

Chapter 6 - Effects of aspartame on the ultra-structure of the fibrin fibres and platelets

RESEARCH QUESTION 4:

Does the morphology of the aspartame treated platelets, platelet aggregation and fibrin fibres when studied with SEM (scanning electron microscopy) differ from those of the control sample, and how?

6.1 INTRODUCTION

Fibrin networks and platelet aggregates can be found at the centre of the blood coagulation system. This fact makes it possible to utilize normal ultra-structural morphology of fibrin fibres and platelet aggregates as an ideal tool for determining the effects of pharmaceutical products on the normal morphology. The previous chapters supplied proof that the rabbit could be utilized as a successful experimental animal model, especially when determining effects of pharmaceutical products on haemostasis and blood coagulation. It was concluded that the rabbit exhibited similar fibrin fibre morphology (major and minor fibres) as the human with the different size of the fibres comparing extremely well with that of human fibrin fibres. The platelet aggregate morphology was also comparable to that of humans. Thus, the rabbit can be used to study normal coagulation and vascular components to determine adverse affects after treatment with pharmaceutical products in their pre-clinical trial periods.

Plasma, platelet and vascular components are activated during an integrated series of haemostatic reactions which forms part of blood coagulation. When endothelium or sub-endothelium is damaged, platelets adhere at the site of injury under the influence of adhesive proteins (Bloom, 1990). As soon as platelets adhere to sub-endothelial components such as collagen, signalling pathways that lead to thromboxane A₂ formation is activated and secretion of platelet granule contents, including adenosine diphosphate (ADP), commence. Both the previously mentioned substances cause platelet aggregation. This process is characterized by a process in which the integrin, glycoprotein IIb/IIIa, becomes a receptor for fibrinogen, forming bridges between adjacent platelets (Packham, 1994). Therefore it can be stated that platelets are able to concentrate and potentiate coagulation reactions on the damaged vessels (Bloom, 1990).

Bloom in 1990 suggested that as soon as factors XII and XI interact with the platelets, the coagulation protease zymogens start undergoing sequential activation. This leads to production of thrombin from prothrombin and fibrinogen can be converted to fibrin, forming a platelet-fibrin haemostatic plug. Thus, fibrin deposition and removal during healing forms part of the fibrinolytic system. In the midst of all the coagulation processes going on during formation of a haemostatic plug, thrombin is generated, which is involved in promoting haemostatic reactions as well as number of protective functions. Serine protease inhibitors (serpins) modulate the activities of thrombin and other serine proteases, which include antithrombin III and heparin cofactor II. The physiological anticoagulant action of glycosaminoglycans at the endothelium and the pharmacological action of heparin are regulated by these proteases. Therefore it can be stated that anticoagulant therapy are driven by the ability to reduce the formation or function of thrombin and other serine proteases (Bloom, 1990).

Morgenstern *et al.* in 2001 developed a model for *in vitro* clot formation which reflects both the contributions of platelets (fibrin fibre internalization and retraction) and of fibres (branching) enabling the retraction. The researchers utilized ultra-structural and immunocytochemical techniques to investigate the constitution of platelet-fibrin contacts, the separation of platelets initially aggregated, and the rearrangement of the platelet cytoskeleton during clot formation. After aggregation, fibrin polymerizing within focal contacts and from degranulating secretory granules contributed to the fibres. The initially formed focal contacts with fibrin obviously persisted during clot formation. The physiological branching of the fibres enabled separation of platelets. The contact associated cytoskeleton formed a constricting and fibre initializing sphere, but later stress fibre like bundles. As retraction progressed, the cytoskeleton changed to stress fibre connecting focal contacts with fibres.

Thus, fibrin networks and platelet morphology form an integral part of normal haemostasis and coagulation and therefore it is of great value for studying effects of pharmaceutical products on normal morphology. The aim of this chapter was therefore to investigate whether aspartame will have an effect on the morphology of the fibrin fibres and the platelets, and if so, how they are affected.

6.2 MATERIALS AND METHODS

6.2.1 Exposure of animals to aspartame

Eight New Zealand white rabbits were kept at the production unit of the University of Limpopo, Medunsa campus. The rabbits were divided into 2 groups: 3 controls and 5 rabbits in the aspartame treatment group. Rabbits in the aspartame-treated group were fed aspartame by means of force-feeding with a syringe into the back of the mouth of the rabbit. Aspartame was utilized in its pure form. The concentrations of aspartame that were studied was calculated according to the projected daily ingestion by humans (34mg/kg body weight) and also at 2 doses considered to be in an abuse range for humans (100 and 150mg/kg body weight) (Stegink *et al.* 1981). Rabbits in the control group only received double distilled water, by means of force-feeding with a syringe into the side of the mouth of the rabbit. Both the control and test groups received the same volume of fluid. The aspartame-treated rabbits received 25 exposures of aspartame over a period of 37 days. No aspartame was fed to the rabbits over weekends.

