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Original Article

Heparin-induced thrombocytopenia-II in hospitalized patients with surgery or deep vein thrombosis

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Abstract: Objectives: Heparin-induced thrombocytopenia (HIT) is clinically the most relevant non-hemorrhagic complication of heparin, which is associated with the increased risk of thrombosis and mortality. This study was conducted to determine platelet activation in HIT-II in hospitalized patients with surgery or deep vein thrombosis (DVT). The clinical outcomes of the patients was also assayed. Methods: In this descriptive/cross-sectional study, 754 heparin-receiving-hospitalized patients with surgery or DVT were evaluated for the incidence of thrombocytopenia 7 days after heparin therapy. Clinical assessment 4Ts and ELISA for heparin-platelet factor 4 (HPF4) antibodies were performed to diagnose HIT-II. Production of platelet microparticles (PMPs), soluble P-selectin (sP-selectin), IL-1, IL-6, and tumor necrosing factor-α (TNF-α) were evaluated in the HIT suspected patients. Results: The frequency of HIT-II was 4.50%. More HIT-II was diagnosed in the elder patients (P = 0.008) and female (P = 0.005). Thrombosis rate was higher in the HIT-II (P = 0.0001). More PMPs, sP-selectin, IL-1, IL-6, and TNF-α was detected in the HIT-II patients. The length of hospital stay was significantly different in HIT-II (P = 0.015). Mortality rate of the HIT-II patients was higher than non-HIT ones (P = 0.0007). Conclusion: Platelet activation in the HIT-II patients mediated more thrombosis formation. It was associated with the increased length of hospital stay and mortality.

Keywords: Thrombocytopenia, P-selectin, platelet microparticles, thrombosis, interleukin-6

Introduction

Heparin-induced thrombocytopenia (HIT) is one of the dangerous side effects of heparin treatment. It could lead to the activation of platelets, thrombosis, and finally to the death. HIT needs to the special attention as an important complication of drug therapy [1, 2]. Two types, I and II, have been defined for HIT based on underlying mechanism, immune or non-immune depended. The first type, known as non-immune heparin associated thrombocytopenia and is not mediated by antibody. HIT-I often occurs 24 hours after heparin admission and has mild symptoms and severity [3]. The platelet count normalizes without any complication or heparin discontinuation in the HIT-I [3, 4].

HIT-II is the immune-mediated one with antibody production against the heparin-platelet factor 4 (HPF4) complex. Platelet factor 4 (PF4), a cationic protein, is stored in the alpha granules of platelets and is released by the platelet activation. Due to its positive charge, PF4 has strong tendency to bind to negatively charged molecules such as glycosaminoglycan and heparin. Formation of HPF4 complex leads to the production of antibodies. Large HPF4 immune complexes are formed both inside the bloodstream and on the surface of platelets [5, 6].

HIT-II is the most significant non-bleeding clinical complication of heparin therapy. Thrombocytopenia might be absolute, platelet count < 150,000/μL, or relative, a platelet count drop about 50% or more from baseline pre-heparin platelet count [4, 7]. Thrombocytopenia occurs at least 4 days after heparin exposure, usually 5 to 14 days [1, 3]. Its prevalence is 0.1 to 5% of heparin-treated-patients [1, 3, 7]. HIT-II diagnosis is based on 4Ts clinical assessment scoring and the HPF4 antibody assay. Enzyme-linked immune sorbent assay (ELISA) is usually used to detect antibodies in patients with 4 or

more scores of 4Ts scoring system. Serotonin Release Assay (SRA) and heparin induced platelet activation assays (HIPA) should be used to confirm the presence of antibodies. But in case of confirmatory test non-availability, OD > 2 in ELISA method could be used to confirm HIT-II [1, 3].

IgM, IgG, and IgA are produced against HPF4 complexes. It has been determined that the binding of IgG antibody to platelet FcγRIIa activates them leading to alteration of membrane, releasing of different components, and production of platelet microparticles (PMPs) [8]. The antibodies active neutrophils and monocytes through their FcγRIIa receptors too. On the other hand, PF4 binds to platelet, neutrophil, monocyte, and endothelial cell glycosaminoglycan, leading to the production of more antibodies. This damages endothelial cells and lead to the production of tissue factor. Furthermore, these antibodies can attach to the surface receptors of monocytes and induce the release of tissue factor. These events along with PMPs production lead to the creation of procoagulant conditions that can persist for days even after discontinuing the heparin admission [1, 9]. Then, thrombosis can occur in 20 to 64% of the patients because of cross-talking of different blood and/or endothelial cells [10].

