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Apoptosis biomarkers (Apaf-1, sFa s, sFa s-L, and caspase-9), albumin, and fetuin-A levels in pulmonary thromboembolic patients

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ORIGINAL ARTICLE

APOPTOSIS BIOMARKERS (APAF-1, SFAS, SFAS-L, AND CASPASE-9), ALBUMIN, AND FETUIN-A LEVELS IN PULMONARY THROMBOEMBOLIC PATIENTS

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ABSTRACT

INTRODUCTION: Pulmonary thromboembolism the third most common medical emergency with mortality two-phase vascular response occurs. Lung ischaemia-reperfusion injury. Lung reperfusion damage is believed to cause cellular damage and apoptosis. The aim of the present study was to evaluate the levels of fetuin-A, albumin, and apoptosis biomarkers (Apaf-1, sFas, and sFasL) among pulmonary thromboembolic patients.

MATERIAL AND METHODS: Blood samples were collected from 45 volunteer patients and 40 healthy control volunteers. Human apoptosis biomarkers (Apaf-1, sFas, sFasL, and caspase-9) and fetuin-A values were measured by ELISA device. Student's t-test or Mann-Whitney U test were used for continuous variables, and categorical variables were compared with the chi-square test to assess the significance of intergroup differences. The mean values of apoptosis biomarkers and acute phase reactants between dead and survival patients were also compared.

RESULTS: While the apoptosis mean values of Apaf-1, sFas, sFasL, and caspase-9 for the control group were 0.12 ± 0.01, 332.1 ± 28.0, 130.4 ± 34.6, and 74.3 ± 2.6, for the patient group they were 0.14 ± 0.02, 509.1 ± 67.6, 139.9 ± 23.7, and 79.4 ± 2.8, respectively. The group differences were significant for all the biomarkers ($p = 0.01$, $p = 0.001$, $p = 0.19$, and $p = 0.01$, respectively). The negative acute phase fetuin-A and albumin levels decreased significantly in the patient groups ($p = 0.01$ and $p = 0.01$, respectively).

CONCLUSİONS: Intrinsic and extrinsic apoptosis pathways are stimulated during pulmonary embolism, and negative acute phase reactants are decreased. There was a correlation with the mortality and Apaf-1, sFas, caspase-9, fetuin, and albumin levels.

KEY WORDS: apoptosis; pulmonary thromboembolism; sFas, sFasL; caspase-9; Apaf-1

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INTRODUCTION

Pulmonary thromboembolism (PE) is the third most common medical emergency with mortality after cardiac and cerebrovascular diseases [1]. In PE at least two-phase vascular response, ischaemia, and reperfusion occurs. Lung ischaemia-reperfusion injury is associated with the alveolar-capillary barrier, and especially with alveolar epithelial cell damage. Thus, pulmonary vascular permeability is increased in relation to the formation of reactive oxygen intermediates, endothelial cell injury, cytokine activation, neutrophil activation, and inflammation response. Lung reperfusion damage is believed to cause cellular damage and apoptosis [2–4].

Apoptosis, known as type I death, was defined in the 1970s. It is accepted that this process is important primarily for tissue homeostasis [5]. Depending on the stimulus, the extrinsic and intrinsic pathways are the main pathways. The intrinsic pathway is activated by intracellular stressors, growth factor deprivation, and oxidative stress. Induced B cell lymphoma-2 family members (Bax, Bak, Bcl-2, and Bcl-xL) by the stressor factors increases the mitochondrial outer membrane permeabilisation and cytochrome c release into the cytoplasm. This process ends with the complex occurred by cysteine protease, caspase-9 adapter protein, and apoptotic protease-activating factor-1 (Apaf-1), known as "apoptosome". Apoptosome then takes procaspase-9, located at the N-terminus of both Apaf-1 and procaspase-9, which induces caspase-3 and cell death [6–8].

Extrinsic apoptosis starts with the activation of the death receptor family proteins: CD95 (Fas/Apo1), TNF-related apoptosis-inducing ligand (TRAIL) receptors, and TNF receptors. This interaction is followed by death-related death domain (FADD) protein and procaspase-8/10, the death-causing signal complex that activates caspase-3, -6, and -7. The activated effector caspases induce cell death or activate the intrinsic pathway, which also causes cell death [9].

The Fas protein is the best-identified member of the 24-member TNF receptor family. The Fas cell receptor that controls cell death in the immune system is found on cytotoxic T cells and natural killer cells. The Fas protein binds to its receptor at the cell surface and provides receptor trimerisation. Activated receptors combine with the FADD receptor molecule. In this way, by stimulating the region near the carboxyl-terminus of the Fas receptor, procaspases are activated and apoptosis begins. As well as Fas and TNF-α, the TRAIL and TRAIL receptors can also induce apoptosis in a similar way [10].