6.2.2 Obtaining blood from rabbits

After the exposure periods for the three aspartame concentrations were complete, 1.5ml of blood was drawn from the 8 New Zealand white rabbits (3 controls and 5 aspartame treated rabbits). Blood was drawn from the marginal ear vein of the rabbits by utilizing a vacutainer needle. Blood was collected into a vacutainer tube containing 166.67 μ l of 0.109M trisodium citrate. The blood was used to form fibrin clots to investigate fibrin and platelet ultra-structure.

6.2.3 Preparation of platelet rich plasma

1.5ml blood was drawn from each rabbit. Ethical clearance was obtained from the University Animal and Ethics committee of the University of Pretoria (H2205) as well as the Animal Ethics Committee of the University of Limpopo, Medunsa campus (AEC 4/2006). Blood from the rabbits were centrifuged at 1000 rpm for 2 minutes, to obtain platelet rich plasma (PRP).

6.2.4 Preparation of fibrin clots

Human thrombin (provided by The South African National Blood Services) was used to prepare fibrin clots (Pretorius *et al.*, 2006). This thrombin, also described in the previous chapter, was added to PRP and the fibrinogen is converted to fibrin and intracellular platelet components.

20µl of the PRP from the rabbits were mixed with 20µl human thrombin. The PRP and thrombin mix was immediately transferred to a 0,2µm millipore membrane with a pipette tip to form the coagulum (fibrin clot) on the membrane, by evenly spreading the coagulum onto the millipore membrane. The millipore membrane was then placed in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 37°C for 10 minutes. This was followed by a washing process where the millipore membranes with the coagula were placed in PBS and magnetically stirred for 2 hours. This was done to remove any blood proteins trapped within the fibrin network (Pretorius *et al.*, 2006).

6.2.5 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 hour. Each millipore membrane was rinsed thrice in phosphate buffer (0.15M PO₄) for 5 minutes before being fixed for 1 hour with Osmium tetroxide (OsO₄) (50% PO₄ buffer at pH=7.4 and 50% OsO₄ from a 1% stock solution). The samples were rinsed thrice with distilled water for 5 minutes and were dehydrated serially in 30%, 50%, 70%, 90% and three times with 100% ethanol. The SEM procedures were completed by critical point drying (Bio-Rad E3000; Watford, England) of the material, mounting and examining the tissue with a JSM-6000F FEGSEM (JEOL, Tokyo, Japan).

6.3 RESULTS AND DISCUSSION

6.3.1 Fibrin networks and platelet aggregates of the controls

Figure 6.1a and 6.1b illustrates the fibrin network and figure 6.2 the platelet aggregate of a control rabbit.

Figure 6.1a: Rabbit fibrin networks with major and minor fibres. Label A – major, thicker fibres; Label B – minor, thinner fibres (x7 000 magnification)