Receiving heparin is identified as a factor contributing to the further worsening of thrombocytopenia. In addition, spontaneous thrombocytopenia also occurred in a significant number of patients, which caused their thrombocytopenia to intensify when they were treated with heparin [1, 10, 11]. Therefore, thrombocytopenia requires accurate and timely diagnosis. This issue can also affect the clinical outcomes of the patients. Considering the widespread use of heparin, especially in hospitalized patients, the researchers of this study decided to investigate platelet activation in HIT-II in hospitalized patients with surgery or deep vein thrombosis (DVT) at Shahid Beheshti and Beasat Hospitals in Hamadan province, the west zone of Iran. HIT-II relationship with the clinical outcomes of the patients was also analyzed.

Material and methods

Study design and outline

DVT and surgery candidate patients admitted to Shahid Beheshti and Beasat hospital in Hamadan from May 2021 to August 2023 who had been administered heparin were included in the study. To normalize the data of two different types of patients, DVT and surgery candidate, and to remove confounding variables, relatively equal numbers were selected from the two groups. The sampling method was performed as a census method and 754 people were selected from the files of eligible patients. The study conducted after officially approval by ethic committee in Hamadan university of medical sciences (research code: 140103171647, ethic code: IR.UMSHA.REC.1401.140). The subjects or their legal guardians signed the consent form. Inclusion criteria were candidate for cardiac surgery, having DVT, heparin exposure, hospitalization for at least 7 days, complete clinical and para-clinical data, and filled consent form. The exclusion criteria were no heparin exposure, other causes of thrombocytopenia, blood disorders, incomplete clinical and para-clinical data, discharging before 7 days from admission, and incomplete consent form. Patient information including sex, age, the type of heparin (heparin or enoxaparin), the reason of heparin administration (prophylaxis or therapeutic), pre-heparin platelet count, post-heparin platelet count, and clinical outcomes such as length of hospitalization, final outcome, mortality, or discharge was extracted from the medical records. Seven days after heparin administration, thrombocytopenia was determined by platelet count < 150,000/μL, or a drop about 50% or more from pre-heparin platelet count [4, 5]. At first, 4Ts clinical assessment scoring was performed to detect HIT-II cases. According to developed 4Ts algorithm. 0, 1, or 2 scores was assigned based on clinical features including Timing of platelet count fall or thrombosis, severity of Thrombocytopenia, presence of Thrombosis, and exclusion of other causes of Thrombocytopenia. Less or equal to 3 points, 4-5 points, and 6-8 points were considered as low, intermediate, and high likelihood of HIT-II, respectively [12]. Then, blood samples were taken from patients with 4 or more scores of 4Ts scoring system for more evaluations.

Anti-HPF4 complex antibody detection

Anti-HPF4 complex antibodies were detected in serum of thrombocytopenic patients with 4 or more scores of 4Ts using ELISA (Hyphen BioMed, France) 7 days after receiving heparin/enoxaparin. The assay was performed accord-

Table 1. Demographic, para-clinical, and clinical information of the hospitalized patients

Parameters	Thrombocytopenia		No	All	*P*-value
	Yes				
	HIT-II	Non-HIT-II			
Age (Years)	61.39±8.11	55.62±7.82	50.33±14.02	53.12±16.82	0.008
Male (%)	11 (1.45)	49 (6.49)	320 (4.24)	380 (50.40)	0.005
Female (%)	23 (3.05)	71 (9.41)	280 (37.1)	374 (49.60)	
DVT (%)	39 (5.17)	36 (4.77)	295 (39.12)	370 (49.07)	0.09
Surgery (%)	49 (6.49)	30 (3.97)	305 (40.45)	384 (50.93)	
Heparin (%)	68 (9.01)	32 (4.24)	426 (56.49)	526 (69.76)	0.01
Enoxaparin (%)	29 (3.84)	25 (3.31)	174 (23.07)	228 (30.23)	
Prophylaxis (%)	31 (4.11)	39 (5.17)	289 (39.52)	359 (47.61)	0.10
Therapeutic (%)	40 (5.30)	44 (5.38)	311 (41.24)	395 (52.38)	
Pre-heparin platelet count $\times 10^3/\mu L$	211.28±70.24	210.78±69.98	216.44±71.91	214.75±79.64	0.07
Post-heparin platelet count $\times 10^3/\mu L$	98.88±31.87	120.45±40.09	208.48±58.15	170.48±48.15	0.0005
D-Dimer ng/mL	3298.18±200.55	2100.59±105.90	405.11±45.90	770.86±63.53	0.003
Thrombosis (%)	12 (1.59)	2 (0.26)	1 (0.13)	15 (1.98)	0.0001

