Ultrastructural comparison of the morphology of three different platelet and fibrin fiber preparations

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Abstract

The aim of the current study was to investigate the ultrastructural morphology of three different sources of fibrin networks and platelets, namely, lypholized human platelet-rich plasma (LPRP), freshly prepared human platelet-rich plasma (FPRP), and human platelet concentrate (HPC). The ultrastructural morphology of the three different fibrin networks was studied using the scanning electron microscope (SEM). Turbidity curves were drawn at 405 nm at room temperature and fibrinogen concentrations were measured. Scanning electron micrographs showed that all clots produced thick major fibrin fibers as well as a well-defined fine fibrin network, which appeared to be a superimposed process that occurred after the major fibrin network was established. These features were decidedly more pronounced in the HPC specimens. Turbidity curves of the three types of plasma showed differences in LPRP and FPRP. Fibrinogen concentrations of all three preparations were in the normal ranges. Because of the great similarity between LPRP, HPC, and FPRP, we suggest that LPRP could be used successfully to study morphological changes in fibrin fibers and platelets, which may occur after exposure to certain therapeutic agents. However, functionality studies such as turbidity curves should concurrently be included. We therefore conclude that from a basic science point of view, LPRP is a valuable research tool and that such results may add information that could be valuable for clinical application.

Activated platelet as well as fibrin network ultrastructural morphology may be an important tool when studying different medical conditions ranging from dysfibrinogenaemias to inflammatory conditions such as asthma. Platelets are activated by a number of stimuli resulting in the expression and/or activation of surface receptors, secretion of vasoactive substances, adhesion, aggregation, and finally thrombus formation (Nemerson, [1988]; Carmeliet and Collen, [1998]; McVey, [1999]; Butenas and Mann, [2002]; Lazarus et al., [2003]). Platelets are inert until they encounter conditions that trigger their activation (Camera et al., [1999]; Furlan, [2002]) and they are also an important source of growth-promoting factors, which are released when thrombin is formed. Platelets that are fragments of the cytoplasm of megakaryocytes are approximately 3 μm long and do not have nuclei. Their cytoplasm is divided into two zones and consists of the outer hyalomere and the inner granulomere, which contains granules. In addition to different types of vesicles, mitochondria, ribosomes, lysosomes, and a little endoplasmatic reticulum are present in the thrombocyte granulomere. The hyalomere contains a cytoskeleton consisting of a circumferential microtubule and submembrane actin filaments support the discoid shape of the resting cell (White, [1987]). Therefore, in medical conditions where platelet internal ultrastructure and perhaps also activation are compromised, ultrastructural changes may be visible when using an electron microscope.

However, ultrastructural changes may also occur in the fibrin network morphology due to abnormalities in the coagulation process. In medical conditions where thrombin [belonging to the trypsin family of serine proteases (Bailey et al., [1951]; Mosesson, [1992]; Lawson et al., [1994]; Furlan, [2002]; Bouma and Mosnier, [2003]; Polack, [2003]) and an important platelet-activating agonist formed during coagulation (Franz, [2002])] as well as other coagulation factors (which are active in the coagulation cascade) are defective, ultrastructural changes to the fibrin network may occur. Coagulation factors are zymogens of serine proteases (Polack, [2003]) and are converted from an inactive form to an active enzyme by limited proteolytic cleavage of one more peptide bonds. Fibrin forms a network that functions to stabilize the primary platelet plug and this fibrin network becomes covalently crosslinked by factor XIIIa that has, in a parallel activation reaction, been produced by the thrombin-induced cleavage of the inactive zymogen factor XIII (Polack, [2003]). Authors such as Ryan et al. ([1999]), Matsuda and Sugo ([2001]), and Weisel and Medved ([2001]) studied the biochemistry of fibrin polymerization and discussed the process in detail. Morphological changes of fibrin networks may therefore occur due to several kinetic and modulating factors present in plasma.

Fibrin structure itself has been shown to play a role in the development of vascular complications. Proneness to the formation of tight and rigid fibrin networks with abnormal architecture has been shown to be independently associated with coronary heart disease (Fatah et al., [1992]) and abnormal gel structure resulting in lower permeability of the network has been observed in type I diabetic patients (Jörneskog, [1996]).

It therefore seems that studying activated platelet and fibrin network morphology may provide insights into vascular diseases and conditions such as allergic asthma and other inflammatory diseases as well as genetic conditions such as dysfibrinogenaemias. Therefore, the question that arises is: how can the ultrastructure of platelets and fibrin networks be studies most effectively? At present, there are three preparations available in the clinical sector in South Africa that are good sources of both platelets and fibrinogen (which can be converted to fibrin) and can be used to investigate this question. The three preparations are lyophilized human platelet-rich plasma (LPRP) and human thrombin (both supplied by the South African Blood Transfusion Services; platelet concentration: 1×10^6 platelets per liter); freshly prepared human platelet-rich plasma (FPRP) from a donor and human thrombin (platelet concentration prepared in the laboratory: 427×10^9 platelets per liter; typical adult count ranges from 140×10^9 top 440×10^9); and human platelet concentrate (HPC) prepared from donors and provided packaged by the South African Blood Transfusion Services (platelet concentration: between 3×10^{11} and 5×10^{11} platelets per liter).

