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Effects of aspartame on the blood coagulation system of the rabbit

by

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ABSTRACT

Aspartame is a dipeptide sweetener that can be found in most of the sugar-free products available on the market today. The FDA approved the use of aspartame, but ever since the safety of the consumption of aspartame has been questioned. Thus the aim of this thesis was to determine the effects of aspartame ingestion on the blood coagulation system and the blood filtering organs (liver and kidneys) of the rabbit.

The protocol for obtaining blood from a rabbit as well as successful administration of aspartame was perfected. The rabbit was proven as best experimental model, when compared to a mouse, for studying the effects of aspartame on coagulation and haemostasis. The effects of aspartame were determined by: 1.) measuring the factors from the different coagulation pathways, namely the common pathway (factors II, V, X and fibrinogen); factors in the intrinsic pathway (factors VIII, IX), as well as factor VII, found in the extrinsic pathway. The *prothrombin time* (PT; measures how long blood takes to form a clot) and activated *partial thromboplastin time* (aPTT; measures recalcification time of plasma) was also measured; 2.) The ultrastructure of the fibrin networks, platelet morphology and endothelial lining were studied; 3.) The histological morphology of the leukocytes, liver and kidney were examined.

Results obtained indicated that F VII, X and VIII were decreased with a prolonged prothrombin time. The concentration of circulating fibrinogen increased significantly, which corroborated with results obtained for the ultrastructure of the fibrin networks. The degree of fibrin fibre formation increased the higher the concentration of aspartame and the degree of platelet aggregation occurring, decreased with the increase of aspartame concentration. It is hypothesized that the amount of circulating serotonin decreased. The endothelial lining of the rabbits were damaged with the nuclei appearing apoptotic. The endothelial lining and their tight junctions play an integral part in the functioning of the BBB, in synchronization with cAMP (complexity of tight junctions, decreased due to decreased amount of serotonin), thus it appeared as though the BBB was compromised. The morphology of the leukocytes were altered, specifically that of the eosinophils and heterophils. The granules inside the eosinophils of the aspartame treated rabbit appeared to have increased and were more clearly visible, while the granules in the heterophils appeared to have decreased. The total number of leukocytes also decreased. The

normal histological morphology of both the liver and kidney were affected by aspartame. Damage to the hepatocytes and their subsequent arrangement were noted. The visceral layer of the capsule of Bowman appeared thickened and the cuboidal epithelium lining the proximal convoluted tubule was also damaged.

The final judgment and conclusion of the results obtained in this thesis regarding the consumption of abuse doses of aspartame, was that aspartame could lead to bleeding disorders (especially in genetically predisposed individuals), suppressed immunity and a compromised BBB. Trouble can occur with formation of the glomerular filtrate and absorption of fluid from the proximal convoluted tubule, which could result in high blood pressure and an increased probability of dehydration respectively.

DECLARATION

I, Petro Humphries hereby declare that this thesis entitled:

"Effects of aspartame on the coagulation system of the rabbit"

which I herewith submit to the University of Pretoria for the Degree of Philosophiae Doctor in Anatomy, is my own original work and has never been submitted for any academic award to any other tertiary institution for any degree.

_____ _____

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LIST OF PUBLICATIONS

Full articles:

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Articles in preparation:

Humphries P., Smit E., Pretorius E. Ultrastructural changes in the aorta of the rabbit after treatment with aspartame.

Humphries P., Smit E., Pretorius E. Changes in the histological morphology of the liver and kidney of the rabbit after long-term ingestion of abuse doses of aspartame.

