Chapter 3 –

Establishing a protocol for successfully obtaining blood samples from rabbits

RESEARCH QUESTION 1:

Which protocol is best suited for successfully obtaining blood samples from a rabbit and how should aspartame be administered to the rabbits in the treatment group to prevent loss of any aspartame?

3.1 INTRODUCTION

Drug development projects utilize primate models of thrombosis, as they are regarded as suitable for predicting parameters of human efficacy and safety (Harker, Hanson and Kelly, 1995). Smaller and more widely available experimental animals have been used as substitutes for preliminary screening of compounds, due to the high costs, deficient access and ethical concerns of utilizing primates. Rabbits are often the species of choice for models of antithrombotic efficacy. Results obtained by these studies are used to extrapolate plasma levels of inhibitor required for therapeutic activity in humans (Sinha *et al*., 2000; Chi *et al*. 1998). Pendurthi, Anderson and James in 1997 showed that the rabbit protein has several non-conserved residues around the active site and in the activation region when comparing the amino acid sequence for rabbit factor X (RafX) and human factor X (HfX). Both the activation kinetics of RafX and proteolytic activity of Rafxa could be affected by an altered protein structure as seen under pathologic conditions of ongoing thrombosis. Measurement of blood flow and thrombus weight in rabbit models can lead to momentous effects by these above-mentioned changes (Edwards *et al*., 2002).

Several *in vivo* models of thrombosis are available for evaluating antithrombotic drug candidates. Therapeutic levels in clinical trials can be better predicted by primate models, as they are seen as having better predictive value, but, their widespread use as a screening tool is excluded for economic, ethical and technical reasons. Therefore, more recently preferred species are rabbits and rats since statistically significant comparisons between control and drug-treated animals can be done relatively easily (Chi, Rebello and Lucchesi, 1998). Differences in rabbit and human prothrombin structure and subsequent physiology are also likely to contribute to variation between animal models. This is in addition to the fX differences found between species. Altered susceptibility to proteolytic cleavage by the prothrombinase complex, as well as different proportions of the two cleavage products (α-thrombin and meizothrombin) have been noted in the two species (Seiffert *et al*., 1999). The interpretation of

thrombus growth rates in rabbits would be further complicated since meizothrombin and α-thrombin are reported to have markedly different platelet activating and fibrinogen cleavage capacities (Doyle and Mann, 1990). Several compromises inherent to utilizing rabbits as an experimental vehicle for oral drug absorption have been observed by Chiou *et al*. (1969). By understanding the similarities and differences between rabbit and human coagulation systems, they can be utilized with great confidence as models although presumed clinical therapeutic doses, derived from *in vivo* results in rabbits, should be evaluated in a conventional manner (Edwards *et al*., 2002).

Experimental animals of choice for pharmaceutical studies are usually rats, mice and rabbits. However, rats and mice are small animals, weighing between 45–300grams and 15-30grams respectively (Rodent weight standards; Backwoods Farm Inc.). This is problematic when taking into account that rats have 50-65ml blood per kilogram body weight and mice 70-80ml blood per kilogram body weight. The rabbit, however, can weigh anything from 2-6kg (New Zealand white rabbits) and have 45-70ml blood per kilogram body weight (Mitruka and Rawnsley, 1977). Blood can be drawn quite easily from the marginal ear vein of the rabbit with minimum invasive procedures required, while blood has to be drawn directly from the heart of the rat and mouse, usually resulting in the death of the animal, because of the volume of blood needed to obtain results. This may lead to difficulty in long term studies, particularly in coagulation studies as blood can only be drawn once from these two animals. Only short term studies are therefore feasible seeing that the animals have to be bled to death to obtain enough plasma to perform coagulation studies.

After carefully considering all the above mentioned factors, the New Zealand white rabbit was chosen as the most appropriate research animal for obtaining blood samples with relative ease. This chapter will therefore focus on the most successful methods for obtaining blood samples from the rabbit. Also, difficulty may occur when administering aspartame orally to rabbits, thus different protocols was tested to obtain a method for successfully administering aspartame to the treatment group rabbits, without loss of any of the aspartame.

3.2 MATERIALS AND METHODS

3.2.1 Obtaining blood samples

Blood was drawn with a needle and syringe, however, the blood tended to clot in the syringe due to the slow rate of obtaining the blood sample. Due to the unsuccessful first procedure, a butterfly was utilized. Blood flow into the tube was also too slow, causing blood clots. Blood drawn by using a vacutainer needle proved to be most successful. The needle was inserted into the marginal ear vein of the rabbit, followed by the removal of the rubber stopper from the opposite side of the vacutainer needle. The vacutainer needle and tube could not be used as is, with the vacuum still in the tube, as the vacuum was too big and it occluded the vein with the negative pressure. Blood dripping/flowing from the needle was then collected in a vacutainer tube which cap was removed (Figure 3.1 and 3.2). The tube contained 0.109M tri-sodium citrate as anti-coagulant. After the blood sample was collected, the tube was lightly tilted to ensure proper mixing with the anti-coagulant.

3.2.2 Tubes utilized

A 15 ml centrifuge tube with a screw cap (NUNC, obtained from Amersham), containing the correct amount of anti-coagulant were used, however, this caused blood clots. A 4.5ml vacutainer tube containing 0.306M tri-sodium citrate proved to be the most successful container, with no clots forming. The amount of trisodium citrate for each tube was prepared as described in diagram 3.1.