On the packaging of the LPRP provided by the South African Blood Services, an expiry date is stipulated for human consumption. The LPRP we prepared was within the expiry date. FPRP was prepared from healthy donors immediately after drawing the blood. HPC is prepared by the South African Blood Services and it is stipulated that the concentrate should be given to patients within 2 weeks of preparation. The HPC was prepared from the platelet concentrate within the given time stipulated by the South African Blood Services; therefore, within 2 weeks after initial preparation.

Because of practical and ethical implications, it would be more feasible to use LPRP or HPC than FPRP, as a human donor is not always available on site to provide the FPRP. However, these can only be used if fibrin network and/or platelet morphology are comparable to that of FPRP.

Furthermore, functional assays are also important determinants for clinical investigations and should also be used in the comparison of LPRP and HPC with FPRP. Therefore, this research question was investigated by studying the morphology of coagulates of the three types of PRPs by using a scanning electron microscope (SEM) in order to determine the differences/similarities between LPRP, HPC, and

FPRP. Turbidity curve measurements were used as a functional assay to provide information regarding the kinetics for fibrin formation as well as fibrin fiber size. The results thereof are used as supportive functional evidence for the fibrin fiber morphology obtained by the SEM work.

Materials and Methods

The South African National Blood Services developed Thrombostim, which contains PRP and lyophilized human thrombin. LPRP is obtained by apheresis from a single regular platelet donor and each individual unit is tested and has to be nonreactive for hepatitis B surface antigen (HbsAg), HIV-1 antibody, HIV-2 antibody, HIV p-24 antigen, hepatitis V virus (HCV) antibody, and antibodies to *Treponema pallidum*. The anticoagulant used in preparing the PRP is ACDA (Citrate Dextrose Solution, Formula A, ACD-A). These tests are performed by licensed assay methods. The LPRP is freeze-dried. Human thrombin is prepared by calcium chloride activation of an euglobulin fraction of plasma obtained by apheresis, also from a single regular donor; this is also tested using the above-mentioned tests for the LPRP. This thrombin is 20 U/ml and is made up in biological buffer containing 0.2% human serum albumin.

When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components (e.g., transforming growth factor, platelet-derived growth factor, and fibroblastic growth factor) are released into the coagulum.

Preparing LPRP, FPRP, and HPC

Vials containing LPRP and human thrombin are stored in a refrigerator (4°C). For experiments, the LPRP are taken from the refrigerator and reconstituted with sterile water and this was allowed to reach room temperature (10-15 min) according to the instructions from the manufacturers of the Thrombostim. This is to allow complete protein rehydration. Then 20 µl LPRP and 20 µl human thrombin (20 U/ml) were mixed on a 0.2 µm millipore membrane to form the coagulate (fibrin clot). This millipore membrane was placed in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 37°C for 10 min. This was followed by a washing process where the millipore membranes with coagulates were placed in PBS and magnetically stirred for 2 hr. This was done to remove any blood proteins trapped within the fibrin network.

FPRP (supplied by donors) was prepared by drawing 40 ml of blood in citrate vials, which was centrifuged at 1,000 rpm for 2 min at 5°C; 20 µl of the FPRP was again mixed with 20 µl human thrombin (supplied by the South African Blood Transfusion Services) at room temperature. The same procedures as described for LPRP were then followed. In the third experiment, 20 µl HPC (supplied by the South African Blood Transfusion Services at room temperature) and 20 µl human thrombin (20 U/ml) was also mixed on a 0.2 µm millipore membrane to form the coagulate (fibrin clot) and the same procedures as described for LPRP was followed. HPC is also initially prepared using ACDA as anticoagulant.

Preparation of Washed Fibrin Clot for SEM

Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's phosphate-buffered saline (DPBS) buffer with a pH of 7.4 for 1 hr. Each fibrin clot was rinsed thrice in phosphate buffer for 5 min before being fixed for 1 hr with 1% osmium tetraoxide (OsO_4). The samples were rinsed thrice with distilled water for 5 min and were dehydrated serially in 30%, 50%, 70%, and 90% and three times with 100% ethanol. The SEM procedures were completed by critical point drying of the material, mounting, and examining the tissue with a Jeol 6000F FEGSEM.

Also, activation of coagulation of all three preparations, using 1 ml of preparation and varying volumes of thrombin, was tested to investigate minimal and maximal doses able to induce aggregation.

Turbidity Curves

Turbidity curves were done at 405 nm at room temperature (25°C) in microplate format using continuous measurements for 2 hr on a Multiscan Ascent (Labsystems OY, Helsinki, Finland). Plasma was clotted with the addition of 20 mM $CaCl_2$ and 0.1 U/ml bovine thrombin (catalog number 101141; MP Biomedicals, Irvine, CA).

Fibrinogen Concentration

Fibrinogen was measured on a Dade Behring BCT using the Multifibren U kit [modification of the Clauss ([1957]) method]. This is a quantitative method for determining fibrinogen levels in plasma.

RESULTS

Clotting Time

Table 1 shows the aggregation time and morphology of the blood clot of LPRP, FPRP, and HPC added to different volumes of thrombin. The clot morphology per time period and per added thrombin volume appeared the same for all three preparations, with 10 µl of thrombin added to 1 ml of preparation, resulting in a flimsy clot with poorly defined and a few fibers distributed unevenly over stub after 5 min of incubation time; 500 and 1,000 µl of thrombin produced a well-defined clot with distinct fibers after a few seconds.