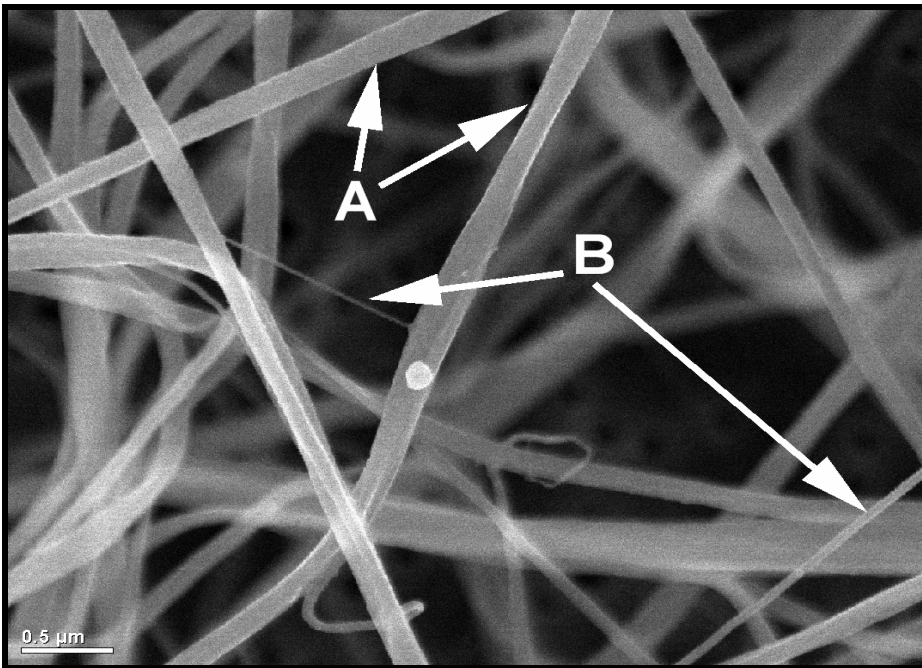


Figure 6.1b: Higher magnification of the fibrin network of the control rabbit, illustrating major fibers (Label A) and minor fibers (Label B) (x10 000 magnification)

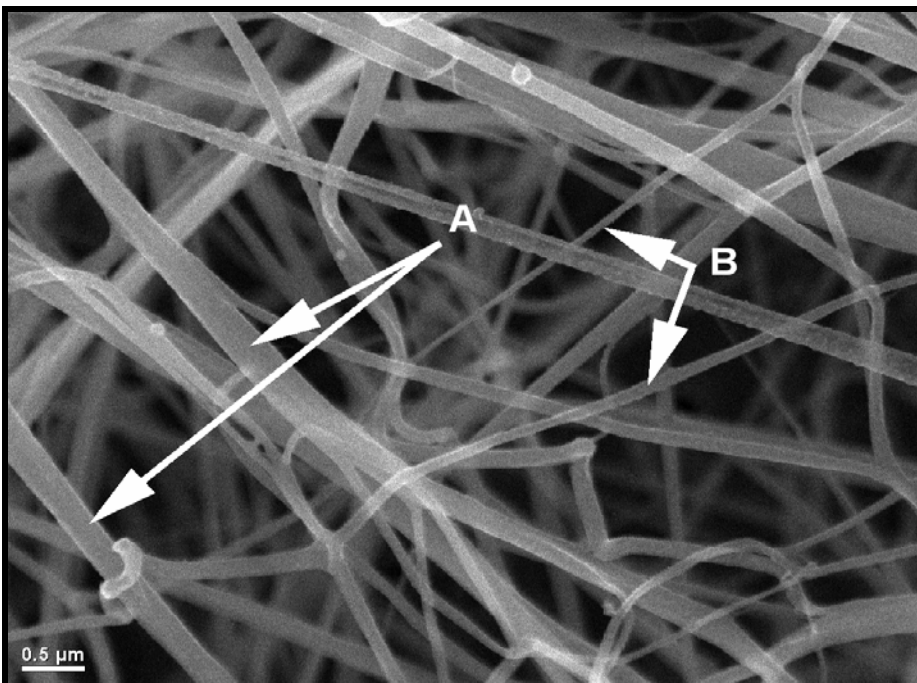
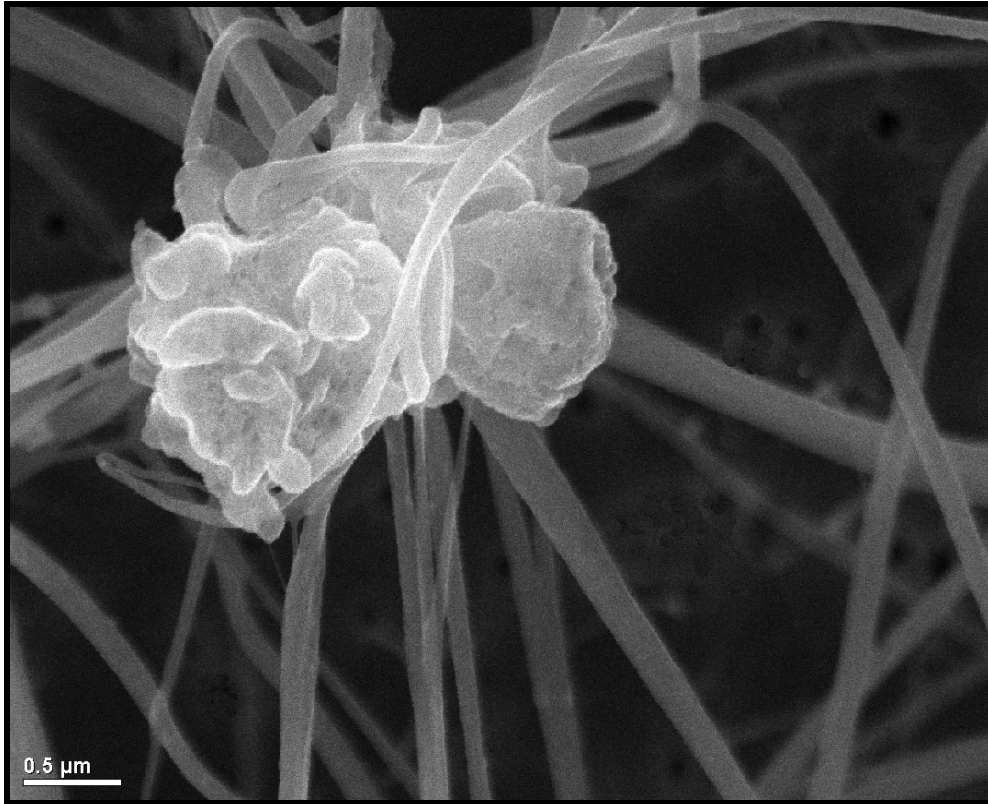