Fetuin-A, also known as human alpha-2-HS-glycoprotein, is mainly derived from the liver parenchymal cells in adults. It has anti-apoptotic and anti-inflammatory activity. While the anti-apoptotic activity is enabled by inhibiting the caspases-3, -8, and -9 subunits that are the main cornerstones in intrinsic and extrinsic apoptosis pathways, the anti-inflammatory is made by macrophage deactivation, antifibrotic activity [11–13].

The aim of the present study was to evaluate the levels of fetuin-A, albumin, and apoptosis biomarkers among PE patients. We hypothesised that while the apoptotic biomarkers would increase due to the increased oxidative stress, fetuin-A and albumin would be decreased due to its utilisation as a protective mediator for the anti-inflammatory effect.

MATERIAL AND METHODS

This prospective case-control study includes patients 45 (26 male and 19 female) with acute PE, who were admitted to the Emergency Department of Cumhuriyet University Hospital from 2018 July to 2019 May. The diagnosis of PE was made with contrast-positive thoracic computed tomography. The control group consisted of 40 patients (24 female, 16 male).

Patients with previous weight management strategies (such as caloric restriction), weight loss surgeries, haematological malignancy, chronic liver disease, diabetes mellitus, or with acute or chronic inflammatory disease were excluded from the study.

The participants were informed about the study and their written approval was obtained. The study protocol was approved by the institutional Ethics Committee of the Sivas Cumhuriyet University (2108-06/17), and consent was obtained from all patients or their relatives.

No human rights were violated during this study, and the study was in accordance with the Declaration of Helsinki.

Blood samples were collected from 45 volunteer patients with pulmonary embolism, who did not receive any treatment before admission at Sivas Cumhuriyet University Medical Faculty Emergency Department. A control group was formed from 40 volunteers who were similar to the patient group according to age and gender.

Approximately 5 mL of venous blood samples were taken from the patient and control groups and added to biochemistry tubes. After 5–10 min at room temperature, the samples were centrifuged at 4000 rpm. The supernatant (serum) formed on top was transferred to Eppendorf tubes and stored at –80°C until the tests were studied.

Plasma fetuin-A levels were measured using a human fetuin-A enzyme-linked immunosorbent assay measurements with Elisa kit for each sample. (ELISA kit Cat no: EK-067-52, lot no: 603894; Phoenix Pharmaceuticals, Belmont, CA, USA).

Human apoptosis biomarkers (Apaf-1, sFas, sFasL, and caspase 9) were also measured by enzyme-linked immunosorbent assay method with the Wuhan Fine Biological Technology Co. Hubei China kit.

Table 1. Apoptosis and biochemical biomarkers between control and patient groups

Parameters	Control group (n = 40)	Patient group (n = 45)	P
	Mean ± SD	Mean ± SD	Student t-test
WBC ($\times 10^3/mm^3$)	7.5 ± 2.0	14.6 ± 2.8	0.01
Glucose (mg/dL)	103.3 ± 18.0	127.4 ± 59.1	0.02
BUN (mg/dL)	17.2 ± 5.9	23.1 ± 5.7	0.01
Creatinine (mg/dL)	0.8 ± 0.2	1.0 ± 0.4	0.01
Calcium (mg/dL)	9.1 ± 0.5	8.8 ± 0.8	0.04
C-reactive protein	7.2 ± 2.9	59.7 ± 23.7	0.01
Albumin (mg/dL)	4.4 ± 0.5	3.4 ± 0.7	0.01
Apaf-1 (ng/mL)	0.12 ± 0.01	0.14 ± 0.02	0.01
sFas (pg/mL)	332.1 ± 28.0	509.1 ± 67.6	0.001
sFasL (pg/mL)	130.4 ± 34.6	139.9 ± 23.7	0.19
Fetuin-A (mg/dL)	11.8 ± 1.8	7.4 ± 1.5	0.01
Caspase-9 (ng/mL)	74.3 ± 2.6	79.4 ± 2.8	0.01

Apaf-1 — apoptotic protease-activating factor-1; BUN — blood urea nitrogen; sFasL — soluble Fas ligand; WBC — white blood cell; SD — standard deviation

Data were analysed with SPSS software version 22.0 for Windows (IBM Corp., New-York, USA). Continuous variables were presented as mean ± standard deviation as appropriate, and categorical variables as numbers (%). Student's t-test or Mann-Whitney U test was used for continuous variables, and categorical variables were compared with the chi-square test to assess the significance of intergroup differences

died or were discharged from the hospital. The apoptosis biomarkers (Apaf-1, sFas, caspase-9) mean levels were significantly higher, whereas albumin and fetuin mean levels were significantly lower in the patient group who died (p = 0.006, p < 0.001, p = 0.01, p = 0.0039, and p = 0.002, respectively) (Tab. 2), which showed us that these biomarkers can be used for prognostication, whereas the sFasL levels did not show any difference between the groups (p = 0.817)

RESULTS

The average age of the control and patient group was 66.33 ± 17.5 and 68.17 ± 16.9, respectively. The control group comprised 24 males and 16 females, and the patient group consisted of 26 males and 19 females. There was no difference according to gender distribution (χ^2 = 0.04, p > 0.05).