3.2.2.1 Preparation of vacutainer tubes

The original tri-sodium citrate inside each of the vacutainer tubes were decanted into a separate tube, therefore pooling the citrate. The required amount of tri-sodium citrate was then pipetted back into clean the vacutainer tubes; this was done to have exactly the correct volume of citrate per volume of blood drawn, as the volume of blood drawn could vary within ml's each time blood was drawn. Too much citrate could possible cause variable results in coagulation studies, while too little citrate will cause clotting of blood. See Diagram 3.1 for calculations. Thus, for 1.5ml blood, 166.67μl tri-sodium citrate was pipetted into the tubes.

Diagram 3.1: Calculating citrate volume per ml of blood

For example:

1 ml tri-sodium citrate : 9ml blood

X ml anti-coagulant : 1.5ml blood

$1 \times 1.5 = X \times 9$

$\frac{1.5}{9} = X$

X = 166.67µl tri-sodium citrate

3.2.2.2 Preparation of aspartame concentrations

The rabbits were weighed (Figure 3) to determine the amount of aspartame given in the study. The amount of aspartame given to each rabbit was calculated according to the concentration of aspartame the rabbits were exposed to. Three concentrations (34, 100 and 150mg aspartame per kg of rabbit) were tested. The concentrations were chosen based on research by Stegink *et al*. in 1981. Thus if the rabbit weighed 3.95kg, this was multiplied with each of the 3 concentrations to calculate the correct amount of aspartame to be given to the rabbits. See diagram 3.2 for calculations.

Diagram 3.2: Calculations of aspartame for each rabbit

Rabbit weighed 4.95kg

= 4.95kg x 34/100/150mg (depending on the concentration being tested)

= 168.3mg/495mg/742.5mg aspartame

Stock solution of aspartame = 5mg/ml

= 0.5g aspartame in 100ml distilled water

Thus to obtain 742.5 mg aspartame:

1485µl

= 5mg/ml ⟶ 10µl

= 742.5mg

Figure 3.1: Utilizing the vacutainer needle for obtaining blood from the marginal ear vein

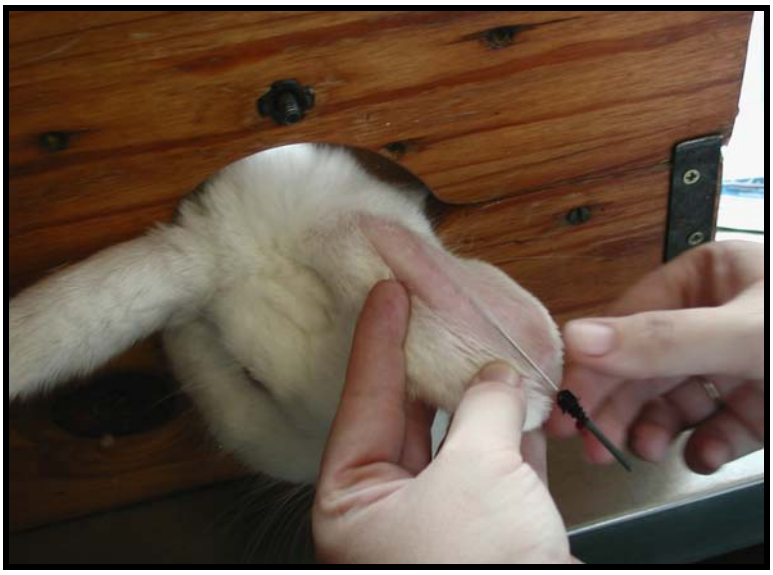


Figure 3.2: Collecting the blood sample into prepared vacutainer tubes by utilizing vacutainer needles

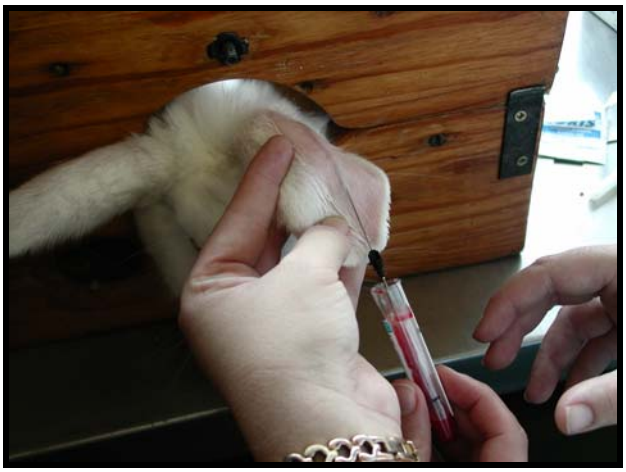


Figure 3.3: Weighing of rabbits for calculation of aspartame concentrations

3.2.3 Vasodilatation for visualization of the blood vessels

Xylene was used initially as indicated by Prof F Clarke, University of Limpopo, Department of Biology (Personal communication), however, it caused the ears of the rabbits to become red and dry. After experimenting with different techniques, the best procedure was to wipe the ears with a cotton ball soaked in water (water leads to 'transparency' of the hair on the ear of the rabbit, promoting visibility of the marginal ear vein). This was followed by wiping the ear with an alcohol swab and flicking of the area surrounding the vein (alcohol is a mild vasodilator together with the flicking-action, increasing visibility of the marginal ear vein) (Figure 3.4).