Figure 6.2: Rabbit platelet aggregate illustrating globular, pseudopodia-like morphology (x10 000 magnification)



6.3.2 Fibrin networks and platelet aggregates of the aspartame treated rabbits

Figure 6.3 illustrates the fibrin network and figure 6.4 the morphology of the platelet aggregate of the rabbit after treatment with 34mg/kg aspartame.

Figure 6.3: Rabbit fibrin network treated with 34mg/kg aspartame. Label A = major fibers still very prominent. Circles = minor fibers have a mat-like formation, visible throughout the picture (x10 000 magnification)

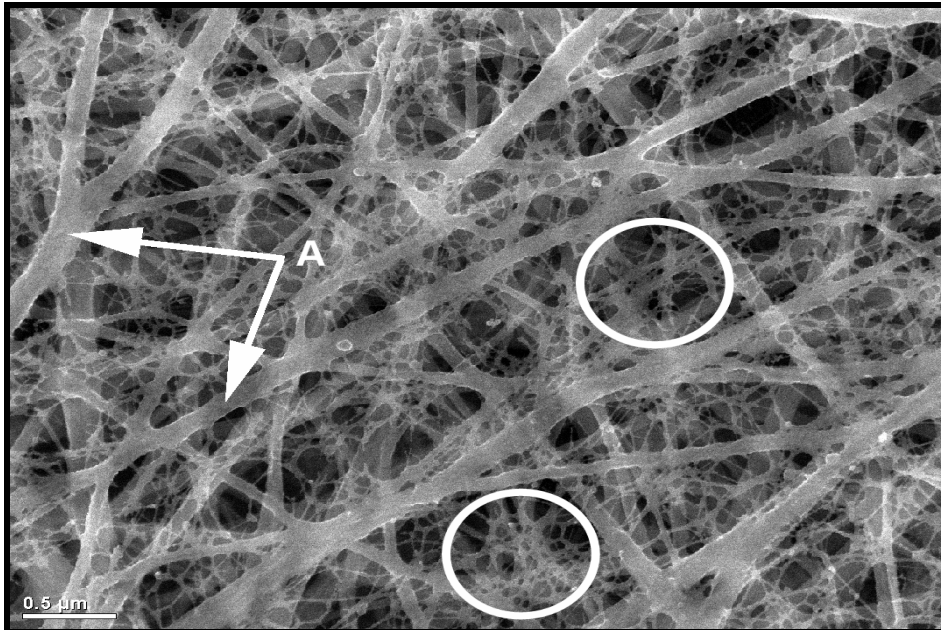


Figure 6.4: Platelet aggregate of a rabbit exposed to the 34mg/kg aspartame. Platelet aggregate more granular appearance, but globular pseudopodia-like morphology still present. (x10 000 magnification)