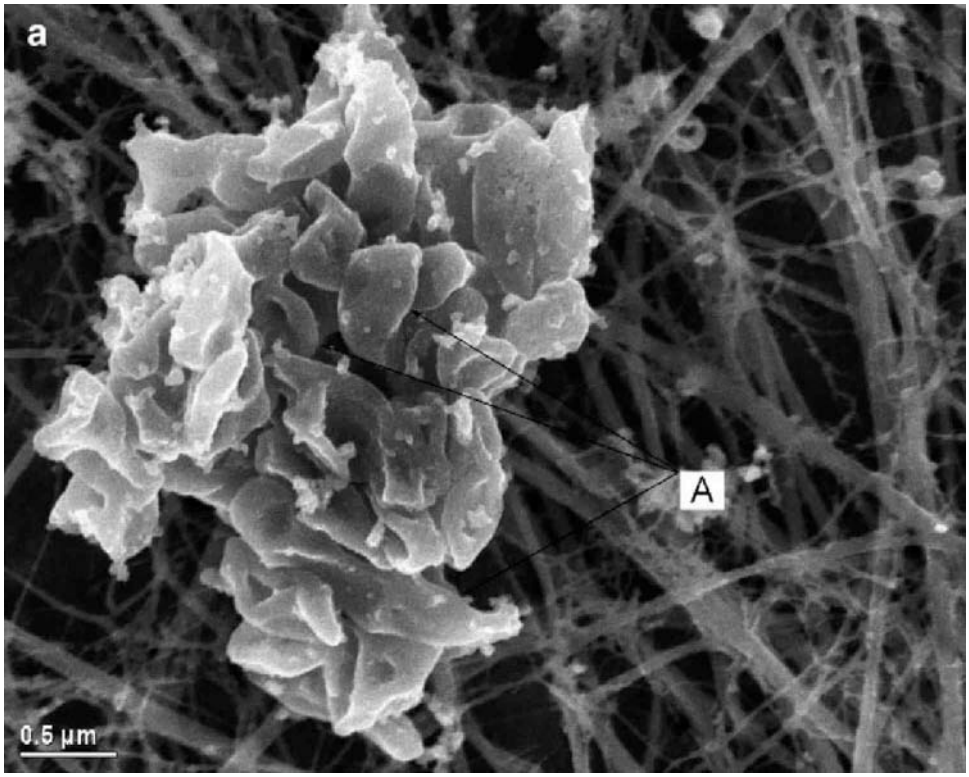
Volume of thrombin in µl	Time to form clot	Morphology of clot
10	5 min	Small clot, flimsy, few fibers
	13 min	Big clot, flimsy, few fibers
15	4 min	Small clot, flimsy, few fibers
	10 min	Big clot, flimsy, few fibers
20	4 min	Small clot, flimsy, few fibers
	10 min	Big clot, flimsy, few fibers
25	3 min	Small clot, flimsy, few fibers
	12 min	Big clot, flimsy, few fibers
50	1 min 30 sec	Big clot, flimsy, few fibers
	5 min	Big clot, flimsy, few fibers
100	20 sec	Big clot, flimsy, medium fiber coverage
	2 min and 30 sec	Big clot, flimsy medium fiber coverage
500	10 sec	Big clot, fibers well-defined, medium fiber coverage
	30 sec	Big clot, fibers well-defined, medium fiber coverage
1000	2 sec	Big clot, fibers well-defined, excellent fiber coverage

Table 1. Aggregation time and morphology of blood clot of LPRP, FPRP and HPC added to different volumes of thrombin

Micrographs

Figure 1 shows SEM photographs of the LPRP fibrin clot, Figure 2 shows SEM photographs of the FPRP fibrin clot, while Figure 3 shows SEM photographs of the HPC fibrin clot.

With all three procedures, fibrin clots were obtained within a few seconds of preparation. Platelets, which appeared as a single entity (body) or in the form of aggregates, were found in LPRP and FPRP (Figs. 1a and 2a). However, very few single platelets or aggregates were identified in the HPC preparations (Fig. 3a). Figures 1a and 2a show more isolated platelets, while Figure 3a shows only small aggregates with poor definition of the individual components. In all three clots, the membranes of the platelets are globular, irregular, and amorphous (label A in Figs. 1a, 2a, and 3a), representing the pseudopodia. Although the platelet clump size of HPC was smaller, no morphological differences in platelet structure were noted when studying the platelets from LPRP, FPRP, and HPC.



When studying the fibrin fibers of the three experiments, two types of fibers were noted, thick fibers as well as finer fibers present between the open spaces formed by the thick fibers. No morphological differences in the thick fibers were noted when comparing SEM photographs of the three experiments. Although fine fibrin fibers were present in all three fibrin clots, the fine fibrin fibers of the LPRP and FPRP were found sparsely distributed among the thicker fibers (Figs. 1b and c and 2b and c). However, in HPC, the fine fibrin fibers formed a dense mat or net between the thicker fibers and also covered them (Figs. 3b and c, label B). Only very rarely were thick fibers without such a net covering (Fig. 3d).