3.2.4 Modified constraint for the rabbits

Rabbits tend to move their heads when they are not constrained properly. The entry point of the needle into the vein is lost when the rabbit moves its head, causing the vein to collapse. Thus, a constraint was build where the rabbits-head is inside a guillotine-like wooden box (Figure 3.5).

3.2.5 Feeding rabbits with aspartame

The aspartame concentrations were prepared in 15 ml centrifuge tube with a screw cap (NUNC, obtained from Amersham) as described above. The aspartame solution was drawn into a 5ml syringe and the needle was removed. A plastic tube was attached to the tip of the syringe and inserted into the back of the mouth of the rabbit. However, the rabbits tended to bite the tubes, causing loss of aspartame. Thus, the tip of the syringe alone was then inserted into the back of the mouth of the rabbit and the solution was slowly transferred into the rabbits' mouth (Figure 3.6). The syringe was moved to ensure that the rabbit swallowed the solution and care was taken that none of the solution were spilled.

Figure 3.4: Preparation of the ear of the rabbit to improve visibility of the marginal ear vein

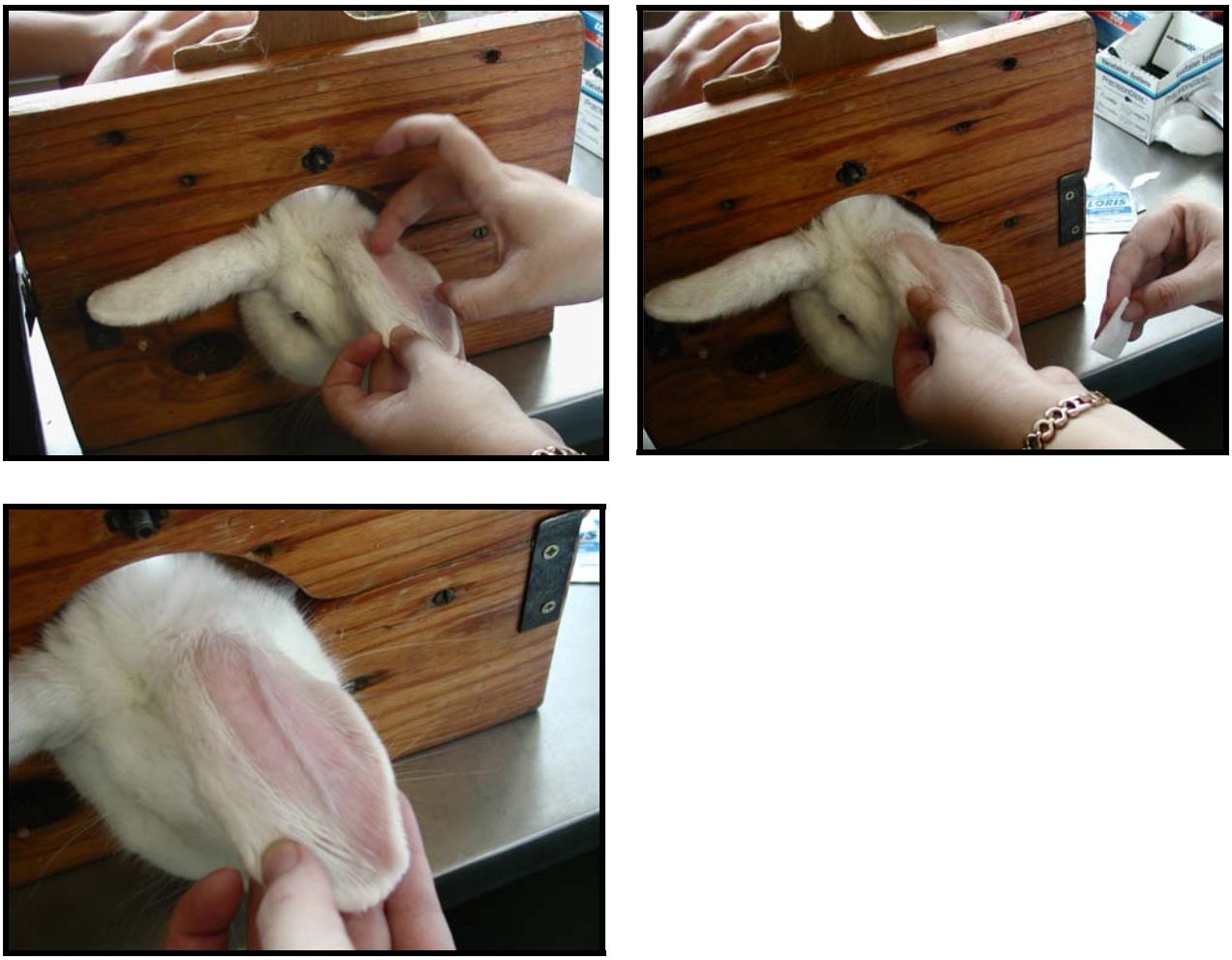


Figure 3.5: Rabbit inside modified constraint before blood is drawn

Figure 3.6: Rabbits being given their aspartame solution

3.3 CONCLUSION

After attempting a number of methods for obtaining blood from the rabbits, it was concluded that blood drawn by using a vacutainer needle proved to be the most successful. The needle was inserted into the marginal ear vein of the rabbit, followed by the removal of the rubber stopper from the opposite side of the vacutainer needle. Blood dripping/flowing from the needle was then collected in a vacutainer tube which cap was removed. A 4.5ml vacutainer tube containing 0.306M tri-sodium citrate proved to be the most successful container, with no clots forming. The correct amount of trisodium citrate was pipetted into the vacutainer tubes (166.67µl trisodium citrate per 1.5ml blood drawn). The marginal ear vein was visualized by flicking with the middle finger and wiping with an ethanol swab. Rabbits were also placed in a restraint because they tended to move their heads upon entry of the needle into the vein, thus the point of entry was lost, causing the vein to collapse.

