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Histopathological Examination with Cd61 Confirms D-Dimer as a Predictor of Diffuse Pulmonary Microthrombi Findings in Covid-19 Patients

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Abstract:- Background: COVID-19 is a global concern that emerged and became a pandemic at March 2020. It is related to dysregulation of immune system causing excessive immune response known as cytokine storm. Other than that there is hypercoagulation that could progress to thrombosis and embolism in various vital organs. Pulmonary embolism is one of the dire consequences of hypercoagulation. CTPA and V/Q scan are the gold standards in pulmonary embolism diagnosis. However in developing countries like Indonesia these facilities are often absent. Thus a simple blood test like D-dimer could aid the detection of pulmonary thrombosis in COVID-19.

Materials and methods: Subjects were 34 patients who died because of COVID-19 in COVID-19 isolation ICU at Dr. Soetomo General Hospital, Surabaya, Indonesia. Clinical information, radiologic and laboratory results were retrieved from medical record files. Histopathological examination was done by routine hematoxylin and eosin staining and CD61 immunohistochemistry staining of lung tissues. Tissues were obtained by core biopsy and examined under 100x and 400x magnification.

Results: All samples examined had positive CD61 results for platelet microthrombi. Focal microthrombi were found in 61.8% of samples while the rest 38.2% had diffuse microthrombi. There was significant difference in D-dimer levels between patiens who had focal and diffuse microthrombi (p value 0.035). Analysis using ROC curve showed D-dimer level cut-off of 3055 ng/ml had 61.5% sensitivity and 71.4% specificity for the findings of diffuse pulmonary microthrombi.

Conclusion: D-dimer values above 3055 ng/ml indicated the presence of diffuse microthrombi in the lungs. D-dimer can be used to aid the diagnosis of diffuse lung microthrombi in COVID-19 patients.

Keywords:- *COVID-19, pulmonary embolism, microthrombi, D-dimer, CD61.*

I. INTRODUCTION

COVID-19 infection is known to be associated with dysregulation of inflammatory response and cytokine storm. This process triggers activation of coagulation cascade and ends up in a state of hypercoagulability causing thrombosis or embolism in vital organs, which worsens the morbidity and mortality of COVID-19 patients. All critically ill patients with or without anticoagulant prophylaxis are at risk for thrombosis or thromboembolism [1]. Immediate medical intervention is required when it happens in the pulmonary vessels. Post mortem examination or computed tomography pulmonary angiogram (CTPA) and V/Q scan remain the gold standards in diagnosing thrombosis or pulmonary embolism.

Limited medical facilities and infrastructures are issues in developing countries like Indonesia. As a result, the authors wished to learn more about thromboembolism in COVID-19 patients' lungs and whether there are any techniques to make pulmonary thrombosis diagnosis easier, particularly in COVID-19 patients. Given that diagnosis equipment is not always readily available, it is worth investigating whether a simple test like D-dimer can aid in the detection of pulmonary thrombosis in COVID-19.

II. MATERIALS AND METHODS

A. Study population

From July to December 2020, samples were taken from COVID-19 patients who died while being treated in the COVID-19 specialized ICU in Dr. Soetomo General Hospital in Surabaya, Indonesia. COVID-19 diagnosis was confirmed

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by ante mortem RT-PCR examination. Clinical information, chest x-ray examination results, and laboratory results were obtained from medical record files. This study had been approved by ethical committee of Dr. Soetomo General Hospital and was carried out with the patient's family's approval via signed informed consent forms.

B. Lung tissue collection, processing and analysis

Lung tissues were retrieved within 60 minutes after the patient was pronounced dead while following WHO COVID-19 handling standards in a COVID-19 mortuary chamber with negative pressure and PPE. Tissue samples were collected from the lungs at multiple locations (7-9 points) using a 14 gauge core biopsy needle by professionals who had performed core biopsy procedures regularly. Ante mortem chest x-ray examination was used as guiding in tissue collection. No patient underwent a CT scan of the thorax due to limited facilities.

Fixation of tissue samples was done using a 10% neutral buffered formalin before. Transportation of the samples to the anatomy pathology laboratory was following laboratory biosecurity standards. After 24 hours, all samples were sectioned at 3-5 µm from paraffin-embedded tissue samples and stained using routine hematoxylin and eosin (H&E) and CD61 immunohistochemistry staining (Biocare). Control true positive and true negative slides were utilized to analyze the readings of CD61 staining. Samples were examined under a light microscope at 100x and 400x magnification. Slides were analyzed by at least two pathology specialists who had experiences and skill in analyzing thoracic pathology.