We analysed the mean values of apoptosis and acute phase reactant biomarkers between the control and patient groups. While apoptosis biomarkers (Apaf-1, sFas, caspase-9) in the patient group were significantly increased, negative acute phase reactants (fetuin-A and albumin) were decreased significantly (p = 0.01, p = 0,001, p = 0.01, p = 0.01, and p = 0.01, respectively). Only the difference of sFasL between the two groups was not significant (p = 0.19) (Tab. 1)

We also analysed the apoptosis biomarkers, albumin, and fetuin levels among the in-patients who

DISCUSSION

The extent of pulmonary cell death, infarct size, is the most important predictor of survival and long-term outcome in patients with PE. After PE cell death can occur due to oedema, inflammation, neutrophil migration, and intraparenchymal haemorrhage. All of the states can be defined as necrosis and apoptosis. While necrosis is the irreversible cell death form due to the loss of cell membrane integrity and ion pump damage, apoptosis is defined as programmed cell death [14].

By electron microscopy, apoptotic cell death is morphologically characterised by overall cellular condensation, shrinkage, and plasma membrane blebbing. Nuclear changes are characterised by chromatin margination and nuclear condensation followed by segmentation and DNA fragmentation. Finally, the apoptotic cell is broken up into smaller membrane-bound apoptotic bodies that are usually

Table 2. Apoptosis biomarkers between dead and survival patients:

Parameters	Survival patients (n = 38)	Non-survival patients (n = 7)	P
	Mean ± SD	Mean ± SD	Mann-Whitney U
Apaf-1 (ng/mL)	0.13 ± 0.02	0.16 ± 0.02	0.006
sFas (pg/mL)	491.5 ± 58.5	595.2 ± 36.7	< 0.001
sFasL (pg/mL)	140.7 ± 26.3	138.3 ± 5.8	0.817
Caspase-9 (ng/mL)	79.0 ± 2.8	81.9 ± 1.3	0.01
Fetuin-A (mg/dL)	7.7 ± 1.4	5.9 ± 0.7	0.002
Albumin (mg/dL)	3.6 ± 0.8	2.9 ± 0.2	0.039

Apaf-1 — apoptotic protease-activating factor-1; sFasL — soluble Fas ligand; SD — standard deviation

phagocytosed by macrophages. An important morphologic distinction from necrosis lies in the fact that apoptotic cell death lacks inflammation [15].

Apoptosis is a clinical state that is induced by many pathways. Pro-oxidants, ionising radiation, protein synthesis inhibitors, apoptotic or physiologic stimulants, and pro-apoptotic genes are some of the pathways. Cell death occurs either by necrosis or apoptosis during an increased level of oxidative stress [16].

In PE pulmonary vascular circulation is occluded and this impairs gas exchange and circulation, which increases reactive oxygen species. İncreased ROS induces lipid peroxidation, inflammatory cytokines released after the migration of macrophage, and neutrophils. Also, there is accelerated apoptotic cell death [17].

Arnalich et al. [18] analysed the plasma levels of mitochondrial and nuclear DNA in patients with pulmonary embolism and compared them with plasma heart-type fatty acid-binding protein and troponin I biomarkers. The plasma mt-DNA and n-DNA concentrations were higher in the massive pulmonary embolism group, compared with the sub-massive and control groups. Also, the plasma levels were increased for mt-DNA 2.3-fold and for n-DNA 1.9-fold among the non-surviving patients, compared with the survivors [18].

This study reveals that cell death is correlated with pulmonary embolism severity. Cell death in PE occurs either by necrosis or apoptosis. İn our study we have determined that the intrinsic and extrinsic apoptosis biomarkers are increased. The increased rate of DNA concentrations can be related to apoptosis.