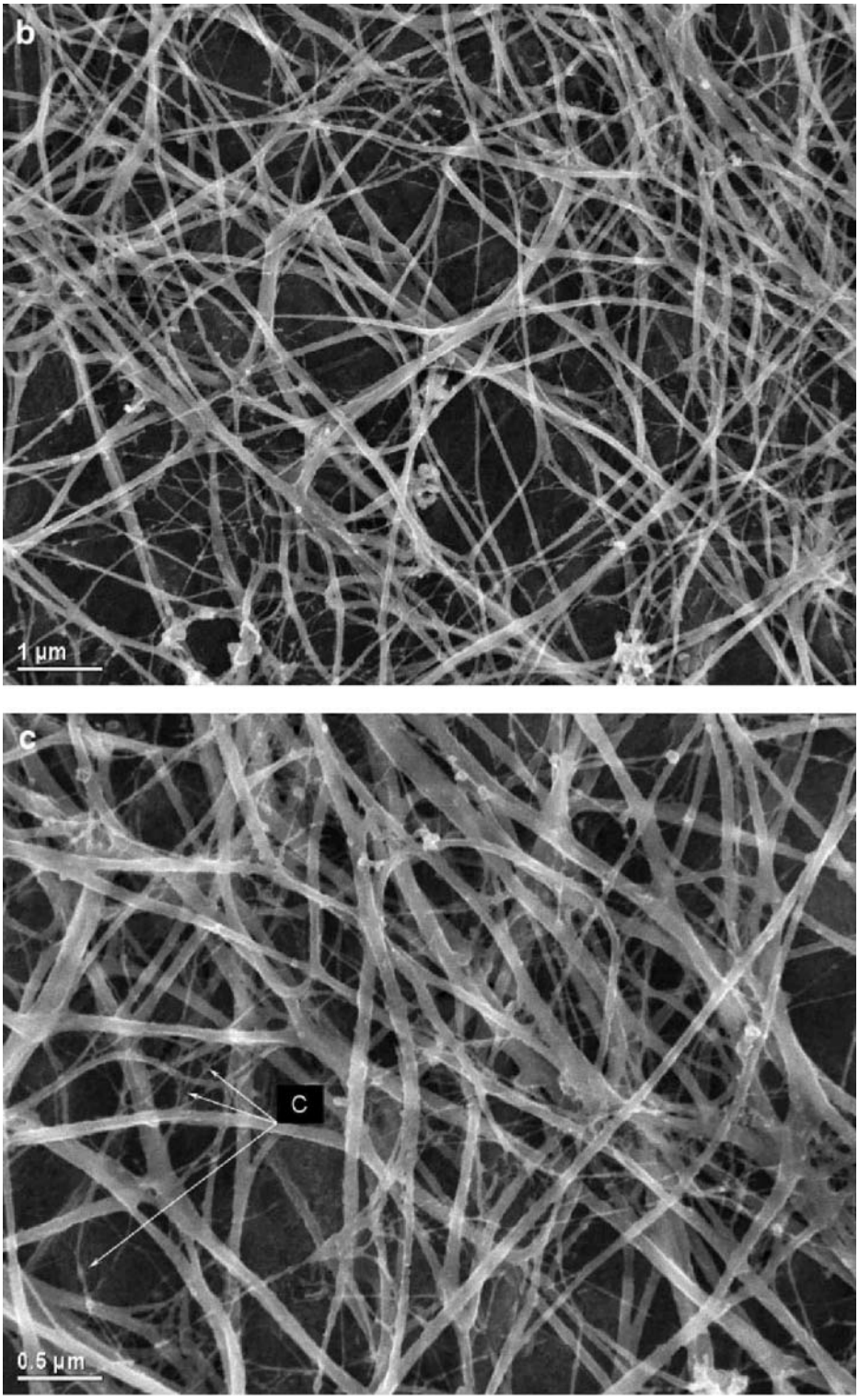
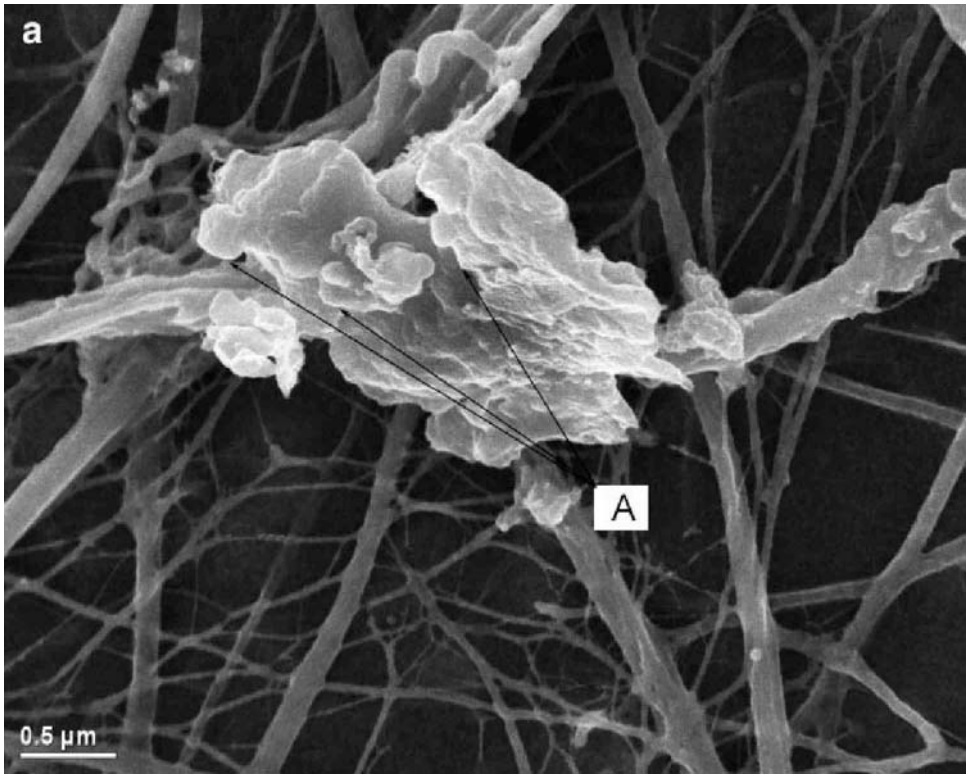