III. RESULT

A. Patient characteristics

This study included 34 deceased COVID-19 confirmed patients. There were 25 men and 9 women. All patients had undergone an ante mortem nasal swab test with RT-PCR, which gave a positive result for SARS CoV-2. All patients were admitted to the COVID-19 isolation ICU, showed a spectrum of ARDS symptoms, and received mechanical ventilation therapy with an average stay of 10 days (10.65 ± 5.40). Symptoms of sepsis and COVID-19 were found in all patients, with majority of SOFA scores above 10. Unfractionated heparin pump at therapeutic dose was given as anticoagulant treatment and D-dimer level was checked periodically. Patient characteristics and D-dimer levels can be seen in table 1.

B. Histopathological findings

The main finding on H&E staining was atypical enlargement of pneumocytes and intraalveolar fibrous plugs with diffuse alveolar damage and hyaline membranes. In terms of vascular changes, there were perivascular or intravascular inflammatory infiltrate, followed by alveolar proteinosis and hemorrhage. Microthrombi in the alveolar capillaries were found in three samples. Interstitial and intraalveolar inflammatory infiltrates, interstitial fibrous changes, and interstitial collagen deposits were also observed. Lung samples were then examined using CD61 immunohistochemistry staining.

Examination under light microscope showed that the findings of CD61 staining were classified semi-quantitatively into focal microthrombi (≤50% visual field) and diffuse microthrombi (>50% visual field) at magnifications up to 400x [2]. This classification was implemented because all samples examined had positive CD61 results for platelet microthrombi. These findings were then compared to the patient's latest D-dimer level prior to death.

Characteristics (mean ± SD)	(N = 34)	Percentages (%)
Microthrombi findings		
focal	21	61.8
diffuse	13	38.2
Age (49.4 ± 12.4)		
<40 years old	7	20.59
40-49 years old	9	26.47
50-59 years old	10	29.41
≥ 60 years old	8	23.53
Sex		
Male	25	73.53
Female	9	26.47
LOS (10.65±5.40)		
≤ 14 days	26	76.47
>14 days	8	23.53
SOFA score (12.26±3.03)		
≤10	8	23.53
>10	26	76.47
Comorbidities		
Hypertension	7	20.59
Diabetes Mellitus (DM)	8	23.53
Both Hypertension and DM	6	17.65
No comorbidity	13	38.24
D-dimer level (3353.82±2411.16)		
<1000 ng/ml	4	11.76
1000-1999 ng/ml	10	29.41
2000-2999 ng/ml	6	17.65
≥3000 ng/ml	14	41.18

Table 1: Patients' characteristics and D-dimer levels

CD61 staining revealed diffuse microthrombi surrounding the alveoli (>50 % visual field). Brownish colored cells indicated positive CD61 staining as seen in figure 2. Cross-section view of the small blood vessels demonstrated a suggestive local thrombotic process (figure 2d). Some of the alveoli were well expanded, and some were collapsed with platelet aggregates surrounding them as seen in figure 3. Furthermore, a picture of the collapsed alveoli with very few microthrombi around them (≤50% of the visual field) can be seen in figure 4.

C. D-dimer levels

The laboratory values used were from the last examination before the patient died. The range of patient's D-dimer level was quite wide with mean ± SD 3353.82 ± 2411.16 ng/ml. Analysis using Mann-Whitney test showed

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median value (min-max) of 1950 (530 – 11320) ng/ml in patients with focal microthrombi and 3950 (1650 – 6940) ng/ml in patients with diffuse microthrombi (p value 0.035).

Statistical analysis was carried out on the D-dimer variable as a predictor of the findings of diffuse microthrombi in COVID-19 patients in this study. Using the receiver operating characteristic (ROC) curve, the area under curve (AUC) was 0.718, which is a moderate level of accuracy in predicting the incidence of diffuse thrombus in the lung. Based on the cut-off curve, a cut-off of 3055 ng/ml was obtained with a sensitivity of 61.5% and specificity of 71.4% for the findings of diffuse pulmonary microthrombi.