Aspartame was given according to the weight of each specific rabbit. A 0.5mg/ml aspartame stock solution was prepared and diluted according to calculations based on the weight of the rabbit and the respective aspartame concentration being tested (34mg/kg, 100mg/kg or 150mg/kg aspartame). Aspartame was successfully deposited into the back of the mouth with a syringe, without loss of any of the aspartame solution.

After the protocol was established successfully for obtaining blood from rabbits and administering aspartame without loss of any of the solution, the investigation of obtaining blood samples was broadened to include mice and humans as the following chapter will include them for establishing an experimental animal model.

It is therefore the aim of this thesis to use the rabbit as animal model, as well as the additional measurement of metabolomics (as described in the introduction), in the form of coagulation factors, and combining this with traditional histology and ultra-structural analysis.

Chapter 4 –

Utilising ultra-structural comparisons of the human, rabbit and mouse fibrin networks and platelet morphology for establishing the best suitable experimental animal model

RESEARCH QUESTION 2:

When comparing the ultra-structure of fibrin networks and platelet morphology of the human, rabbit and mice, can the rabbit be successfully used and implemented as a model for studying the blood coagulation system?

4.1 INTRODUCTION

Due to the complex nature of coagulation in the human and the numerous ethical concerns regarding human subjects in experimental research, it is sometimes difficult to study coagulation in the human. Human subjects, who present with coagulation disorders, generally also suffer from other diseased states for which they already receive treatment, which in turn can interfere with the coagulation system. This being the case, human subjects can therefore be seen as subjective when used in trials. Pre-clinical trial periods are characterized by using of experimental animal models to determine the effects of the pharmaceutical products on the animals.

Animal models are used in nearly every field of biomedical research and numerous information regarding the treatment of diseases and the effect of medication, has involved using different laboratory animal models. Advances include the development of a vaccine for polio, antibiotics for infectious diseases like pneumonia and insight into the treatment of chronic illnesses like diabetes. The murine model e.g. is used successfully because mice allow for a variety of *in vivo* immunological applications (Bice *et al*., 2000). A few types of animal models include orphan disease models; induced disease models; transgenic disease models; spontaneous or genetic disease models and negative disease models (http://www.uac.arizona.edu//notes/classes/animalmodels/animalmodels03.html#CLASSIFICATION%20OF%20DISEASE). Animals used in these models vary, however, probably the most frequently used animals are rodents. However, in this study we used the rabbit model, which can possible be classified as a good example of an induced disease model.

The previous chapter concluded that the rabbit can be used successfully to measure metabolomics, in the form of coagulation factors and to perform ultra-structural analysis. Due to a lack of knowledge regarding the ultra-structure of platelets and fibrin networks of species other than humans, the current study was done to determine the morphology of fibrin and platelets of mice and rabbits using the scanning electron microscope to determine which of the two species would be the most successful experimental animal model.

4.2 MATERIALS AND METHODS

4.2.1 Obtaining blood from rabbits

0.5ml of blood was drawn from 3 New Zealand white rabbits (controls) at the production unit of the University of Limpopo, Medunsa campus. Blood was drawn from the marginal ear vein of the rabbits by utilizing a vacutainer needle. Blood was collected into a vacutainer tube containing 166.67μl of 0.109M trisodium citrate. The blood was used to from fibrin clots to investigate fibrin and platelet ultra-structure.

4.2.2 Obtaining blood from humans

40 ml of blood was drawn from 6 individuals in citrate tubes. This blood was used to form fibrin clots to investigate fibrin and platelet ultra-structure (the blood was not pooled).

4.2.3 Obtaining blood from the mouse

Balb/c mice were kept at Onderstepoort Animal Facilities. Mice not exposed to any products were euthanized and blood drawn directly from the heart, by inserting the needle into the thorax. Citrate was removed from citrate tubes and pooled, because of the inconsistency of the amount of blood drawn from each mouse. The syringe was flushed with citrate, in order to prevent clots from forming while drawing the blood. For each 100μl of blood drawn from the mouse, an assistant added 11μl of citrate to an empty citrate tube. When the blood was drawn, it was transferred to this citrate tube. Between 300μl and 500μl of blood was obtained from each mouse. The blood of six mice was pooled for the experiment. The pooled blood was used to from fibrin clots to investigate fibrin and platelet ultra-structure.

4.2.4 Preparation of platelet rich plasma

40 ml of blood was drawn in citrate vials from each healthy human donor (Ethical Clearance number 151/2006); 300µl of blood was drawn from 6 Balb-C mice (totalling 1800ul) and blood was pooled. Ethical clearance was obtained from the University Animal and Ethics committee (H2905); 500µl blood was drawn from each rabbit (Animal Use and Care Committee of the University of Pretoria (H2205); Animal Ethics Committee of the University of Limpopo, Medunsa campus (AEC 4/2006)). . For every 100µl of blood that was drawn from the rabbits and mice, 11µl citrate was added. Blood from the three groups was centrifuged at 1000 rpm for 2 minutes, to obtain platelet rich plasma (PRP).