HIT-II (≥ 4 score for 4Ts and HPF4 antibody positive with OD > 2). Non-HIT-II (< 4 score for 4Ts and HPF4 antibody negative/with OD 0.4-2). HIT-II: Heparin induce thrombocytopenia-II, ELISA: Enzyme immune sorbent assay, HPF4: Heparin-platelet factor-4 antibody.

ing to manufacture suggested protocol. Optical density (OD) > 0.4 was considered as cut off to determine ELISA positive result and presence of antibody. Due to non-availability of confirmatory test, OD > 2 was utilized to identify HIT-II patients [1, 3].

PMPs isolation and characterization

PMPs isolation was performed on HPF-4 antibody positive patient and healthy people plasma by ultracentrifugation. The samples were centrifuged in various time and force conditions [13]. Isolated PMPs were characterized by dynamic light scatter (DLS) (Malvern, UK) and scanning electron microscopy (SEM). Flowcytometry (Life Technologies Attune Nxtm, USA) was performed for expression of CD63 and CD62p surface markers (Abcam, UK).

IL-1, IL-6, TNF-α, and soluble P-selectin (sP-selectin)

For more evaluation, the inflammatory cytokines such as IL-1, IL-6, and TNF-α as well as sP-selectin were measured in the sera of HPF-4 antibody positive patient and healthy people by sandwich-based ELISA. sP-selectin kit (MyBioSource, USA) kindly gave as a gift on behalf of Dr. Deyhim from High Institute for Research and Education in Transfusion Medicine. IL-1, IL-6, and TNF-α (RayBiotech, USA) were assayed according to manufacturing instructions.

Statistical analysis

The data was analyzed using SPSS version 26 software. The data description was performed using descriptive statistics, expressing the mean and standard deviation for quantitative variables, and the ratio and percentage for qualitative variables. The difference between before and after heparin administration was analyzed by paired t-test. The independent t-test was used to assess the difference between the two groups. Additionally, the Mann-Whitney non-parametric test and the Chi-square test were used. *P*-value less than 0.05 was considered as significance level of difference.

Results

Demographic and clinical information of the patients

In this study, 754 heparin receiving patients with DVT and surgery were followed for thrombocytopenia. The mean and standard deviation (SD) of the patients age were 53.12±16.82 years. In terms of gender, 380 (50.4%) were men and 374 (49.6%) were women. Detailed information of the subjects was presented in the **Table 1**. 154 patients (20.42%) had thrombocytopenia at day 7. Thirty eight patients (5.03%) were scored as ≥ 4 by 4Ts clinical assessment. HPF4 antibodies were detected in all of these patients (OD > 0.4). The OD was > 2