Figure 1. A: SEM photograph showing platelet aggregate from LPRP fibrin clot (magnification = 10,000×) as well as fibrin fibers. Label A denotes pseudopodia. B: SEM photograph showing fibrin fibers from LPRP fibrin clot (magnification = 5,000×). C: Higher-magnification SEM photograph showing fibrin major and minor fibrin threads fibers from LPRP fibrin clot (magnification = 10,000×). Label C denotes globular protein areas on minor fibers.



Turbidity Curves and Fibrinogen Concentrations

Figure 4 shows the turbidity curve experiments. Differences were found in the turbidity curves of the LPRP and the FPRP. Turbidity curves of the HPC could not be obtained due to platelet retraction that prevented uniform clot formation in the wells. The fibrinogen concentrations were as follows: HPC demonstrated the highest fibrinogen concentration, namely, 3.5 mg/ml, while the fibrinogen concentrations of FPRP and LPRP were more similar, with FPRP equaling 2.5 mg/ml and LPRP equaling a fibrinogen concentration of 2.45 mg/ml.

DISCUSSION

This is the first report where the ultrastructural morphologies of LPRP, FPRP, and HPC are compared and discussed. Clinically, it seems as if LPRP is used more frequently in countries like South Africa and America, as it can be stored long term and in a small space and can be easily transported, making it perhaps more accessible than FPRP and HPC. However, also from a research perspective, LPRP could have fundamental therapeutic implications, as it provides researchers with an easily accessible source for studying platelets as well as clot structure, particularly when studying the effect of pharmaceutical products on platelet and clot morphology.