IV. DISCUSSION

This study initially aimed to prove whether there are microthrombi in the lungs, in accordance with the COVID-19 theory of hypercoagulation and immunothrombosis [3]. In this study, microthrombi were observed in all samples. Borczuk et al, conducted a COVID-19 autopsy study. Focal and diffuse microthrombi were found in 84% of the patients and 77% of the samples with pulmonary microthrombi had large vessel thrombi [2]. Microscopic platelet fibrin thrombosis was found in 80-100% of reported COVID-19 deaths [4].

The core biopsy method was used to obtain lung tissue in this study. Due to facilities and cultural boundaries, an autopsy could not be performed. Several other studies had used the core biopsy approach to analyze lung tissue of COVID-19 patients in other countries. The total number of samples examined in this study was 34 samples. Results of H&E staining were consistent with the characteristics of diffuse alveolar damage (DAD) [5][6][7][8]. Microvascular thrombi and cases of pulmonary infarcts suggestive pulmonary vascular thrombus obstruction were found [7][8]. H&E staining showed few thrombi in the pulmonary microvasculature.

In this study, CD61 immunohistochemistry staining was used to identify microthrombi. Platelets and megakaryocytes express transmembrane protein CD61 (integrin beta three chains). CD61 can detect thrombus formation by binding to prothrombotic molecules such as fibrinogen, fibronectin, plasminogen, prothrombin, and von Willebrand factor [9]. CD61 is useful in detecting acute thrombus. Positive COVID-19 cases showed significant increase in CD61 staining compared to control lung tissue ($p < 0.0001$) and influenza ($p < 0.01$). Overall, the predominant pattern of CD61 staining was seen in the form of intravascular aggregates, which were located within the intraalveolar capillaries and smaller vessels. CD61-positive tissues are identified primarily in the capillaries, and in areas with inflammation, the neutrophil inflammation was usually negative [10]. Borzcuk et al, found large vessel thrombi in 42% and fibrin microthrombi or platelet component (CD61 positive) in 84% of 68 autopsies. The morphology of microthrombi is somewhat different from that of large thrombi that are visible. Microthrombi contain fibrins and platelets and are mostly detected in small arteries <1.0 mm in size. Some thrombi contain large numbers of platelets and appear as intravascular granular material highlighted by the CD61 immuno histochemical staining [2].