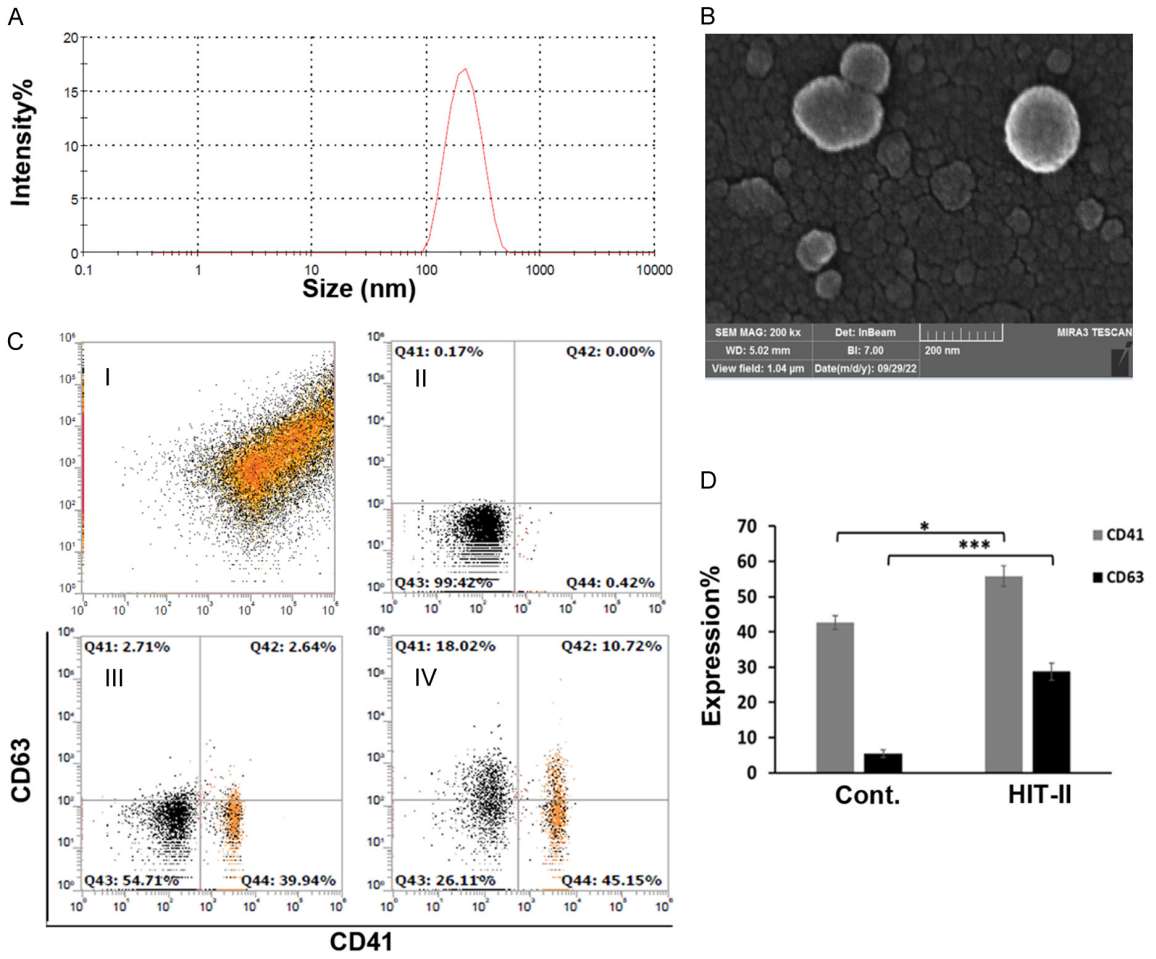

Figure 1. Platelet microparticles (PMPs) characterization. A. Dynamic light scatter (DLS). B. Scanning electron microscopy (SEM). C. Flowcytometry. I. Gating. II. Isotype control. III. Expression of CD41 and CD63 in healthy control. IV. Expression of CD41 and CD63 in HIT-II. D. Quantification of flowcytometry results. *P < 0.05 and ***P < 0.001. HIT-II: Heparin-induced thrombocytopenia-II.

in 34 patients (4.50%). Because of non-available confirmatory test and according to American Society of Hematology [1, 3] these patients were defined as HIT-II cases. More laboratory assessments were performed to evaluate platelet activation and inflammatory status of them. They were managed by clinicians for HIT-II treatment and were followed for any thrombosis events. Twelve (35.29%) of HIT-II patients, 1.59% of all patients, showed evidence of thrombosis after 10 days of receiving heparin. As it is demonstrated in **Table 1**, HIT-II occurred more in the women and elder patients (P = 0.005 and P = 0.008, respectively). There was no significant difference between DVT patients and surgery candidates in terms of HIT-II occurrence (P = 0.09). The incidence of HIT-II in the patients receiving enoxaparin was significantly lower than that of ones receiving heparin (P = 0.01). D-Dimer levels were detected as higher in the HIT-II patients (P = 0.003).

Activated platelets shed more PMPs in HIT-II patients

PMPs were isolated from HIT-II patients and healthy controls. DLS and SEM results confirmed the PMPs isolation, average size 200 nm (**Figure 1A** and **1B**). The expression of CD63 and CD41 on PMPs was measured by flowcytometry. Platelets of HIT-II patients produced more PMPs in comparison to healthy controls. CD63 lysosome-associated membrane protein (LAMP), as platelet activation marker, expressed more on PMPs isolated from HIT-II (**Figure 1C** and **1D**) (P = 0.0006).