4.2.5 Preparation of fibrin clots

Human thrombin (provided by The South African National Blood Services) was used to prepare fibrin clots from human blood (Pretorius *et al*., 2006) as well as blood from mice and rabbits. 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.

20µl of the PRP human donors was mixed with 20µl human thrombin. Because of the small volumes of blood from the mice and rabbits, 10µl of PRP from the mice and rabbits were mixed with 10µl of human thrombin. The PRP and thrombin mix was immediately transferred with a pipette tip to a 0,2µm millipore membrane to form the coagulum (fibrin clot) on the membrane. This millipore membrane was then placed in a Petri dish on filter paper dampened with phosphate buffered saline (PBS) to create a humid environment and placed at 37ºC 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 120 minutes. This was done to remove any blood proteins trapped within the fibrin network (Pretorius *et al*., 2006).

4.2.6 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 fibrin clot was rinsed thrice in phosphate buffer

(0.15M PO_4 at pH=7.4) for 5 minutes before being fixed for 1 hour with Osmium tetraoxide (OsO_4) (solution of 50% PO_4 buffer at pH=7.4 and 50% OsO_4 from 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).

4.3 RESULTS

Figure 4.1a and 4.1b show the fibrin network of a human fibrin clot and platelet.

Figure 4.1a: Human fibrin with major and minor fibres. Label A = major, thicker fibres; Label B = minor, thinner fibres

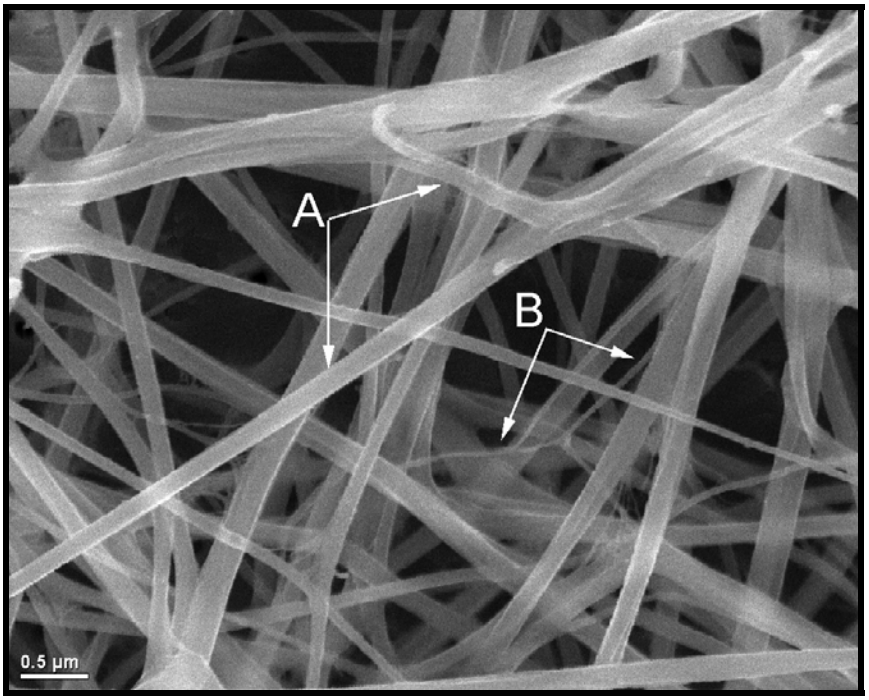


Figure 4.1b: Human platelet aggregate showing globular, pseudopodia-like morphology

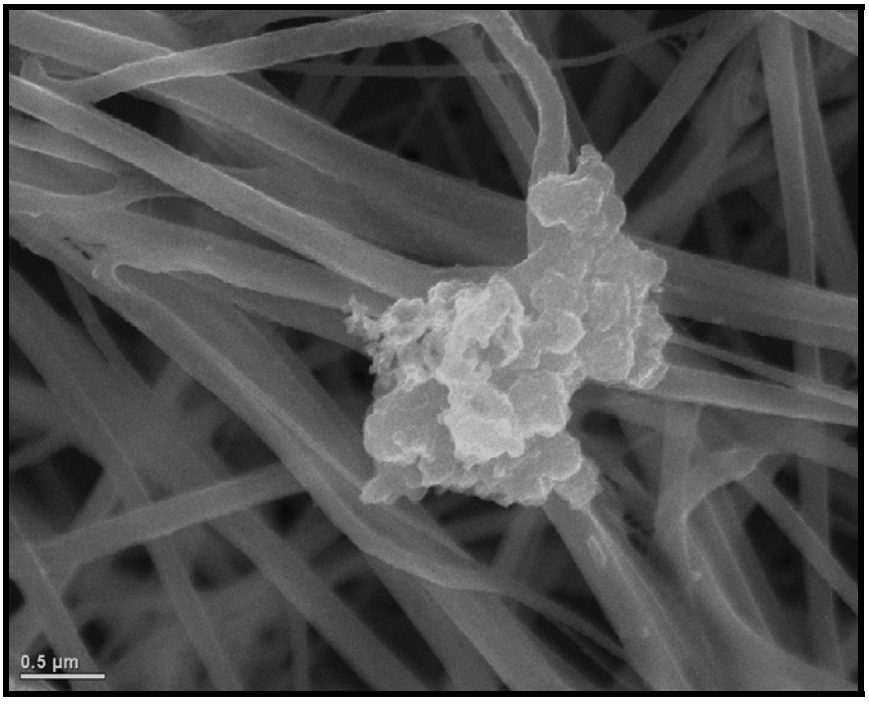


Figure 4.2a and 4.2b show the fibrin network and clot of rabbit blood.