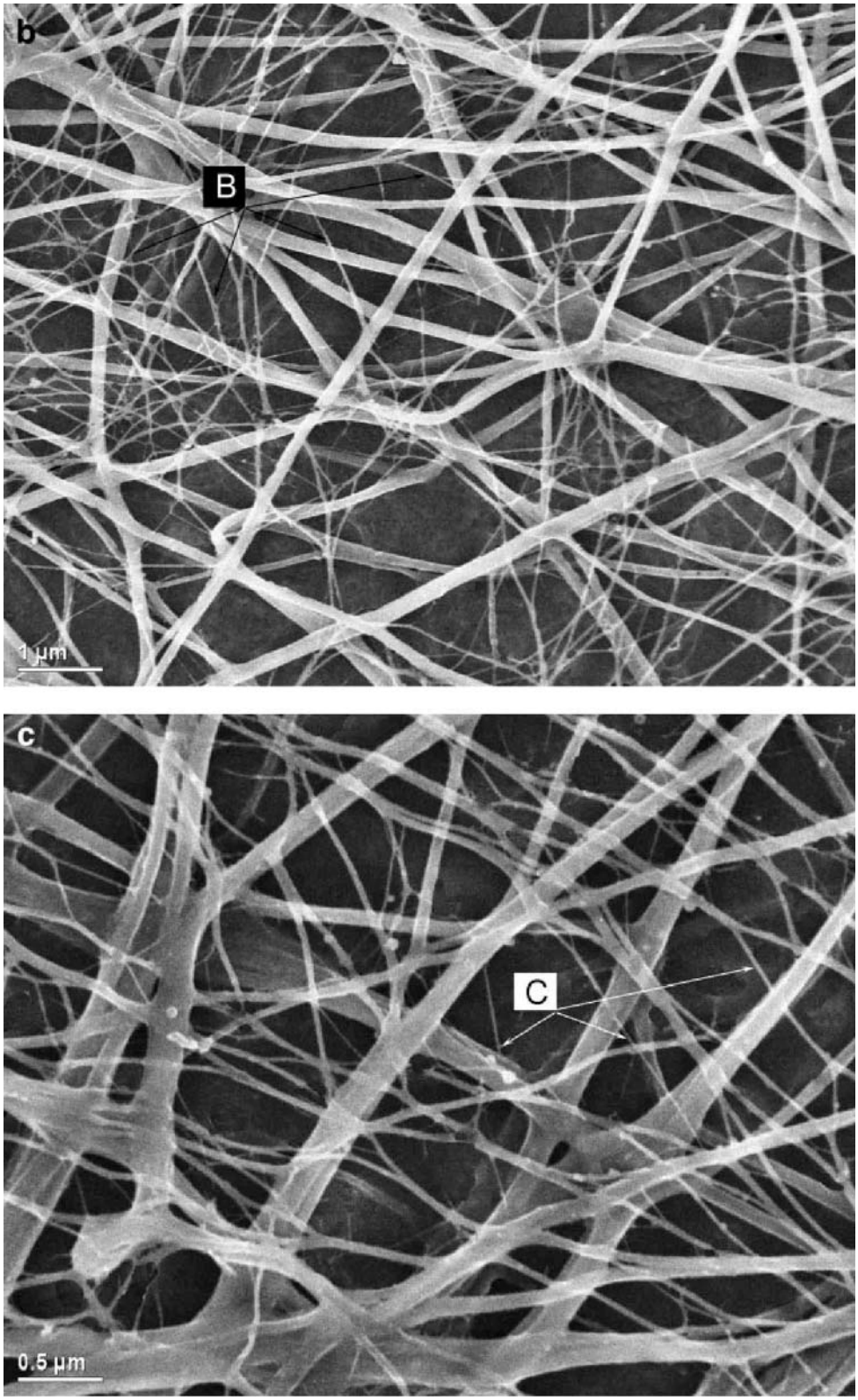


Figure 2. A: SEM photograph showing platelet aggregate from FPRP fibrin clot (magnification = 10,000×) as well as fibrin fibers. Label A denotes pseudopodia. B: SEM photograph showing fibrin fibers from FPRP fibrin clot (magnification = 5,000×). Label C denotes minor fine fibrin fibers with smooth appearance. C: Higher-magnification SEM photograph showing fibrin major and minor fibrin threads fibers from FPRP fibrin clot (magnification = 10,000×). Label C denotes minor fine fibrin fibers with smooth appearance. Photographs A and B from Pretorius et al. ([2006]), courtesy of Taylor and Francis.