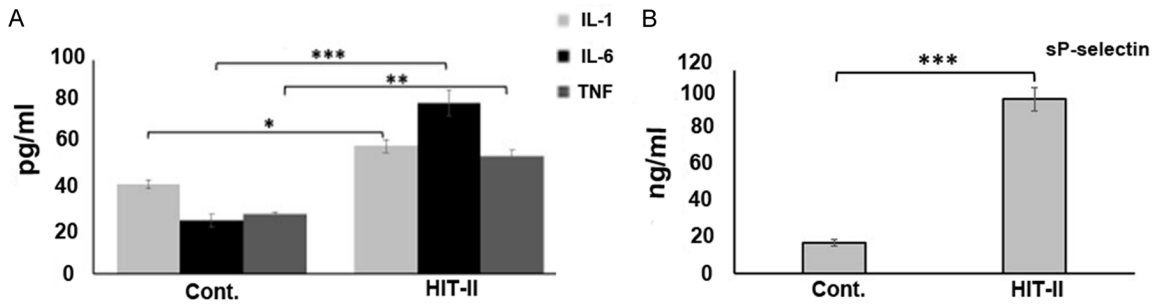

Figure 2. Evaluation of IL-1, IL-6, TNF-α, and soluble P-selectin by ELISA. A. The levels of inflammatory cytokines increased in HIT-II. B. More amount of sP-selectin was detected in the HIT-II. *P < 0.05, **P < 0.01, and ***P < 0.001. HIT-II: Heparin-induced thrombocytopenia-II, IL-1: Interleukin-1, IL-6: Interleukin-6, TNF-α: Tumor necrosing factor-α, sP-selectin: Soluble P-selectin.

Table 2. The length of hospital stay and mortality of the hospitalized patients

Parameters	Thrombocytopenia			All	*P*-value
	Yes		No		
	HIT-II	Non-HIT-II			
Length of hospital stay (Days)	19.36±10.52	16.83±12.60	9.45±6.20	10.86±5.52	0.015
Discharge (%)	39 (5.17)	63 (8.35)	528 (70.02)	630 (83.55)	0.0007
Death (%)	35 (4.64)	19 (2.51)	70 (9.28)	124 (16.44)	

HIT-II (≥ 4 score for 4Ts and HPF4 antibody positive with OD > 2). Non-HIT-II (< 4 score for 4Ts and HPF4 antibody negative/with OD 0.4-2). HIT-II: Heparin induce thrombocytopenia-II, ELISA: Enzyme immune sorbent assay, HPF4: Heparin-platelet factor-4 antibody.

The levels of serum IL-1, IL-6, TNF-α, and sP-selectin was higher in HIT-II patients

IL-1, IL-6, TNF-α, and sP-selectin were measured in the sera of HIT-II and healthy controls by ELISA. IL-1, IL-6, and TNF-α values were higher in HIT-II patients (**Figure 2A**) (*P* = 0.03, 0.0004, and 0.002 respectively). sP-selectin was also increased in the sera of HIT-II vs control (**Figure 2B**) (*P* = 0.0008).

The length of hospital stay and mortality was higher in HIT-II patients

The length of hospital stay and discharge/death rate of subjects was determined as clinical outcomes. According to the presented date in **Table 2**, the mean and SD of the hospital stay days of patients with HIT-II were significantly different from non-HIT-II ones (*P* = 0.015). In terms of hospitalization outcomes, 83.55% discharged and 16.44% death was recorded for all patients. The mortality rate in HIT-II patients was higher than in patients who were not diagnosed as HIT-II (*P* = 0.0007).

Discussion

In the current study, the incidence of thrombocytopenia was 20.42%% in heparin-receiving patients. 4.50% of the patients were diagnosed as HIT-II following by 4Ts assessment and ELISA HPF4 antibody assay. Using of ELISA for HIT diagnosis in combination to 4Ts increases the sensitivity and predictive value for platelet activation and aggregation [1, 14]. But some studies conclude that excluding of 4 or less score might lead to ruling out HIT cases [2, 14]. In the study conducted by Ahmadinejad, the frequency of thrombocytopenia caused by heparin, based on platelet count, was 15%, and those confirmed based on HPF4 antibodies was 5.4% [15]. The incidence of thrombocytopenia in patients treated with long-term heparin (4 days or more) was reported as 43.8% by Olivera et al. in which the cases were diagnosed with clinical assessment method [16]. In the current study, 4Ts clinical assessment along with ELISA HPF4 antibody assay were performed to define the HIT patients. Shah reported 0.6% prevalence for HIT in the DVT patients [17]. The prevalence of HIT has

been reported from 0.1% up to 5% in the different studies [1-4]. The fluctuation in the HIT incidence might be due to the difference in the diagnostic method, the duration of heparin prescription, the type of administered heparin (unfractionated heparin, UFH, or low-molecular-weight heparin, LMWH), prior exposure to the heparin, the underlying diseases of the patients, and host-related factors including gender, age, and race. Dhakal revealed that cardiopulmonary bypass followed by hemodialysis is associated with relatively high rate of HIT. Blacks and other races were more likely to develop HIT compared with whites [18]. In the present study, all subjected had the Persian descent and the results were reported without any racial consideration.

