Differences in fibrin fiber diameters in healthy individuals and thromboembolic ischemic stroke patients

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Cerebrovascular disease is one of the leading causes of death and the cause of long-term adult disability. An important characteristic of thromboembolic ischemic stroke is a prothrombotic or hypercoagulable state and altered fibrin clot structure, whereas a resistance to fibrinolysis is also present. An expansive fibrin network is created when adding thrombin, and in stroke, the network appears thickened, netted and matted, compared with that of healthy individuals. Although this is clearly visible in micrographs of patients, there is a need to quantify the changes. The current study, therefore, investigates fibrin fiber diameters in stroke patients and compares it to healthy individuals. The fiber diameters were measured in nanometres, with University of Texas Health Science Center at San Antonio (UTHSCSA) Image Tool. A total of 100 measurements were done for each of the 12 patients in the healthy control group, and the same number of measurements was done for 12 stroke patients. These measurements were statistically analysed with NCSS 2007, using a significance level of 0.05. Normality was assessed with the Shapiro–Wilk W test and the thickest and thinnest fiber of each individual in the two groups was quantified and differences between groups were assessed with the Student's *t*-test. Results showed that there is a statistical difference in fibrin fiber thickness during thromboembolic ischemic stroke. We conclude that the changed coagulation and hemostasis, typically associated with stroke, causes a statistically relevant change in fibrin thickness, and that this netted and matted network is more resistant to lyses.

Introduction

Cerebrovascular disease is the third leading cause of death in the United States [1] and stroke is the leading cause of long-term adult disability [2]. Worldwide, ischemic stroke accounts for approximately 85% of stroke cases [2]. Hypercoagulation and microthrombi formation, which lead to microvascular occlusion and reduction in blood flow, are important events associated with stroke [3–6]. Impaired fibrinolysis also play an important role and research has shown that in ischemic stroke patients, there are significantly longer euglobulin clot lysis times, as well as higher levels of plasminogen activator inhibitor-1 and tissue-type plasminogen activator antigen [7].

An important characteristic of thromboembolic ischemic stroke is a prothrombotic or hypercoagulable state and altered fibrin clot structure. Also, in cryptogenic stroke and other ischemic strokes, a resistance to fibrinolysis is present [8].

In 2010, Undas *et al.* [8] compared acute ischemic stroke to controls, and showed that acute ischemic stroke patients produced clots that had 30.5% less porous network, were less susceptible to fibrinolysis and that the clots were 20.5% more compact with a 17.1% higher clot mass. Also, there was an increased (by 10.2%) overall fiber thickness with 8% shorter lag phase of fibrin formation. In 2011, Pretorius *et al.* [9] showed that in thromboembolic ischemic stroke, fibrin networks have a changed ultrastructure. Fibrin networks were created within 24 h of stroke, using citrated blood, after the addition of thrombin. An expansive fibrin network is created when adding thrombin, and in stroke, the network appears thickened, netted and matted, compared to that of healthy individuals. The question now arose whether the changes in fibrin fiber network can be quantified. The current study, therefore, investigates fibrin fiber diameters in stroke patients and compares it to healthy individuals.

Materials and methods

Samples

In our healthy individual research pool, we have hundreds of micrographs of fibrin networks. Whole blood was collected from individuals who did not have any of the following exclusion criteria: cardiovascular disease, anticoagulant medication, smoking, hormonal contraception or hormone replacement therapy, asthma, diabetes and inflammatory diseases, as all these conditions are known to influence the ultrastructure of fibrin networks [10,11]. From this research pool, 12 individuals were

chosen and they represent typical fibrin morphology seen in healthy individuals. In our ongoing study of thromboembolic ischemic stroke patients, we have analysed 25 individuals. They were diagnosed according to the Trial of Org 10172 in Acute Stroke Treatment criteria [9]. From this sample, micrographs of 12 patients were chosen for the current study. Initially, fibrin networks were prepared by collecting whole blood from the patients within 24 h after the onset of stroke. The blood was processed to form fibrin networks and micrographs were taken of the networks. The micrographs were methodically viewed to establish the consistency of the ultrastructure, and one representative micrograph of each individual was chosen for further analysis.