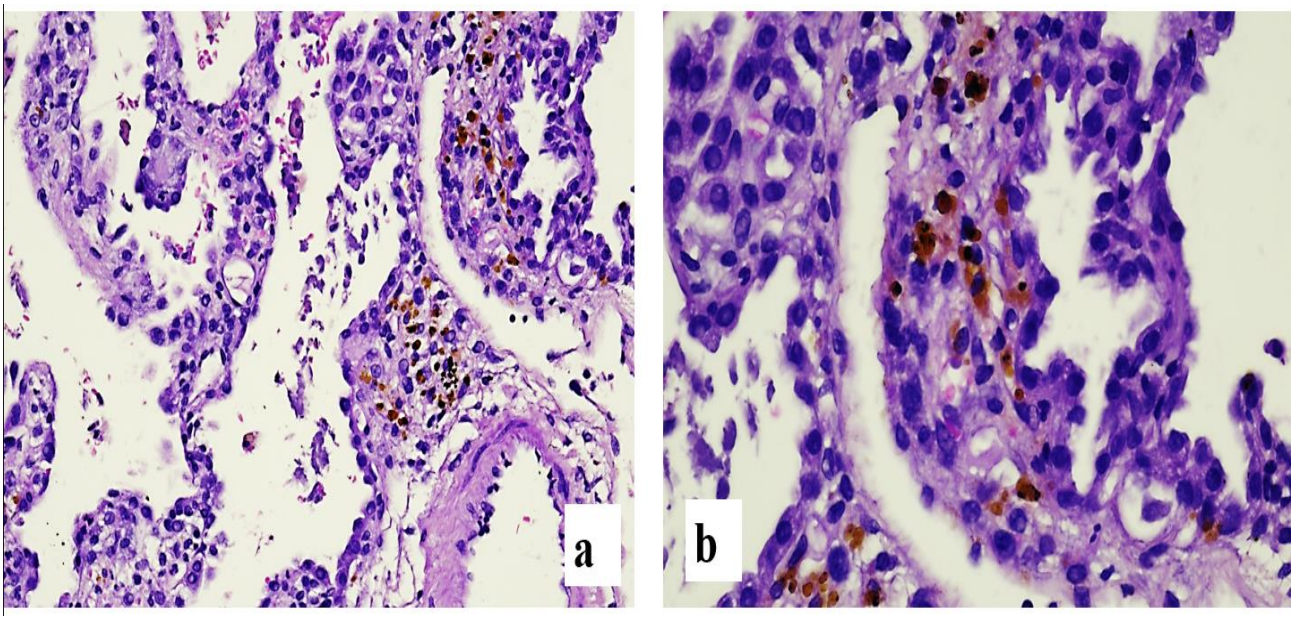

Fig. 1: H&E and CD61 staining showed microthombi around the alveoli. (Figure 1a. At 200x magnification H&E staining showed inflammation-associated hemosiderin pigment, 1b. At 400x magnification of the same section H&E staining showed hemosiderin pigment identified around blood-vessel. This area would be confirmed by CD61 to look for microthrombi.)

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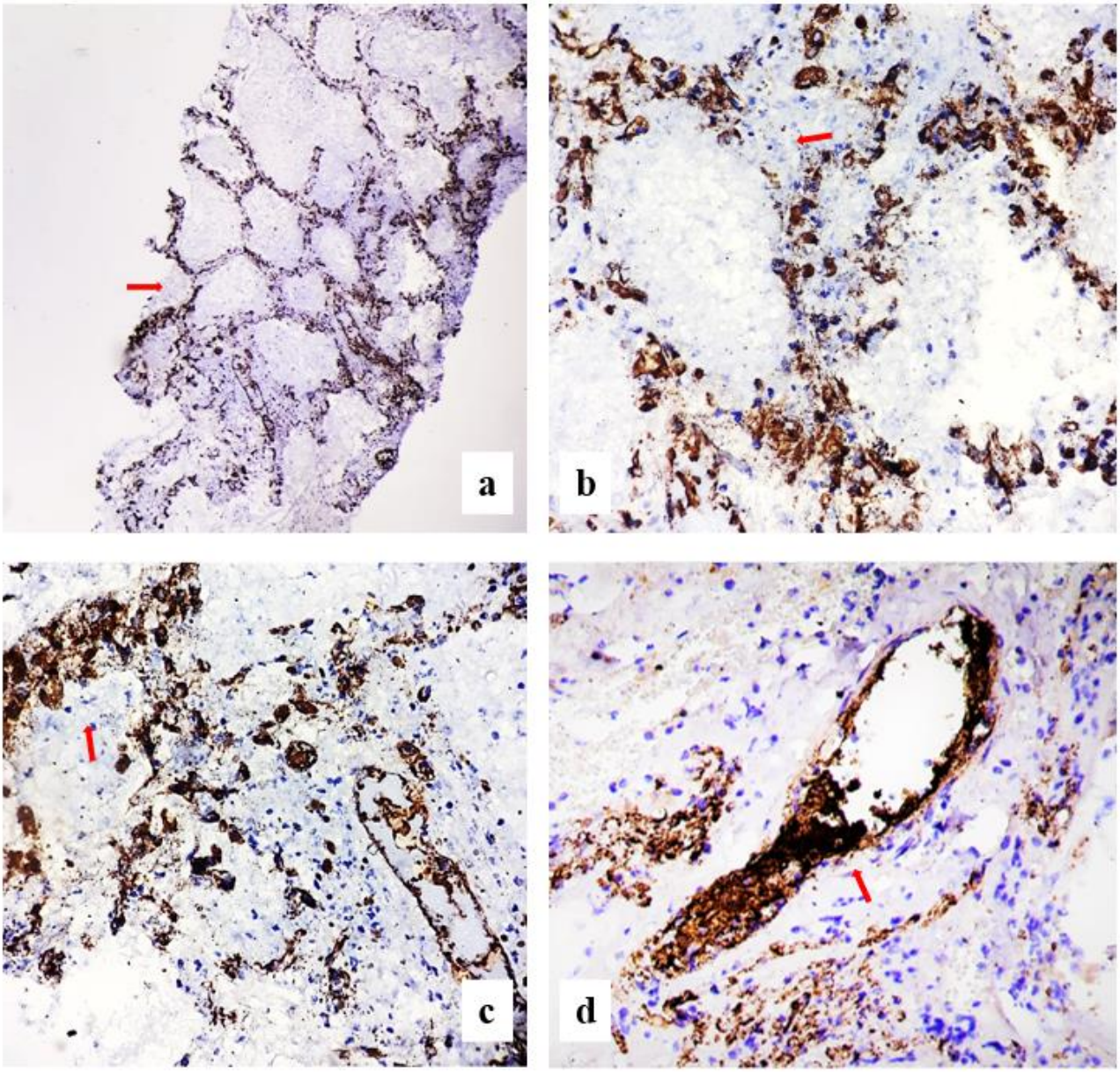