Our study showed that the HIT incidence was higher in the elder patients and females. In Dhakal's and Olivera's studies, it was observed that increasing age increases the risk of HIT [16, 18]. Host-related factors including sex (female) and age (older age) are mentioned as risk factors lead to increase HIT rate [19, 20]. In contrast, Warkentin reported significantly lower incidence of serologically confirmed HIT in women than men [21]. According to the findings of Dhaka's work, women have lower risk of developing thrombocytopenia [18]. Other risk factors and comorbidities might be possible reason for the increased thrombocytopenia in older age which needs further investigation.

Occurrence of HIT in patients receiving heparin was significantly higher than in patients receiving enoxaparin. LMWH has many advantages over UFH such as lower HIT incidence [2, 4]. Enoxaparin is an anticoagulant drug with low molecular weight, fast effect, and long-life period. Its antiplatelet effect is greater and leads to decrease in thrombin production and activity [22]. Farasatinasab showed that admission of LMWH significantly decreased HIT frequency from 40.5% to 2.3% [23]. However, Kim compared the incidence of HIT caused by heparin and enoxaparin. No significant difference was observed between the two drugs with regard to the incidence of HIT [24]. Variation in the duration of heparin admission, re-exposure to the heparin, the patient underlying diseases might explain the diversity of reported results.

According to our data, HIT patient platelets produced more PMPs that confirmed more platelet activation. Different markers including CD41, CD62p, and CD63, are evaluated for confirmation of platelet PMPs production and platelet activation [25-27]. In consist with our results, Campello detected increased PMPs levels in the plasma of HIT patients in comparison to healthy control by flowcytometry measurement of CD62p and CD41 [27]. Papalambros reported more sP-selectin plasma level and platelet activation in peripheral arterial occlusive disease versus DVT [28]. Amin evaluated the sP-selectin in the serum of HIT patients by ELISA. They concluded more sP-selectin correlated with more risk of thrombosis in these patients [29].

IL-1, IL-6, and TNF-α levels increased in HIT patients in comparison to healthy controls. Poredos determined the increased pro-inflammatory cytokines, IL-1 and IL-6, and TNF-α and the decreased anti-inflammatory cytokine, IL-10, in the idiopathic venous thrombosis patients [30]. Zhang demonstrated the negative correlation between IL-6 level and microRNA-338-5p in DVT patients. Increased IL-6 and decreased microRNA-338-5p indicated poor prognosis and more thrombosis events in these patients [31].

Our results specified more thrombosis events in the HIT patients. Various occurrence of thrombosis has been reported in the HIT patients. Several factors including patient-related and/or diagnostic/therapeutic-related factors involve in thrombosis process. High titer of HPF4 antibodies (greater OD in ELISA method) along with PMPs production lead to activation of endothelial cells as well as activation of white blood cells [2, 4]. PMPs-containing IL-1, IL-6, and TNF-α mediate neutrophil, monocyte, and endothelial cell activation [10]. Cross-talking of different blood cells with endothelial cells develops inflammatory and pro-coagulant conditions in the HIT status [10]. It accompanied by more thrombus formation and negatively effects on patient outcomes.

In the present study, more length of hospital stay and death were recorded in HIT patients. Liu identified heparin admission and HIT occurrence as risk factors for disease severity. HIT associates with a surge in the intensive care unit (ICU) hospitalization and mortality [11]. Shah recorded more death but not more hospital stay days for HIT patients with DVT history

[17]. HIT associates with the increased mortality rate, length of hospitalization, and needs to the intensive care in the hospitalized patients [2, 4, 32].

We should state that non-availability of confirmatory or functional tests such as SRA, investigation on two different types of patients, DVT and surgery candidate, and difference in heparin therapy in terms of heparin type or dose were the main limitations of the study. Further researches with more normalization in the study subjects, more sample size, and more defined HIT patients with up dated guide lines should be conducted to address these points and improve validity and reliability.

Conclusion

Platelet activation and PMPs production develop inflammatory and pro-coagulant status and results in more thrombus formation in the HIT patients. Mortality and length of hospital stay increase in these patients. The evaluation of pro-inflammatory and pro-coagulant markers could be useful for monitoring of mentioned patients.

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Disclosure of conflict of interest

None.

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