Preparation of fibrin clots

Blood was centrifuged at 227 rpm for 2 min to obtain platelet-rich plasma (PRP). Thrombin (provided by the South African National Blood Services) was used to prepare fibrin clots. The thrombin is at a concentration of 20 U/ml and was prepared in a biological buffer containing 0.2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components, for example, transforming growth factor, platelet-derived growth factor and fibroblastic growth factor are released into the coagulum. Ten microliter of human PRP was mixed with 10 μl of human thrombin. The PRP and thrombin mix was immediately transferred with a pipette tip to a 0.2 μm Millipore membrane to form the coagulum (fibrin clot) on the membrane. This Millipore membrane was then placed in a Petri dish on filter paper damped with phosphate-buffered saline (PBS; to create a humid environment) and kept at 37°C for 10 min. This step was followed by a washing process in which the Millipore membranes with the coagula were placed in PBS and magnetically stirred for 20 min. This step is necessary to remove any blood proteins trapped within the fibrin network.

Preparation of washed fibrin clot for scanning electron microscopy

Washed fibrin clots were fixed in 2.5% gluteraldehyde in Dulbecco's PBS in a 0.075 mol/l phosphate buffer at a pH of 7.4 for 1 hour. Each fibrin clot was rinsed thrice in phosphate buffer for 5 min before being fixed for 30 min with 1% osmium tetraoxide. The samples were again rinsed thrice with PBS for 5 min and were then dehydrated serially in 30, 50, 70, 90% ethanol and three times with 100% ethanol. The scanning electron microscopy (SEM) procedures were completed by critical point drying of the samples, mounting, coating with carbon and examination of the tissue with a ZEISS ULTRA and FEG scanning electron microscope.

Statistical analysis

After visualization of the fibrin networks using SEM, micrographs were taken at 40 000 times machine magnification. A representative photograph of each sample was selected, a 10×10 grid was drawn onto the picture (grids shown in Fig. 1) and the picture was enlarged to a degree that the thinnest fibers could be measured easily. One fiber was randomly selected out of each block of the grid, to ensure that a fiber was not measured twice, and that fibers were systematically measured to prevent observer bias; this resulted in 100 measurements per individual photograph. The diameter was measured in nanometres, with UTHSCSA Image Tool version 3.00. A total of 1200 measurements were done for the healthy control group; the same number of measurements was done using micrographs of stroke patients. Measurements were repeated by two observers. These measurements were statistically analysed with NCSS 2007 version 07.1.20 (NCSS LLC, Kaysville, Utah, USA), using a significance level of 0.05. Normality was assessed with the Shapiro–Wilk W test of the two groups, and frequency histograms were constructed to display the distribution of fiber diameters graphically. The thickest and thinnest fiber of each individual in the two groups was quantified, and made into four groups: maximum of the control group, minimum of the control