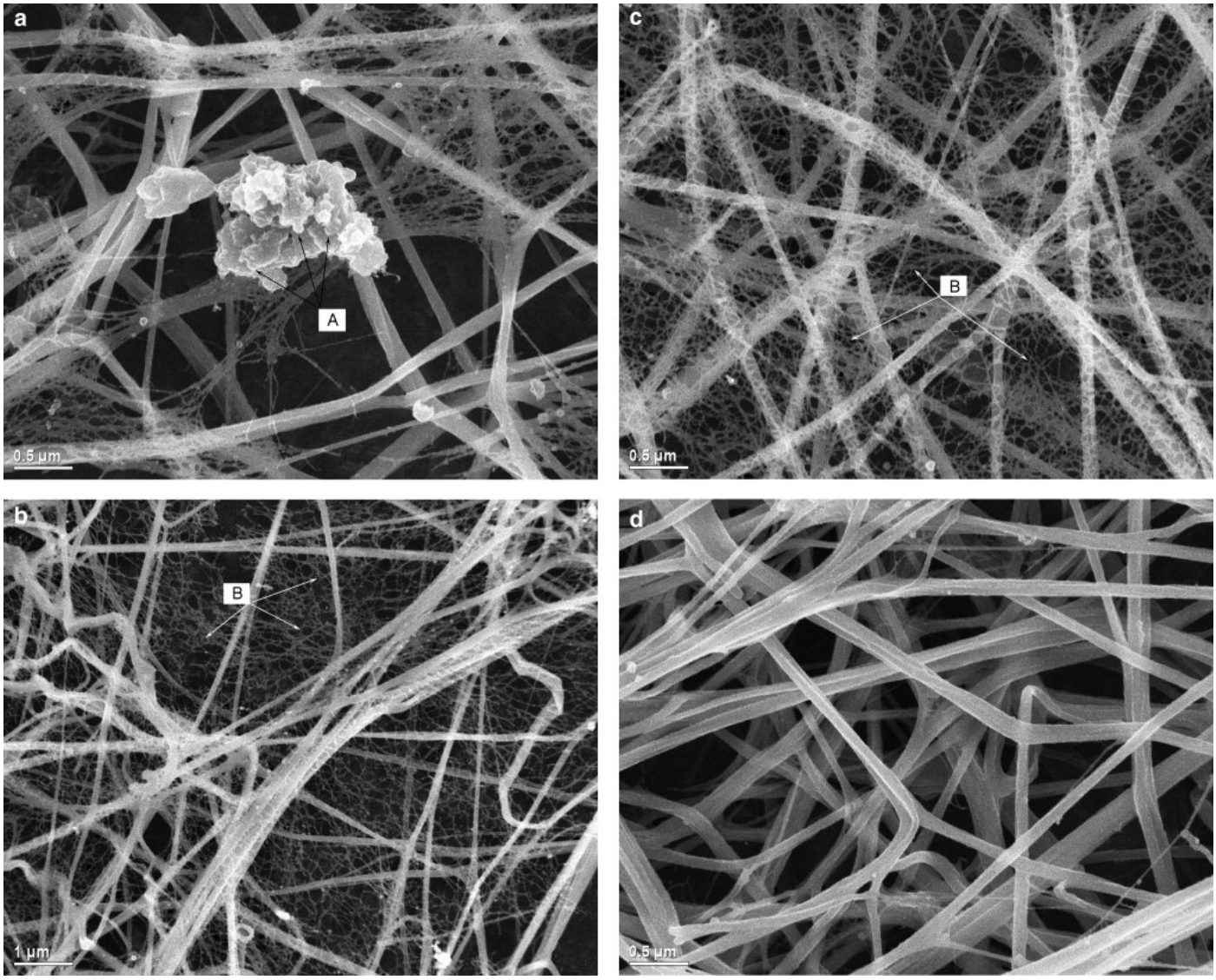


Figure 3. A: SEM photograph showing platelet aggregate from HPC fibrin clot (magnification = 10,000×) as well as fibrin fibers. Label A denotes pseudopodia. B: SEM photograph showing fibrin fibers from HPC fibrin clot (magnification = 5,000×). Label B denotes minor fibrin fibers with smooth appearance forming a secondary net over major fibrin fibers. C: Higher-magnification SEM photograph showing fibrin fibers from HPC fibrin clot (magnification = 10,000×). Label B denotes minor fibrin fibers with smooth appearance forming a secondary net over major fibrin fibers. D: Higher-magnification SEM photograph showing fibrin fibers from HPC fibrin clot showing areas without minor fibrin network (magnification = 10,000×). Only very rarely will thick fibers be without such a net covering.

As mentioned previously, no morphological differences in platelet aggregate structure were noted when studying the platelets from LPRP, FPRP, and HPC. However, there might well have been damage to the platelets during lyophilization, as the process may damage the platelet membrane by denaturing the proteins and leaving platelet fragments and microparticles. However, although this damage may occur during the lyophilization process, once the LPRP preparations were clotted, the current research shows that the platelet fragments or microparticles that may be produced by the lyophilization would clump or fuse together into the aggregates, forming aggregates that have similar morphology to FPRP and HPC aggregates. Therefore, if the platelets were damaged during the initial lyophilization, it is not noticeable

in the aggregates. Also, it seems as if the physiological process directing the formation of the fibers was undamaged, as similar major and minor fiber networks were found in LPRP as well as FPRP.

When comparing the HPC fiber network to that of the other two networks, a more pronounced and striking presence of the fine minor fiber network is seen in the HPC. This might be related to the high initial concentration of platelets in the HPC (3-5 × 10^{11} platelets per liter), where local procoagulant activity may well be enhanced by amplification of the initiating prothrombotic stimulus, which leads to an almost explosive thrombin generation with consequent increased fibrinogenesis on the platelet surface. Thus, because more platelets are available, there are therefore more coagulation factors available for consequent fibrin formation and polymerization. The HPC also had a higher fibrinogen concentration (3.5 mg/ml) compared to the FPRP (2.5 mg/ml) and LPRP (2.45 mg/ml), which likely contributes to the observed difference in clot structures. Furthermore, it seems as if the fine net was formed secondarily to the first process, therefore after the thick fibers were formed. This is suggested because the net covers the thick fibers.

Another plausible explanation for the lower platelet concentration and smaller aggregates, seen after activation, as well as the altered fibrin network morphology may also be the effect of the additives in the HPC on the platelets and fibrinogen. It has previously been reported that there is a 40% reduction in the maximal binding of fibrinogen to platelets in response to thrombin in platelet concentrates after 7-day storage (Lozano et al., [1997]). This decreased binding of fibrinogen to the GP IIb/IIIa platelet membrane receptor may explain the smaller number of platelets and smaller aggregates observed in the HPC micrographs as this fibrinogen binding to platelets is responsible for the aggregation and adhesion of platelets. According to Lozano et al. ([1997]), this reduced fibrinogen platelet binding was caused by the impaired ability of platelets to become activated by thrombin as storage time increases. It may, however, also be caused by dextrose monohydrate that is added to the HPC as a substrate for platelet metabolism, which causes glycation of many of the blood proteins. The final concentration of the dextrose monohydrate in the HPC is 123.6 mmol/l, a concentration much higher than plasma glucose levels found in diabetic patients, who already show increased glycation of proteins. Therefore, it is likely that glycation of proteins occurred in the HPC. There is evidence for the glycation of both the GP IIb/IIIa receptor as well as fibrinogen in diabetic patients. Keating et al., ([2004]) found that there is reduced fibrinogen binding to the GP IIb/IIIa receptor in diabetic patients for the first 10 min after platelet activation. They concluded that the initial decreased rate of fibrinogen binding may be due to glycation of the GP IIb/IIIa receptor. Glycation of fibrinogen itself may, however, also be responsible for the reduced fibrinogen-platelet binding as well as the altered fibrin network structure observed in the HPC. It is well documented that diabetics show increased fibrinogen glycation and that this glycation results in altered functional ability of fibrinogen (Brownlee et al., [1983]; Nair et al., [1991]; Dunn and Ariëns, [2004]). Furthermore, the fibrin network structure of diabetics is also altered. One of the reasons given for the altered fibrin network structure is glycation of fibrinogen (Nair et al., [1991]; Jörneskog et al., [1996]). Nair et al., ([1991]), Jërneskog et al. ([1996]), and Dunn et al. ([2005]) found that the network structure of diabetics is less permeable than healthy controls. The more pronounced presence of the fine minor network in the HPC is in agreement with this, as the presence of the minor network has been shown to decrease the permeability of the fibrin network (Shah et al., [1982]). Therefore, glycation of plasma proteins due to the high dextrose monohydrate content of the HPC may explain both the platelet aggregates seen and the altered fibrin network structure decreased.