Figure 4.2a: Rabbit fibrin network with major and minor fibres. Label A = major, thicker fibres; Label B = minor, thinner fibres

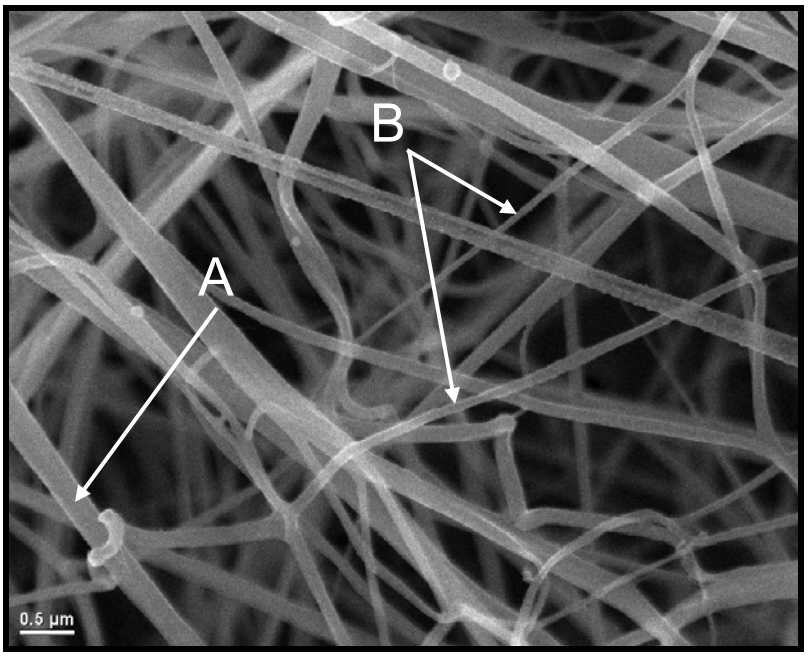


Figure 4.2b: Rabbit platelet aggregate showing globular, pseudopodia-like morphology

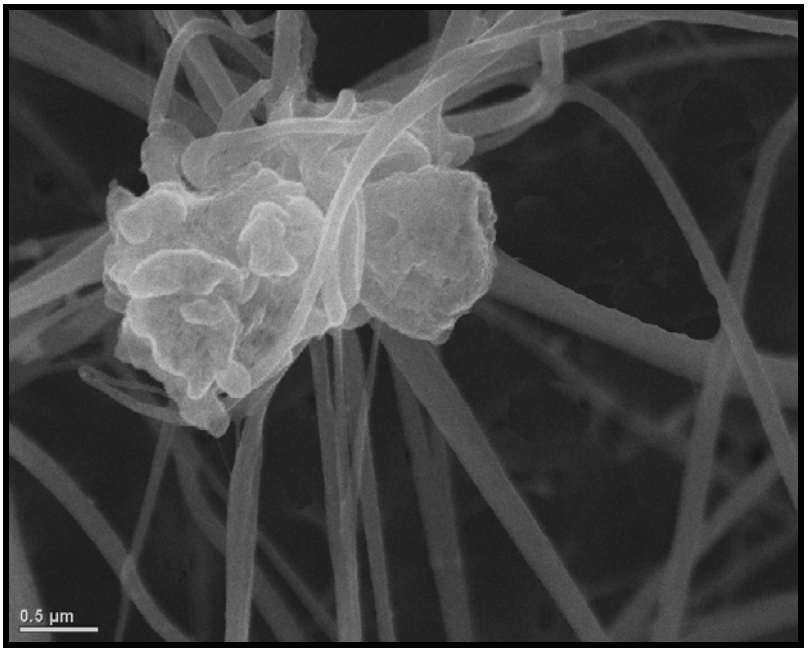


Figure 4.3a and 4.3b show the fibrin network and platelet aggregate of the mouse.

Figure 4.3a: Mouse fibrin with fine major and minor fibres and areas of mat-like coverage. Label A = major, thicker fibres; Label B = minor, thinner fibres. Label C inside block = mat-like fibre formation