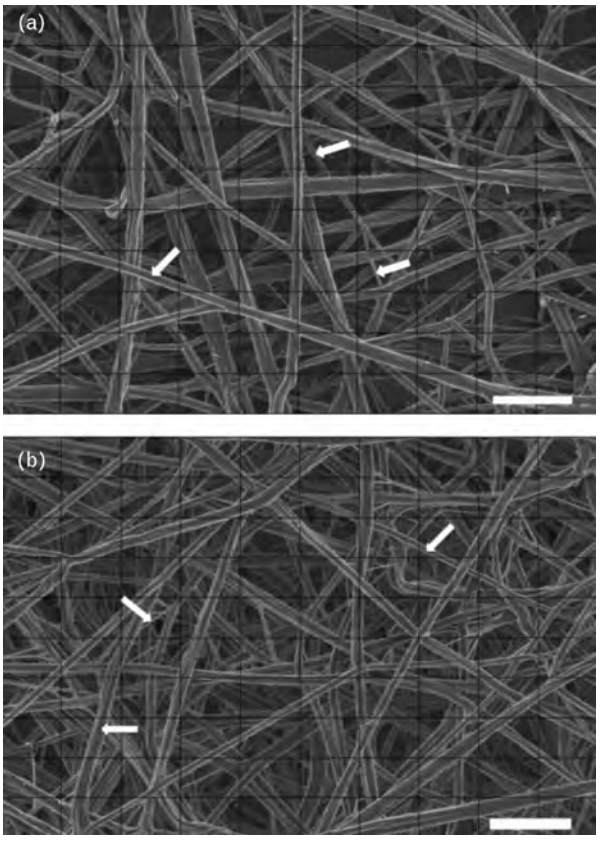

Fig. 1. Micrographs from two healthy individuals showing grids used to identify fibers for measurement. Arrows indicate examples of fibers that were measured. All scale bars = 1 μm.

group, maximum of the stroke group and minimum of the stroke group. The difference between group first and third groups, as well as the difference between group second and fourth groups were assessed with the Student's *t*-test.

Results

Figure 1a and b show micrographs of individuals that are representative of the control group. Typically, fibrin networks of healthy individuals form thick, major fibers with fine minor fibers dispersed amongst the major fibers. Figure 2a–f shows micrographs from the patient stroke group. As previously noted by Pretorius *et al.* [9], in ischemic stroke patients, fibrin fiber arrangement changes after the addition of thrombin to citrated blood, to form a matted and netted fibrin mass. As can be seen in Fig. 3 (which shows histograms of the frequency distributions of fiber diameters) the stroke patient group has a much larger amount of thin fiber counts than the control group, with very few thick major fibers.