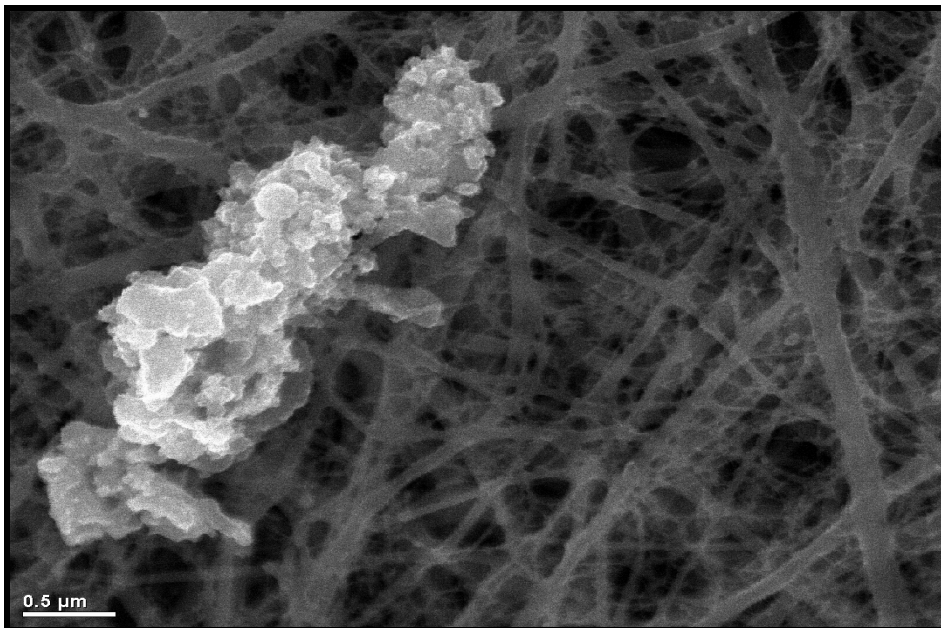


Figure 6.5 illustrates the fibrin network and figure 6.6 the morphology of the platelet aggregate of the rabbit after treatment with 100mg/kg aspartame.

Figure 6.5: Rabbit fibrin network after treatment with 100mg/kg aspartame. Major fibers (Label A) and minor fibers (Label B) can be distinguished. Minor fibers form mat-like formation (x10 000 magnification)

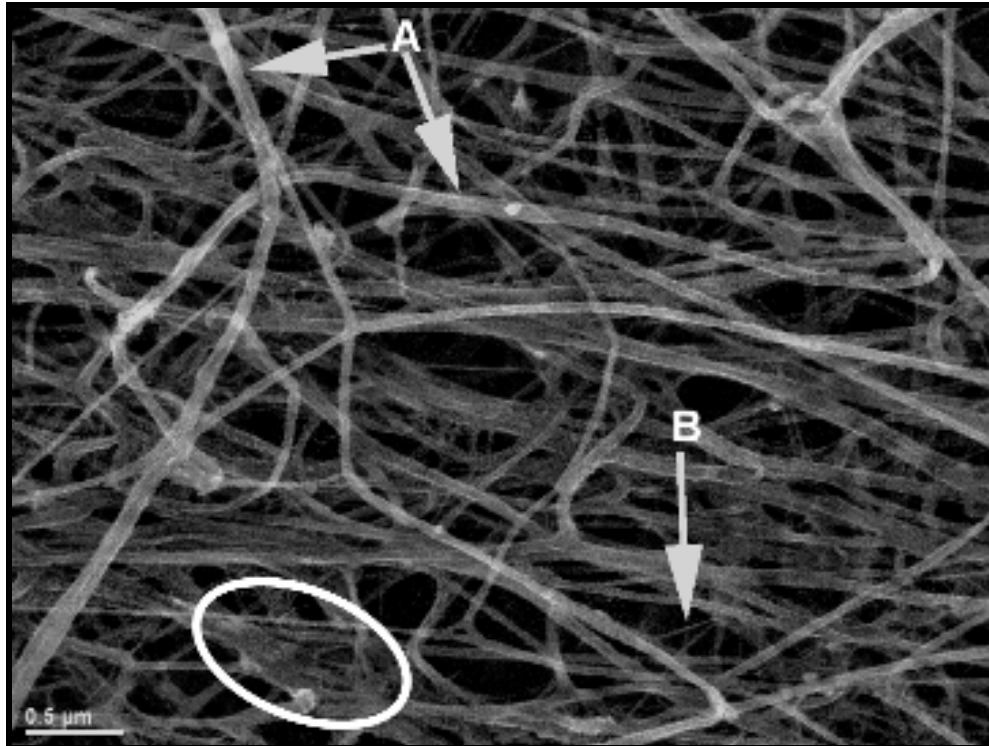


Figure 6.6: Platelet aggregate of a rabbit treated with 100mg/kg aspartame. Platelet aggregate show globular, pseudopodia-like morphology with disheveled membranes (x10 000 magnification)