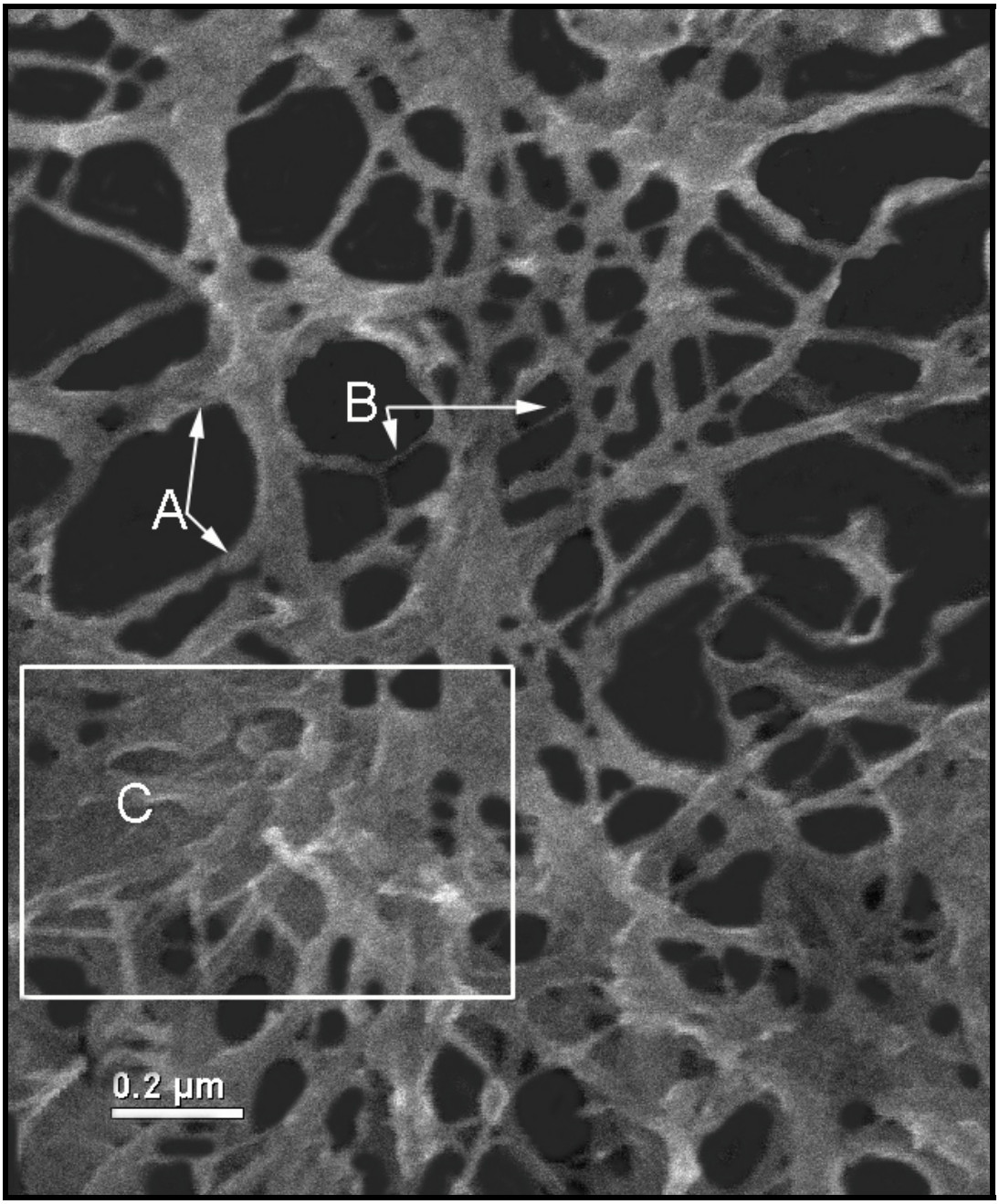
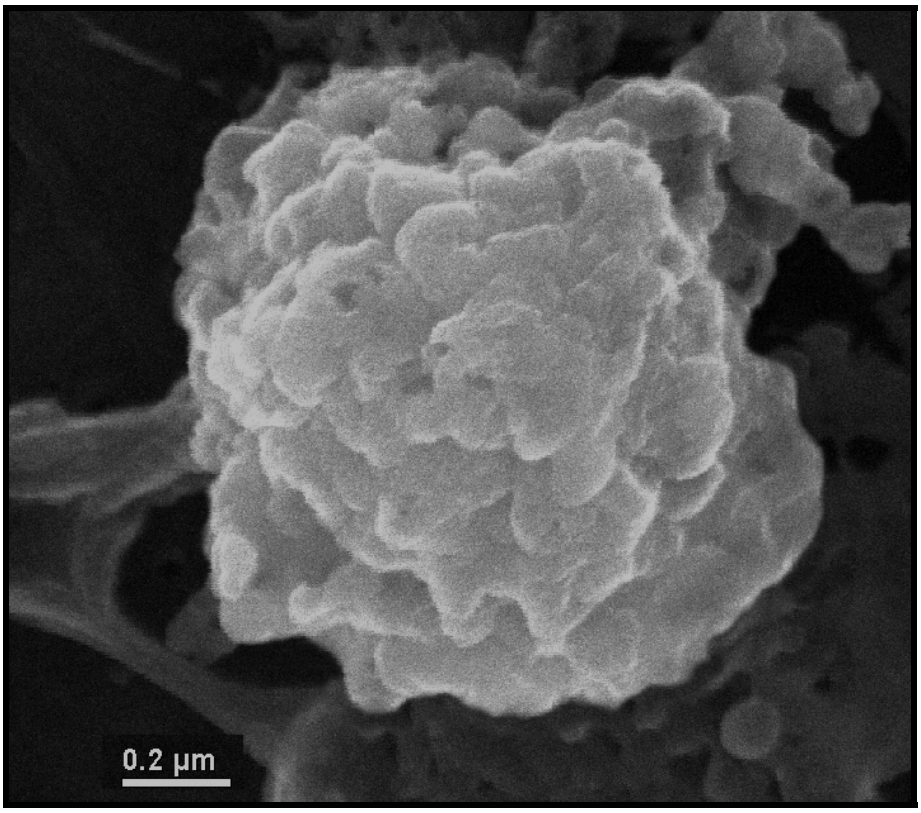