Fig. 2: CD61 immunohistochemistry staining showed diffuse microthrombi of lung tissue. (Figure 2a. At 100x magnification microthrombi appear in almost all perialveolar areas that appear to be well-inflated, 2b. At 400x magnification the well inflated perialveolar area surrounded with microthrombi, 2c. Diffuse microthrombi in damaged/collapsed alveolar tissue, 2d. Aggregates of platelets on the walls of blood vessels. Aggregates appeared to adhere to the wall, suggesting that thrombosis process developed locally.)

Another study using CD61 identified multiorgan megakaryocytes and platelet fibrin thrombi, indicating that megakaryocytes and platelets might play a role in COVID-19 diffuse microvascular thrombosis. Most thrombi were found in the pulmonary arteries, arterioles, and microvasculature rather than the systemic arteries. The megakaryocytes and platelets in the microvasculature could still be linked to microvascular thrombosis caused by platelet activation [10][11].

COVID-19 patients with severe symptoms had higher D-dimer levels at hospital admission. The D-dimer levels of non-survivors were also higher according to a meta-analysis of 29 studies (4328 patients). As a result, in COVID-19 patients, an elevated D-dimer can be used as a marker of severity and mortality [12]. Higher D-dimer level is also linked to higher risk of thromboembolism [13].

The presence of D-dimer in the blood does not indicate the specific location of thrombus formation, and it can be elevated in a variety of other medical conditions. However, the finding of this study was consistent with the COVID-19 theory, which stated that the primary thrombotic process occurs from the lungs (pulmonary intravascular coagulation) [3]. The capacity of the lungs to degrade fibrin is well known. Simultaneous process of coagulation and anticoalgulation causes the formation of a fibrin degradation fragment known as the D-dimer, which is then released into the bloodstream and detected on examination [14].

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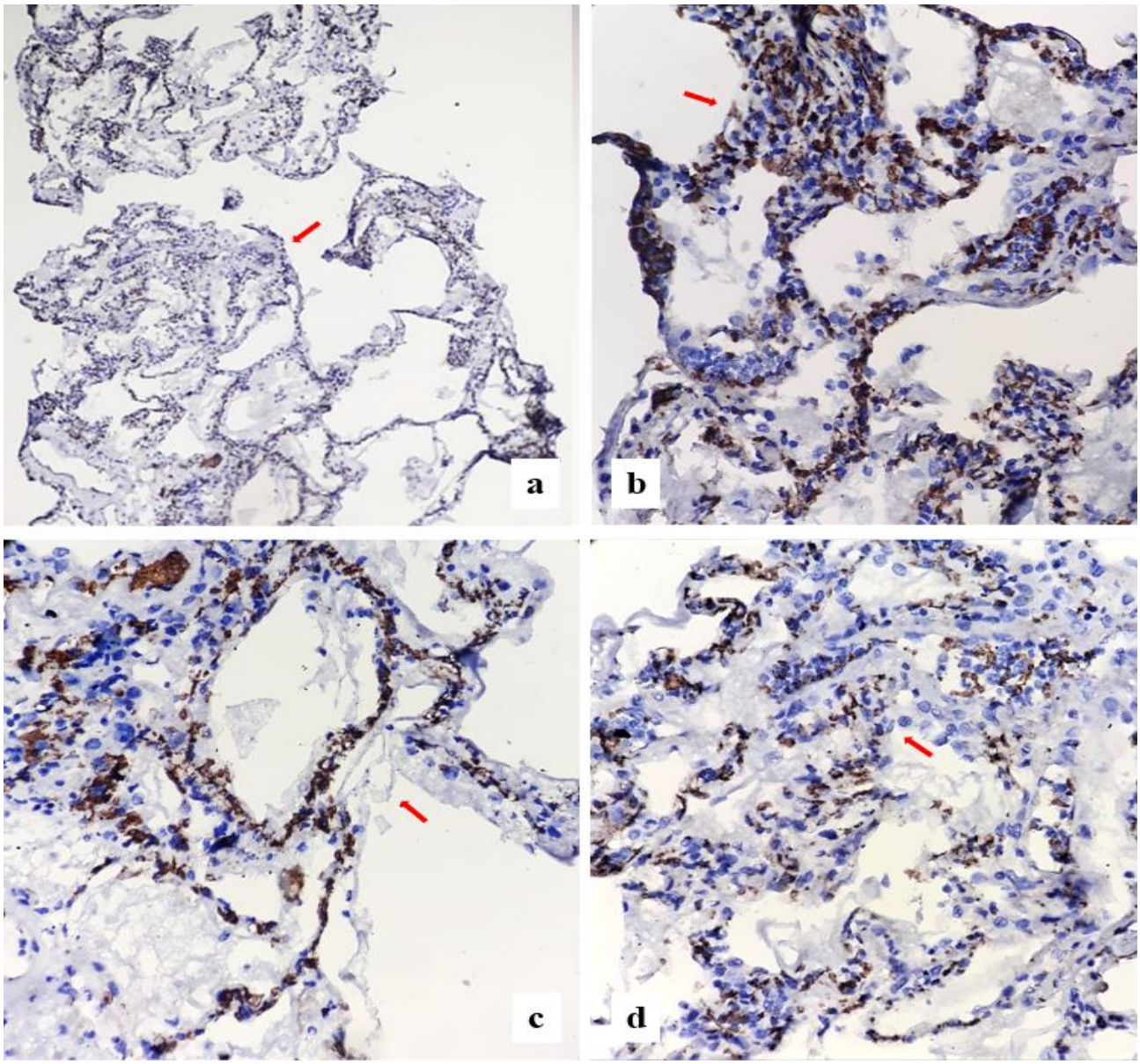