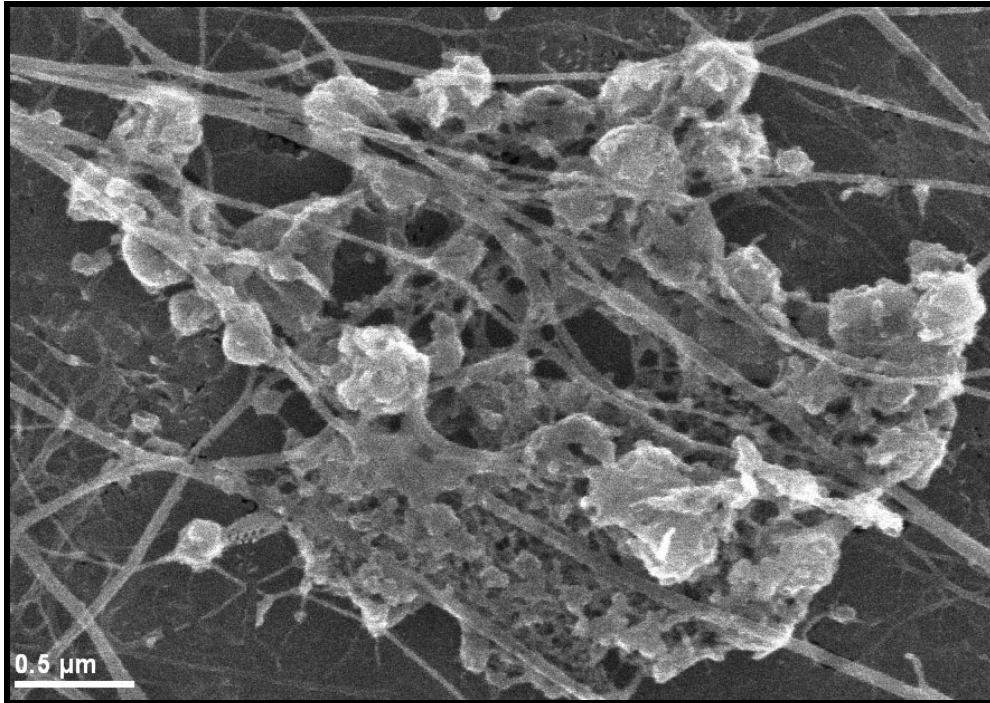


Figure 6.7 illustrates the rabbit fibrin network and figure 6.8 the platelet aggregate morphology after treatment with 150mg/kg aspartame.

Figure 6.7: Rabbit fibrin network after treatment with 150mg/kg aspartame. Label A = major fibres; Label B inside block = minor fibres forming dense mat-like coverage over major fibres (x10 000 magnification)