FasL expression is regulated by oxidants that are generated in different clinicopathological states like PE-related inflammation and ischaemia/reperfusion injury [19]. Suzuki et al. [20] investigated the rela-

tionship between oxidative stress and sFasl levels in human umbilical vein endothelial cells. The oxidative stress was provided by exposing endothelial cells to nontoxic concentrations (1–100 μM) H_2O_2 and cigarette smoke extracts (0.5–1%). The H_2O_2 increased the FasL level significantly to 0.1–0.3 ng/mL according to the exposure level, which was determined by cell-monolayer-based spectrofluorimetry and flow cytometry. Also, FasL increased significantly after cigarette smoke extracts [20]. İt is known that Fas/FasL is increased to an inflammatory response. Adly et al. [21] analysed the sFas and sFasL levels in sickle cell disease. They compared the levels of sFas and sFasL among the patient and control group. sFas and sFas/sFasL ratio were significantly higher in SCD patients compared with the control group (p < 0.001), whereas the sFasL was significantly lower in patients (p = 0.022). In conclusion of the study, they revealed that the apoptosis biomarkers were significantly higher in patients with pulmonary hypertension or nephropathy [21]. Cardinal et al. [22] researched apoptotic sFas levels among patients with chest pain for the diagnostic accuracy of acute coronary syndromes. sFas baseline levels increased the diagnostic accuracy for acute coronary syndromes. The sFas and sFasL levels were significantly higher in the patient group as a sign for the activated apoptosis pathway in pulmonary thromboembolism.

In the intrinsic pathway after the activation of Bax proteins, cytochrome c and Apaf-1 are released from the mitochondria. The release of Apaf-1 induces caspase-9 and the subsequent caspase cascade. The result is the apoptosis of the cell. Forgiarini et al. [23] determined that an ischaemia period longer than 45 minutes increased the caspase cascade activated the apoptosis. Wang et al. [24] confirmed that caspase-9 and Apaf levels were increased and

apoptosis occurred in acute myocardial infarction. They exhibited a new compound (ZYZ-488) with cardioprotective properties by reducing the apoptosis rate. They examined the apoptosis biomarker levels, caspase-9, and Apaf-1 by treating the mice with ZYZ-488. Apoptosis biomarker levels induced by ischaemia in myocardial infarction decreased after the treatment, and the ejection fraction was better [24].

In our study, the Apaf-1 and caspase-9 levels were increased significantly among the patient group, which explains the increased apoptosis rate (Tab. 1)

Fetuin-A is known as a protein with pro- and anti-inflammatory features. İt induces the cytokines expression and macrophages as a positive acute-phase protein. Whereas in a disease like sepsis, trauma, cerebral ischaemic diseases acts as a negative acute-phase protein [25, 26].

Peng Ma and Yi-Chao Feng [27] investigated the fetuin-A serum levels in inflammatory bowel diseases among 139 patients with Crohn's disease, 114 patients with ulcerative colitis, and 46 healthy persons. They revealed that fetuin-A levels were significantly lower in the people with active inflammatory bowel diseases when compared with healthy and nonactive inflammatory bowel diseases [27]. Minas et al. [11] analysed the correlation of fetuin-A and chronic pulmonary disease severity, a progressive inflammatory disease. They determined that lower levels of fetuin-A were positively correlated with pulmonary function tests and disease severity [11].

An inflammatory process takes part in PE is also after the pulmonary arteries are occluded that has decreased the fetuin-A levels in our study. Also, the mean fetuin-A levels were significantly lower in the patients who died. These results established that fetuin can be used as a prognostic marker in PE cases.

Albumin is the main source for plasma thiols. Parlak et al. [28] evaluated the relationship between dynamic thiol/disulphide levels in pulmonary embolism patients and healthy groups. They demonstrated that the plasma thiol/disulphide rate was decreased in the patients with pulmonary embolism, which may indicate that the albumin level is also reduced [28].

Hayıroğlu et al. [29] evaluated the prognostic nutritional index in PE. According to the classification, prognostic nutritional index patients in tertile 1 was 8.1 times higher. Albumin levels in tertile 1 were also significantly lower than in tertile 3. As a result, they demonstrated that serum albumin-based prognostic nutritional index was an independent prognostic factor for mortality in patients with PE [29]. Our results for negative acute-phase reactant albumin were similar to these studies, and there was a significant decrease in serum albumin level for non-survival patients, which indicated it as a prognostic biomarker for PE disease.

Strengths and limitations
The strength of this study is that it is the first study to represent a significant association between apoptosis biomarkers, fetuin-A, albumin, and PE. The results indicate that this biomarker can be used for prognosis determination. Nevertheless, there are some limitations to our current study. First, all of our patient group was recruited from a single-centre large sample with a limited patient number, and hence a multicentre study with a large number of patients is needed.

CONCLUSIONS
Pulmonary thromboembolism is not only a disease whose progression is dependent on necrosis and inflammation; intrinsic and extrinsic apoptosis pathways are stimulated during the PE process, and negative acute phase reactants levels are decreased. These biomarkers can be used as auxiliary diagnostic and prognostic biomarkers.

Acknowledgements

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