.8	0.861
iNOS (uM)	147.6 ± 70.5	121.8 ± 61.2	0.263

ROS: reactive oxygen species; MIF: macrophage migration inhibitory factor; TNF: tumor necrosis factor; IL: interleukin; iNOS: inducible nitric oxide synthase. *p<0.05.

(5.1 ± 5.8 vs. 15.0 ± 26.1, P = 0.004) (**Table 3**; **Figure 3**).

When subjects were classified based on AIS (11 points or more, no more than 11 points), no significant differences were found between the dose groups.

When the patients were classified based on KTAS, ROS and TNF-α the levels showed significant differences between the groups. (P = 0.001 and 0.008, respectively) (data not shown).

Discussion

Trauma patients accounts for approximately 30% of all emergency department visits [11]. Multiple traumas are highly important medically and socially. Despite the extensive treatment for multiple traumas, a high mortality rate is common problem of multiple trauma patients. Appropriate oxygen supply-without hyperoxia or hypoxia-has an effect on the systemic inflammatory response to trauma. A free airway and an appropriate oxygen supply are considered to be of the highest priority in the treatment of trauma patients. They are emphasized as initial treatments before hospital admission. In addition, until a trauma patient undergoes surgical treatment and recovers, secondary complications should be prevented through the maintenance of body homeostasis. Therefore, the control of inflammatory function is fundamental. Trauma and injuries induce inflammation with features such as cytokine release, neutrophil activation, and microvascular adhesion [12, 13].

This study prospectively investigated multiple trauma patients who had visited a tertiary hospital and analyzed the clinical results of the patients in the early treatment phase as well as the laboratory results. Patient prognosis and

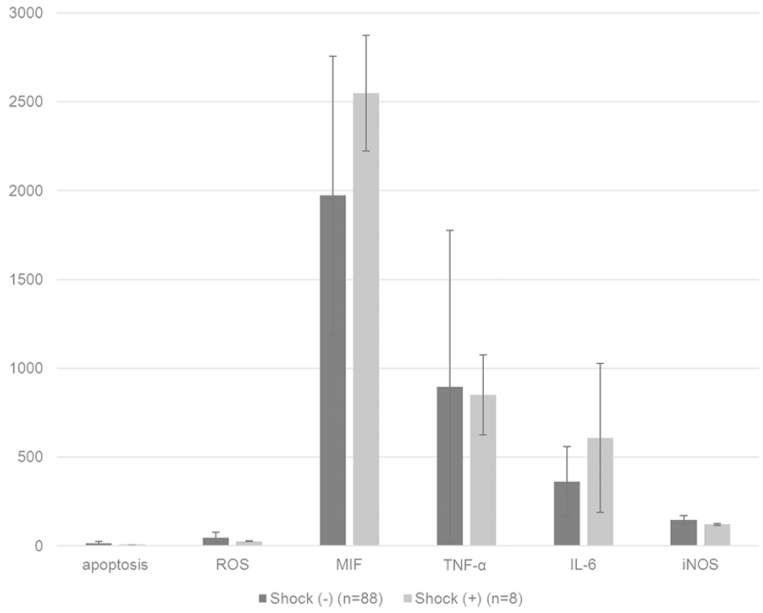

Figure 3. Comparison between shock group and non-shock group.

severity were very diverse, and significant differences could not be shown by classifying patients according to GCS, mental status, or AIS score. However, when targeting trauma patients who required hospitalization, laboratory tests found that levels of inflammatory cytokines such as TNF-α were increased in ICU-hospitalized patients.

TNF-α is a central regulator of the immune-inflammatory response after trauma, with a short half-life in the plasma [14]. Initial blood samples were collected from multiple trauma patients in the emergency department for TNF-α analysis. The effects of TNF-α are controversial, and most of the TNF research in patients with multiple injuries focuses on the clinical course of those in the ICU. It has been reported that consistently high levels of TNF-α are associated with poor outcomes, despite the initiation of treatment [15].

Martin et al. [16] examined the TNF-α and IL-6 levels in trauma patients and found high IL-6 but normal TNF-α concentrations. In addition, septicemic patients were found to have high concentrations of TNF-α and IL-6, which correlated with fatal results. This indicates that septicemic patients had higher activation of the immune-inflammatory system than did trauma patients and that an increased IL-6 level is an index of nosocomial infection in trauma patients.

As the main mechanism underlying early MOF, SIRS is caused by monocytes, PMNs, endothelial cells within the blood vessels, and various inflammatory mediators. In particular, PMNs were found to be an important cell type that triggers organ damage after shock resuscitation [10]. PMNs are short-lived cells, surviving *in vitro* for about 8-12 h before apoptosis. However, the lifespan of PMNs is markedly increased during traumatic injury [17]. PMN cell apoptosis in multiple trauma patients has not yet been studied, and the mechanism is not clear.

Paunel-Gorgulu et al. revealed that PMNs isolated from patients with multiple injuries were found to express significantly greater amounts of the anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1) and significantly lower levels of the proapoptotic protein Bax, a balance that would favor the stabilization of the mitochondrial membrane potential and cell survival [18, 19].

PMNs play an important role in managing the defense mechanisms through antigen presentation of pathogens. PMNs not only manage the cellular rotation through apoptosis but also trigger tissue damage through ischemia-reperfusion in cases of damage or septicemia [20]. Although PMNs serve as an important defense mechanism, uncontrolled long-term survival of PMNs can continuously trigger inflammation; therefore, it is important to maintain control over the cellular lifespan in the setting of inflammatory conditions [11].

The present study found that apoptosis of PMNs was reduced especially in patients with hemorrhagic shock in the emergency department according to initial laboratory data. Although in the analysis of all patients, PMNs apoptosis did not show any meaningful results, PMNs apoptosis seemed to be relative to SIRS due to hemorrhagic shock.

This study has some limitations. First, our study is limited by the small sample size of cohorts and was performed in a single tertiary center; therefore, these results need to be confirmed in studies with larger cohorts. Additionally, it was not feasible to continuously analyze PMN apoptosis and inflammatory cytokines serially in a single trauma patient.

Despite these limitations, our study has important implications since it reveals the effects of TNF-α and apoptosis of PMNs on the prognosis of patients with multiple traumas. Further prospective studies of larger populations and in collaboration with other centers are needed to verify these results.

Conclusions

This study prospectively investigated multiple-trauma patients who had visited a tertiary hospital and analyzed the clinical results of the patients in the early phase as well as the laboratory results. When targeting trauma patients who required hospitalization, laboratory tests found that the levels of inflammatory cytokines such as TNF-α were increased in ICU-hospitalized patients. PMNs apoptosis was reduced in emergency department patients with hemorrhagic shock according to initial laboratory data.

Acknowledgements

This study was supported by a grant from Korea University (K1710681).

Disclosure of conflict of interest

None.

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