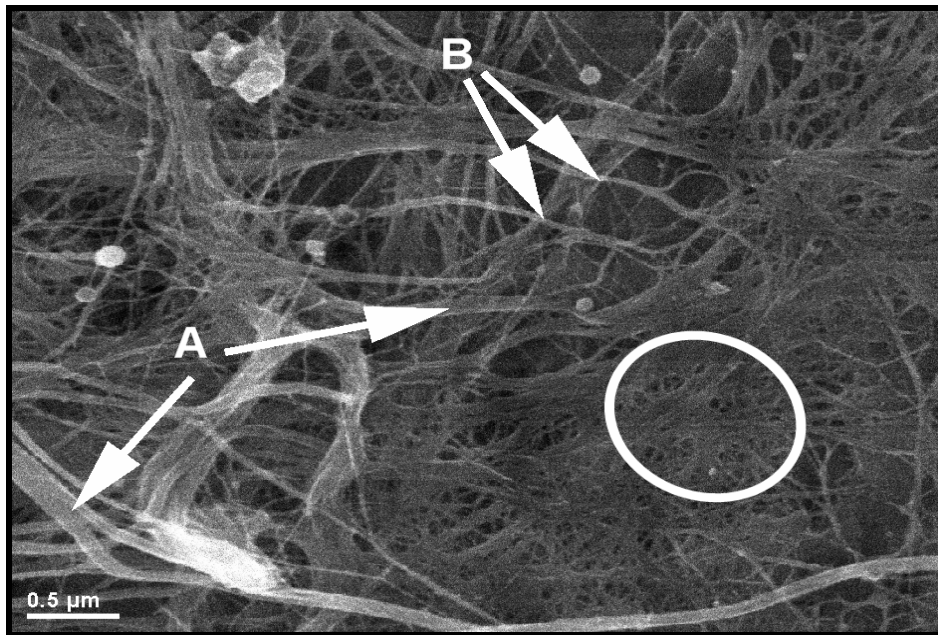
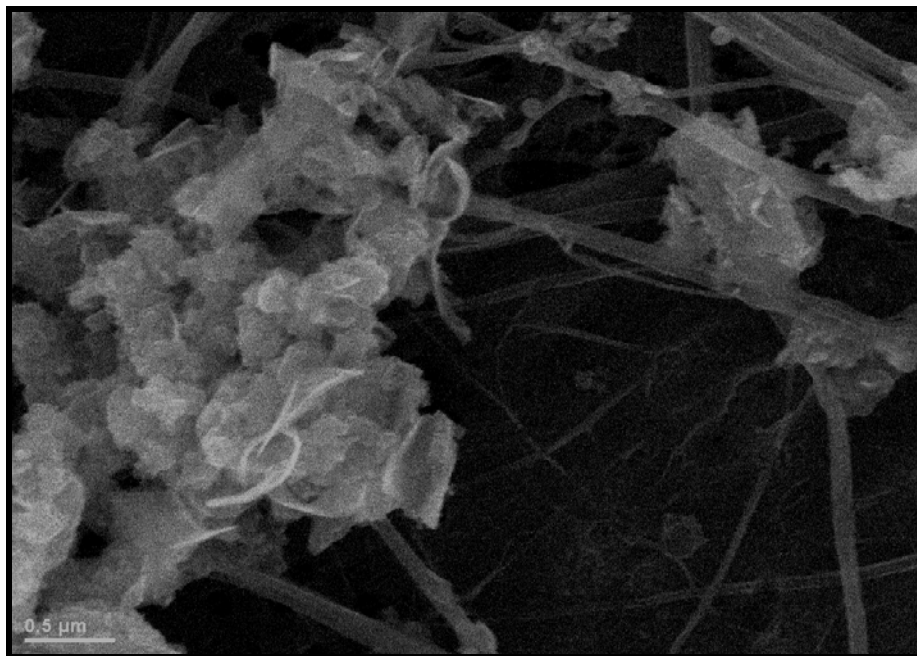


Figure 6.8: Platelet aggregate of a rabbit treated with 150mg/kg aspartame (x10 000 magnification)



The effects of aspartame were determined by comparing the normal ultra-structure of the fibrin fibres and platelets of the control rabbits with those of rabbits treated with aspartame. It appears as though aspartame had a major effect on the normal arrangement of the fibrin fibres and the morphology of the platelets. The control fibrin networks (Figure 6.1a and 6.1b) and the aspartame treatment groups' fibrin networks (Figure 6.3; Figure 6.5 and Figure 6.7) were compared with each other. The control exhibited clearly distinguishable major and minor fibres (Pretorius *et al.*, 2006). The major thicker fibres were more prominent with the minor fibres being smaller and more to the back of the major fibres (Figure 6.1a and 6.1b). This differed from the results obtained for the aspartame treated fibrin clots. The fibrin networks obtained after treatment with 34mg/kg aspartame exhibited the prominent major fibres, but the minor fibres had formed a mat-like formation over the major fibres (Figure 6.3). The degree to which the minor fibres were affected after treatment with 100mg/kg aspartame (Figure 6.5) was not as severe as after treatment with the 34mg/kg aspartame (Figure 6.3). The major fibres were still visible over the minor fibres and it appeared as though the minor fibres started to fuse (Figure 6.5). The fibrin fibres obtained after treatment with 150mg/kg aspartame exhibited the same effect as seen for the lowest concentration, only at a higher level. The mat-like formation of the minor fibres was more densely packed, starting to form a continuous cover over the major fibres (Figure 6.5). The major fibres were still present, but it appeared as if the fibres were starting to fuse together.

A comparison between the platelet aggregates of the control rabbits and aspartame treated rabbits also had differing results. The platelet aggregate of the controls (Figure 6.2) were compared to the platelet aggregates of the 34mg/kg (Figure 6.4), 100mg/kg (Figure 6.6) and 150mg/kg (Figure 6.8) aspartame treated groups. The platelet aggregate of the control exhibited normal globular, pseudopodia-like morphology and smooth platelet membranes were visible (Figure 6.2). This was not the case for the platelet aggregates after treatment with the different concentrations of aspartame. The globular, pseudopodia-like morphology could still be distinguished, but the platelets appeared more granular the higher the concentration of aspartame. The membranes of the platelets after treatment with the 100 mg/kg and 150mg/kg aspartame also appeared more dishevelled than the membranes of the controls or the platelets after treatment with the lowest concentration of aspartame. Due to cell membrane damage after

treatment with aspartame, the granular appearance of the platelet aggregate could be caused by the exposed granules of the individual platelets.