Fig. 3: CD61 staining revealed diffuse pulmonary microthrombi around some collapsed alveoli. (Figure 3a. At 100x magnification there were a multitude of CD61 positive cells around the collapsed alveoli, 3b. At 400x magnification microthrombi were noticed in the perialveolar area which partially collapsed and partially expanded 3c. At 400x magnification there were visible microthrombi around well-inflated alveoli, 3d. At 400x magnification microthrombi were found in the collapsed perialveolar capillaries area.)

D-dimer levels were higher in COVID-19 patients with pulmonary thrombosis or embolism. Anticoagulant therapy was not proven to be associated with pulmonary thrombus, and D-dimer levels greater than 2660 mcg/L had 100% sensitivity and 67% specificity for findings of pulmonary thrombosis or embolism on CT scan [15]. The D-dimer value are quite valuable for detecting venous thromboembolism. With an increase in D-dimer, the likelihood ratio of venous thromboembolism increases [16]. D-dimer has a high negative predictive value when it applies to exclude the possibility of pulmonary embolism. A D-dimer limit greater than 2152 ng/ml significantly increased the risk of developing pulmonary embolism in non-COVID-19 conditions, with a positive predictive value of 53% and a negative predictive value of 82% [17].

In this study, CD61 immunohistochemistry examination yielded 100% positive findings of microthrombi in lung tissue, which were then classified into focal and diffuse categories. We used the D-dimer parameter to distinguish between focal and diffuse microthrombi in the lungs of critically ill COVID-19 patients, not to identify thrombi. With a sensitivity of 61.5% and specificity of 71.4%, D-dimer values above 3055 ng/ml indicated the presence of diffuse microthrombi in the lungs.