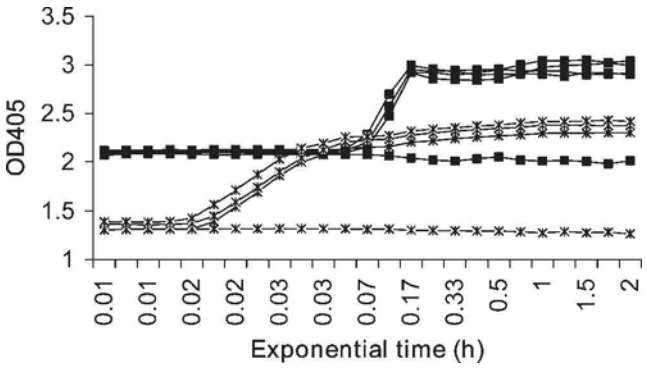


Figure 4. Turbidity curves of four FPRP samples (square) and three LPRP (asterisk) samples, clotted with 20 mM $CaCl_2$ and 0.1 U/ml thrombin, continuously measured at 405 nm, 25°C. The two lines that remain horizontal for the total 2-hr period represent unclotted plasma (negative controls).

Also, in the 10,000× magnification showing the fine fibers of LPRP and FPRP (Figs. 2c and 3c), the individual thin fibers of LPRP possessed thicker globular areas (Fig. 2c, label C), while those of FPRP were smooth (Fig. 3c, label C). The globular areas on some of the fine fibers in the LPRP might be because the LPRP is freeze-dried and therefore causes changes in fibrin polymerization, resulting in some proteins still being attached to the network. Pieters et al. ([2002]) studied the effect of freeze-drying, freezing and frozen storage of blood plasma on fibrin network characteristics. They noted that when plasma is freeze-dried, it undergoes both freezing and dehydration and that both processes cause the unfolding of proteins and consequent aggregation, which leads to possible loss of activity upon rehydration (Vemuri et al., [1994]; Allison et al., [1998]). Their results indicated that there was no difference between the mass-length ratio of the fibers, compaction, and fibrin content values of the clots made from freeze-dried and fresh plasma, nor was there any difference between the fibrinogen concentration of the freeze-dried and fresh samples. However, the authors found that the permeability of the clots prepared from freeze-dried plasma was significantly lower that that of the fresh samples. Furthermore, they mentioned that redissolving of freeze-dried samples can lead to increased protein-protein interactions. If there are more fibrinogen-fibrinogen interactions even before cleavage of the fibrinopeptides, there will initially be more small oligomers (Weisel and Nagaswami, [1992]). Possible structure-function alterations of the fibrinogen molecule due to the freeze-drying may explain why in the current research we find globular areas on some of the fine fiber network of the LPRP not seen in the FPRP. However, another reason for the presence of more pronounced globular areas might be the fact that, due to the lyophilization process, platelets could be damaged, leaving platelet fragments and microparticles and that the globular areas seen in the micrographs might actually be remnants or microparticles of damaged platelets.

Although morphologically there seems to be only small differences in the clot structure of the LPRP and FPRP, there seems to be some functional differences. Results from the turbidity curve experiments (Fig. 4) indicate differences in the turbidity curves of the LPRP and the FPRP. Turbidity curves of platelet-rich plasma should be interpreted with caution as the plasma clots may not be uniform due to the presence of the platelets. The increase in optical density due to clotting in the LPRP and FPRP samples is similar, although the baseline levels differ. This is in agreement with the SEM photographs indicating similar fiber sizes for the two preparations. The reason for the difference in baseline is probably due to the difference in platelet concentration of the LPRP and FPRP, since the presence of the platelets causes the plasma to become murky. As mentioned before, the LPRP and FPRP had similar fibrinogen concentrations. The LPRP samples, however, had a much shorter lag phase (1 min) than the FPRP samples (3 min). This may be the result of damage to the platelets that occurred during freeze-drying. If the platelets were

damaged by freeze-drying, increased membrane surface area from breakup of platelets could affect the clotting cascade, explaining the shorter lag phase. However, morphologically, no damage was noted in the LPRP with SEM studies.

Morphologically, LPRP shows great similarities with FPRP and fibrinogen levels are very similar. An important piece of research information is that, although the lyophilization process may cause platelet fragmentation and microparticles, the physiology of these platelet fragments does not seem to be damaged during the lyophilization process. This is suggested as the fragments still successfully form aggregates as well as fibers with morphology typically found in FPRP. These results suggest that, from a research perspective, LPRP can successfully be used in testing the effects of pharmaceutical products on platelet and clot morphology to determine the effect of the products on clot stability and morphology. As mentioned earlier, because of practical and ethical implications, it would be more feasible to use the more readily accessible LPRP or HPC in research investigating morphological changes in platelet and fibrin fiber morphology in the presence of, e.g., antiasthmatic drugs or platelet- and fibrin-related diseases. It would seem that structural changes in the platelet-fibrin networks may have fundamental diagnostic and therapeutic implications. However, it is interesting to note that the turbidity curves do not fully support these similarities in LPRP and FPRP; therefore, functionality tests should concurrently be performed. We conclude that, from a basic science point of view, LPRP is a valuable research tool and that such results may add information that could be valuable for clinical application. However, more research using the three preparations is suggested to determine precise clinical relevance.

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