Figure 4.3b: Mouse platelet aggregate showing globular, pseudopodia-like morphology



Little differences were found between human and rabbit fibrin networks (Figure 4.1a and 4.2a) as well as platelets (Figure 4.1b and 4.2b). Human fibrin fibres show two distinct morphological types, namely major fibres and minor fibres (Pretorius *et al*., 2006). The major fibres tend to be most prominent, with the minor fibres much thinner and appearing between the major, thicker fibres (Figure 4.1a; Labels A and B). This was also found in the rabbit fibre network (Figure 4.2a; Labels A and B). Human fibre thickness varies from 0.15µm to 0,35µm. Rabbit major fibre thickness varies from 0,16µm to 0,33µm (Figure 4.2a).

The mouse fibrin network varied considerably from that of both human and rabbit fibrin. Although the clot does indeed appear to have both thinner and thicker fibres present (Figure 4.3a; Labels A and B), the thicker fibres have an approximate diameter of 0.03µm, forming a very fine fibrin network. This fine network sometimes has a mat-like appearance, where the fibres seemed to have formed on top of each other (Figure 4.3a; Label C inside block). It was interesting to note that, in order to take a SEM micrograph that show the fibres of the human/rabbit versus mouse fibrin, the SEM magnification for the human/rabbit clot was 6000 to 10 000x, while that of the mouse blood was between 30 000x and 40 000x.

Because platelet rich plasma was used for all three types of clots, platelets were still present trapped between fibrin fibres, or lying on top of the network. Figure 4.1b, 4.2b, 4.3b show the platelets of the three different species. Because of the coagulation process single platelets are bound to each other to form aggregates of platelets. Size of platelet aggregates therefore cannot be used to suggest differences between the three species. When comparing the ultra-structure of the platelet aggregates between the three species, no differences was noted. In all three species the aggregates have a rounded, globular pseudopodia-like appearance with the membranes of the aggregates forming a smooth surface.

4.4 DISCUSSION

Platelets and fibrin play and important role in the coagulation process where they are involved in the maintenance of haemostasis as well as the initiation of repair following tissue injury (Herd and Page, 1994). Platelets are an important source of growth-promoting factors and *in vivo* their

activation requires the initiation of a blood coagulation cascade when subendothelial *tissue factor* is exposed to the blood flow following either damage or activation of the endothelium (Nemerson, 1988; Carmeliet and Collen, 1998; McVey, 1999; Butenas and Mann, 2002). However, fibrin dysfunction is associated with the development of vascular complications, while proneness to the formation of tight and rigid fibrin networks is independently associated with thrombotic disease. There are also genetic disease complexes that cause inborn errors of fibrinogen structure and are by definition congenital dysfibrinogenaemias. This anomaly results in a disturbance of the interchain disulphide bond, an ultra-structural defect which interferes with fibrin polymerization (Pretorius *et al*. 2006). Furthermore, if platelets and fibrin function is disturbed by medication it may lead to either bleeding or clotting problems.

Thus, owing to the fact that the morphology of platelets and fibrin networks forms and integral part of normal coagulation and haemostasis, knowledge of normal morphology provides a valuable diagnostic tool to use when pharmaceutical products are tested during their pre-clinical trial periods. Thus, insight into normal haemostasis can therefore be used to distinguish dysfunctional haemostasis. However, the analytic value of the morphology and ultra-structure of platelets and fibrin networks are often underestimated.

Although a few studies have shown the value of transmission electron microscopy (TEM) to investigate not only human, but also animal ultra-structure, not many researchers have been looking at the SEM ultra-structure. Animal studies used animals ranging from the rhinoceros, elephant and bovine species (Zucker-Franklin *et al*. 1985; Ebbeling *et al*. 1992; Lui *et al*. 1998).

Cheryk *et al*. (1998) studied the ultra-structure of platelets from the Asian elephant (*Elephas maximus*) before and after activation with the agonist platelet activating factor (PAF) using the TEM. du Plessis and Stevens, in 2002 studied internal ultra-structure of the African elephant, and du Plessis *et al*., 1996 studied buffalo, *Syncerus caffer* platelet morphology; both studies used the TEM.

4.5 CONCLUSION

After obtaining a reproducible method for obtaining blood samples, as discussed in the previous chapter, human blood were compared to that of mice and rabbits. Both mice and rabbits are used frequently in testing of pharmaceutical products, in pre-clinical trial research. Blood can be drawn repeatedly with relative ease from the rabbit, without the need to kill the animals, making long term studies possible. Mice on the other hand, have to be killed to obtain enough blood for performing test, making long term studies impossible.

From the current research, it would seem as if the rabbit model provides a better option to study, particularly coagulation and haemostasis processes, as the fibrin network and the platelet aggregate ultra-structure seems comparable to that of humans. Also, both major and minor fibre thickness are well comparable to that of human tissue.

This chapter concludes by suggesting that the rabbit model complies with the following requirements:

- It can be used in a long-term study
- Blood can be drawn regularly without harming the animals
- Animals are easily handled
- Their coagulation system is comparable with that of humans, making it a suitable model in the study of coagulation metabolomics and the change of different factors due to the intake of aspartame.