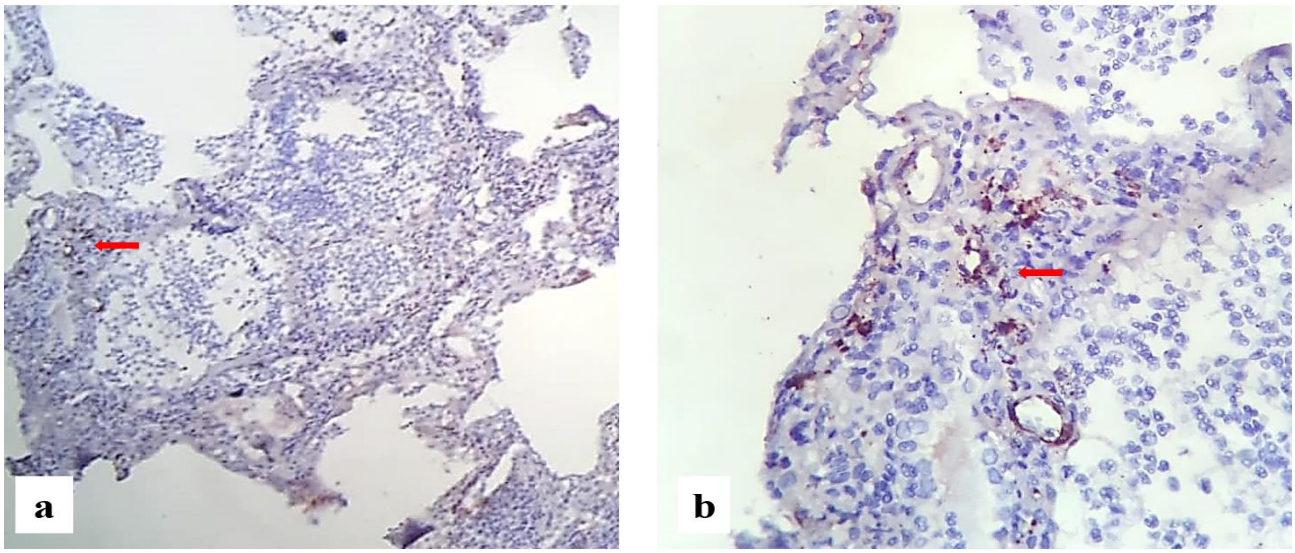

Fig. 4: CD61 staining revealed focal pulmonary microthrombi. (Figure 4a. At 100x magnification there were few CD61 positive cells in partially collapsed alveoli (red arrows), 4b. At 400x magnification microthrombi found in the collapsed alveoli.)

Pulmonary intravascular coagulopathy can progress to disseminated intravascular coagulation (DIC) in critically ill COVID-19 patients, and DIC usually occurs near death. The majority of SOFA scores above 10 in this study population also indicated multiorgan dysfunction. Thrombocytopenia and low fibrinogen are classic symptoms of DIC, as are

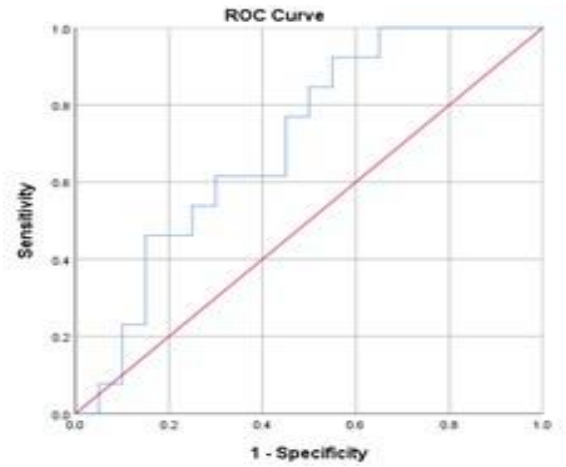

Fig. 5: ROC curve of D-dimer levels in patients with diffuse microthrombi.

increased fibrin degradation products like D-dimer [3]. Fibrinogen levels were not routinely checked in all patients, but we analyzed platelet levels in all patients. Mean platelet count were 231,095.24 ± 138,778.21 /µl in patients with focal microthrombi and 350,076.92 ± 198,231.96 /µl in patients with diffuse microthrombi.

It was seen that severe thrombocytopenia was not common in our study population, and platelet levels were significantly associated with the findings of focal and diffuse microthrombi on the independent T-test (p value 0.048). This finding supported the pulmonary intravascular coagulopathy concept that the thrombotic process in critically ill COVID-19 patients occurs primarily in the lungs. Even though D-dimer level cannot be used as a single predictor of thrombi, it can be used to help identify the presence of diffuse microthrombi in these patients.

Limitations of this study were the limited number of samples, potential of bias when using non-specific parameters like that was the D-dimer level, limited diagnostic facilities, including CT scans and fibrinogen assays. Different results may be obtained if comparison were done with non-COVID-19 patients.

We did not evaluate the use of antiplatelet agents in this study since they were not included in our center's routine COVID-19 treatment regimens. Given the high suspicion of platelet activation in the findings of platelet-rich microthrombi, research on the use of antiplatelet agents in critically ill COVID-19 patients and their association with pulmonary thrombosis may be feasible.

V. CONCLUSION

D-dimer values above 3055 ng/ml is suggestive of diffuse microthrombi in the lungs. Although D-dimer level alone cannot be used as a predictor of microthrombi, it can be used to help identify the presence of diffuse microthrombi.

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