The platelet specific storage granules are α and dense granules. The α -granules contain proteins such as platelet factor 4, β thromboglobulin, platelet derived growth factor, fibrinogen, fibrinectin, thrombospondin, plasminogen activator inhibitor I and von Willebrand Factor (vWF). Dense bodies are rich in serotonin, adenosine diphosphate (ADP) and calcium. Platelets also produce and secrete pharmacologically active substances such as thromboxane and platelet activating factor. Platelet adhesion, activation and aggregation are regulated by specific glycoproteins on the platelet cell surface (Blockmans *et al.*, 1995). Thrombin mediates shape change and the release of granule contents. It also activates the glycoprotein IIb-IIIa receptor, which results in the binding of fibrinogen and vWF (Macfarlane *et al.*, 2001). Thus, from the results reported in chapter 5 on the coagulation profile, the amount of circulating thrombin would be decreased after treatment with aspartame due to the decrease in the amounts of circulating factors (F VII, X and VIII). This indirectly causes a decreased release of the granule contents of the platelets which results in a decrease in the extent of platelet aggregation, as seen in the results obtained for the platelet aggregation after treatment with aspartame was complete (Figures 6.6 and 6.8). Serotonin, contained within the dense granules, also improves binding of two adjacent platelets. After serotonin is released from one platelet, it adheres to a receptor on the membrane of another platelet in close proximity, forming bonds that lead to platelet aggregation. However, it has been stated that aspartame also decreases the amount of serotonin (Humphries *et al.*, *In Press*). Thus, it could be stated that platelet aggregation was also hindered in this way by aspartame treatment.

6.4 CONCLUSION

From the results obtained in this chapter it would appear as if the fibrin fibre and platelet aggregate morphology could be used as a useful tool for determining the effects of pharmaceutical products on normal morphology. The normal morphology of the fibrin networks and platelet aggregates were altered by the treatment with aspartame, indicating that aspartame influences coagulation.

Due to the subsequent changes of the fibrin fibre morphology and the degree of fibrin fibre network formation, it is hypothesized that the amount of fibrinogen increased after treatment with aspartame. A definite trend of increased coagulation could be observed between the control samples and the aspartame treated samples. It seemed as though the major fibres started to fuse after treatment with aspartame. The degree to which the major fibres started to fuse became more pronounced the higher the concentration of aspartame treatment. The effects of the aspartame were more distinct when looking at the minor fibre morphology. The minor fibres were present in the controls, but they were less prominent than the major fibres. After treatment with 34mg/kg aspartame, the minor fibres started to form a mat-like formation on top of the major fibres. The degree to which the minor fibres formed a mat-like formation over the major fibres in the 100mg/kg aspartame treated group, decreased. A possible explanation for this phenomenon could be that the rabbits activated a defence mechanism, which in turn triggered an adaptation in their coagulation system to the concentration increase (from 34mg/kg to 100mg/kg aspartame). The highest concentration (150mg/kg) however was too great a shock to their system, leading to a mat-like formation that were even more densely packed, almost covering the major fibres.

Platelet aggregate size cannot be used as a diagnostic tool for determining effects of pharmaceutical products on normal coagulation as the size of the aggregate are determined by the number of platelets that bind together during aggregation. The morphology of the platelet aggregate however can be used to indicate adverse effects after treatment with pharmaceutical products. The platelet aggregates of the controls resembled normal, healthy platelet aggregates, where the platelet aggregates had globular, pseudopodia-like morphology with smooth membranes (Pretorius *et al.*, 2006). After treatment with the aspartame, the platelet aggregates still exhibited globular pseudopodia-like morphology, but the platelets aggregates became more granular (possibly α and dense granules) with membranes being more dishevelled. Platelet aggregation was hindered, which could be attributed to low concentrations of circulating thrombin (chapter 5).

Thus, it could be concluded that aspartame adversely affected normal fibrin fibre and platelet aggregate morphology and that coagulation and haemostasis can therefore be negatively influenced by utilizing products containing this artificial sweetener.