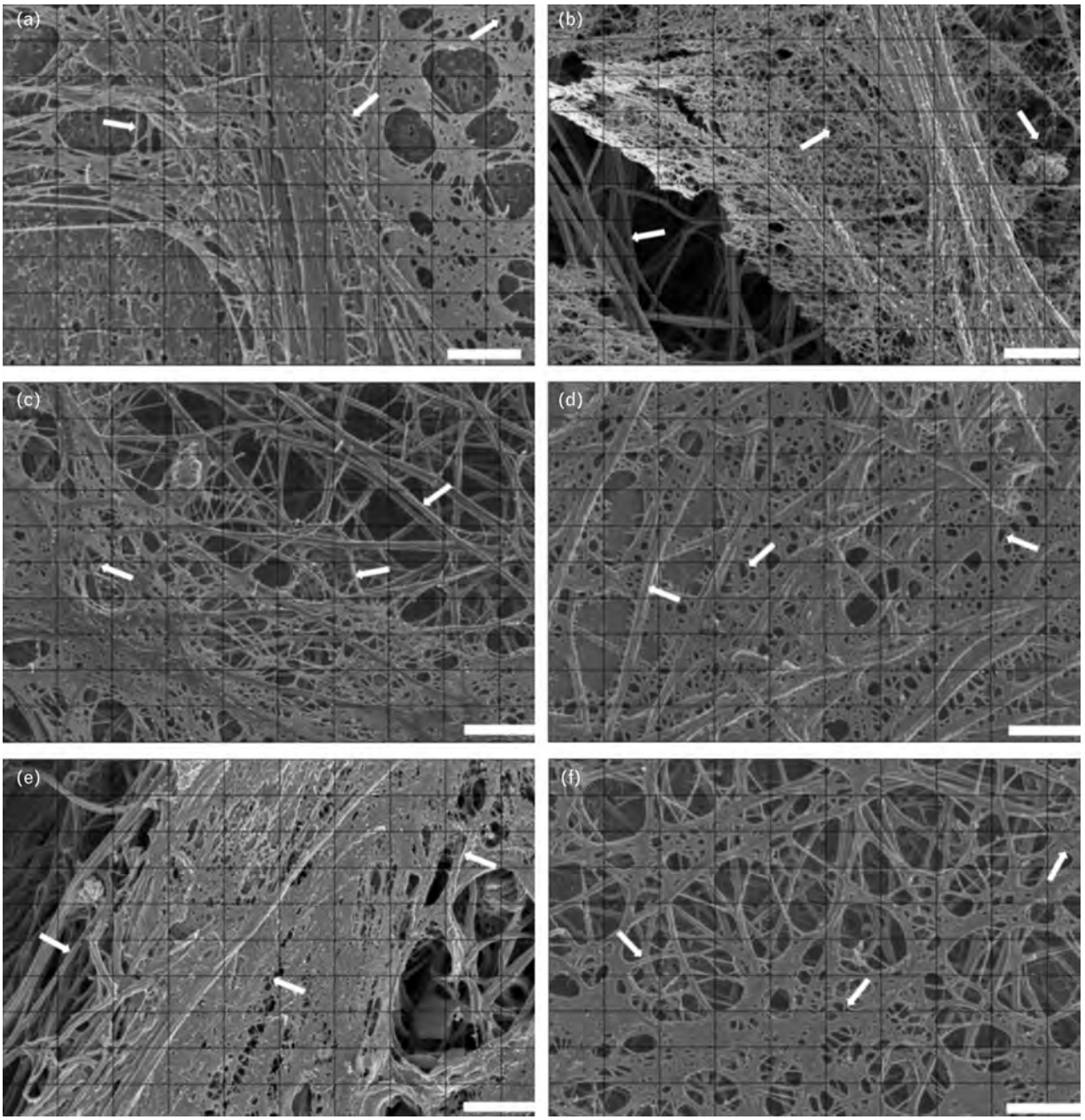

Fig. 2. Micrograph examples of six different thromboembolic stroke patients showing grids used to identify fibers for measurement. Arrows indicate examples of fibers that were measured. All scale bars = 1 μm.

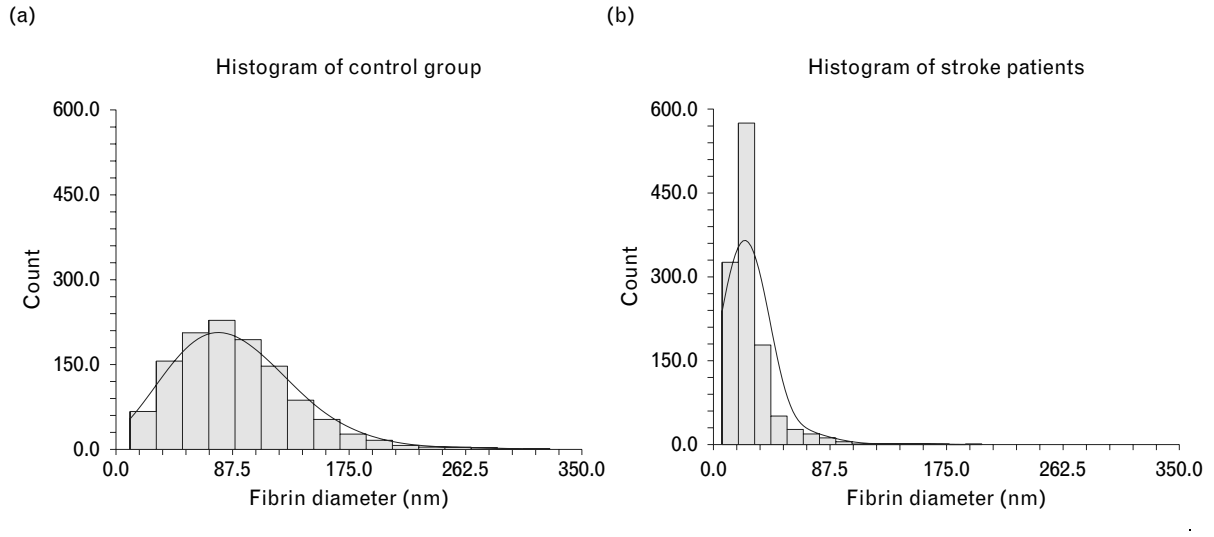

Fig. 3. Histograms illustrating the frequency distribution of (a) healthy individuals and (b) stroke patients' fibrin fiber diameters.

Table 1 shows the mean and SD of the overall measurements, and also of the maximum and minimum groups. In the current analysis, the Shapiro–Wilk W test was used to assess normality; both groups showed that the fibrin diameters were not normally distributed, in which both the control and stroke patient group had *P*-values of less than 0.000. It is expected that the groups will not show a normal distribution, as a fibrin clot consists of thick, major and thin minor fibers [12]. Frequency histograms were plotted in NCSS for each group to give a visual display of the distribution of fiber diameters (Fig. 3).

Discussion

Fibrin structure and stability and abnormal fibrin structure is a novel link between inflammation and thrombosis [13]. Previous research also suggested that fibrin clots have increased fiber diameter and density in stroke patients [8,14]. Also in their 2010 study, Undas *et al.* [14] suggested that scanning electron microscopy of fibrin clots could provide direct data on fibrin thickness and pore structure.

For the current analysis, micrographs of 12 healthy individuals and 12 stroke patients were chosen. The 12 control micrographs were chosen from a database consisting of thousands of control micrographs and these micrographs, therefore, represent typical morphology for controls. Currently, we have a micrograph database for 30 ischemic stroke patients. These micrographs also represent a typical morphology for ischemic stroke patients. A grid was overlaid over each micrograph, and one fiber measured per area grid-block (100 measurements per micrograph; Figs 1 and 2). This assured a nonbiased choosing of fibers, systematically over whole area of micrograph. The number of measurements from each of the two samples showed statistical significant results between the two groups. The distribution of both groups shows a 'merged' distribution pattern in which there are no two clear peaks showing the thick and thin fibers (Fig. 3); this suggests a possible intermediate thickness fiber because in a group in which there are only thick and thin fibers, a bimodal distribution pattern would be expected.

Table 1 Analysis of fibrin fiber diameters of thromboembolic stroke patients and healthy individuals

	Control group	Stroke group
Total mean ± SD (nm)	89.87 ± 44.16	27.37 ± 17.03
Maximum mean ± SD (nm)	227.28 ± 55.03	95.54 ± 47.43
Minimum mean ± SD (nm)	23.69 ± 9.48	10.95 ± 3.46

In the control group, the single largest measurement of each individual was taken and classified in a 'maximum' group, whereas the single smallest measurement of each individual was classified as a 'minimum' group. The same was done for the stroke patients, and significant difference was then determined using the Students' *t*-test between maximum control and stroke groups, and between minimum control and stroke groups. Table 1 shows the mean and SD of the overall measurements, and also of the maximum and minimum groups. Between group analyses of the maximum measurements resulted in a *P*-value of 0.000, which suggest that there is a significant difference between the control and stroke patients' thickest fibers. Between group analyses of the minimum measurements resulted in a *P*-value of 0.002, which suggest that there is a significant difference between the control and stroke patients' thinnest fibers.

Currently, the use of morphology to investigate clots is debated in the literature. In an editorial in the journal *Stroke* in 2011, the authors discussed the increasing use of mechanical embolectomy devices [15]. This methodology allows for retrieving fresh clots from patients with acute stroke and successive morphological characterization. It, therefore, seems that visualization of clot structure in ischemic stroke might give us insights into the disease and possible treatment regimes [15]. The present research might add to the knowledge of clot structure and function and stimulate further research, as it shows that there is a statistical difference in fibrin fiber thickness during thromboembolic ischemic stroke. Previous research has suggested that in conditions that affect the pathophysiology, fibrin polymerizes differently, compared with the typical process present in healthy individuals [16]. Clots in healthy and diseased individuals may, therefore, differ in structure as well as physical and chemical properties; and these properties may change due to the internal biological environment of the individual. Here we show that in stroke, fibrin polymerization is changed and this caused a layered, matted fibrin clot structure. In 2010, Undas *et al.* [14] showed that there are unfavourably altered fibrin clot properties in stroke. Here we suggest that the changed coagulation and hemostasis typically associated with stroke, causes a statistically relevant change in fibrin thickness.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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