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LIST OF DIAGRAMS

LIST OF ABBREVIATIONS AND SYMBOLS

ADP	-	Adenosine diphosphate
α	-	Alpha
α_2-PI	-	α_2-plasmin inhibitor
ANOVA	-	Analysis of variance
APM	-	Aspartame
aPTT	-	Activated partial thromboplastin time
Arg-Gly	-	Arginine-glycine
AT III	-	Antithrombin III
ATP	-	Adenosine triphosphate
β	-	Beta
BBB	-	Blood brain barrier
Ca^{2+}	-	Calcium
$(Ca^{2+})i$	-	Intracellular calcium
$CaCl_2$	-	Calcium chloride
CARR	-	Carrageenan
cAMP	-	Cyclic adenosine 3,5-monophosphate
CNS	-	Central nervous system
CO_2	-	Carbon dioxide
Cl^-	-	Chlorine ion
^{o}	-	Degree
^oC	-	Degrees Celsius

DIC	-	Disturbed intravascular coagulation
DKP	-	Diketopiperazine
DPBS	-	Dulbecco's phosphate buffered saline
F IIa	-	Activated F II
F IXa	-	Activated F IX
F Va	-	Activated F V
F VIIa	-	Activated F VII
F VIIIa	-	Activated F VIII
F Xa	-	Activated F X
F XIIIa	-	Activated F XIII
FDA	-	Food and Drug Administration
FPA	-	Fibrinopeptide A
FPB	-	Fibrinopeptide B
14C	-	14 Carbon
5-FU	-	5-Fluorouracil
g	-	Gram
g/L	-	Gram per litre
H^+	-	Hydrogen
HfX	-	Human factor X
HMWK	-	High molecular weight kininogen
INR	-	International Normalized Ratio
ISI	-	International Sensitivity Index

K^+	-	Potassium
L-aspartyl-L-phenylalanine methyl ester	-	Aspartame
M	-	Molar
mg	-	Milligram
mg/kg	-	Milligram per kilogram
ml	-	Millilitre
mM	-	Millimolar
mmol/L	-	Millimolar per litre
MS	-	Mass spectrometry
µl	-	Microlitres
µm	-	Micrometre
n	-	Number of values used to obtain mean
Na/K	-	Sodium/Potassium
Na^+	-	Sodium
NaOH	-	Sodium hydroxide
NMR	-	Nuclear magnetic resonance
OIT	-	Optimal incubation time
OsO_4	-	Osmium tetraoxide
P	-	Level of significance
P/T-Ph	-	Platelet or tissue phospholipids
PAI-1	-	Plasminogen activator inhibitor 1
PBS	-	Phosphate buffered saline

PK	-	Prekallikrein
PKU	-	Phenylketonuria
PO_4	-	Phosphate buffer
PRP	-	Platelet rich plasma
PT	-	Prothrombin time
PTT	-	Partial prothrombin time
RafX	-	Rabbit factor X
RafXa	-	Activated rabbit factor X
RNA	-	Ribonucleic acid
rpm	-	Resolutions per minute
RuO_4	-	Ruthenium oxide vapour
SEM	-	Scanning electron microscope
TAFI	-	Thrombin-activatible fibrinolysis inhibitor
TEM	-	Transmission electron microscope
TF	-	Tissue factor
TFPI	-	Tissue factor pathway inhibitor
tPA	-	Tissue plasminogen activator
U/ml	-	Units per millilitre
uPA	-	Urokinase plasminogen activator
vWF	-	von Willebrand factor
γ	-	Gamma

Chapter 1 - Introduction

1.1 INTRODUCTION

The artificial dipeptide sweetener aspartame (APM; L-aspartyl-L-phenylalanine methyl ester) is present in many products, especially unsweetened or sugar free products. These products are frequently utilised by people trying to lose weight or patients with diabetes. Concern relating to the possible adverse effects have been raised due to aspartame's metabolic components, which is produced during its breakdown, namely phenylalanine, aspartic acid (aspartate), diketopiperazine (DKP) and methanol (Trocho *et al*., 1998) . This product has great controversy regarding its safety for human consumption including possible neurologic and behavioural side effects. Anecdotal reports on the toxic effects of aspartame are numerous, and various issues continue to be raised today, more than 20 years after aspartame had been approved by the FDA. The acceptable daily intake of aspartame as approved by the World Health Organization is 40mg/kg/day, while the amount approved by the FDA is 50mg/kg body weight per day (Butchko and Kotsonis, 1991). The effects of aspartame have been studied on various species, including humans, rats, mice and rabbits. Results obtained from these studies underline the need for further experimentation with this product, as the results obtained vary from severe adverse effects to none observed. Thus, taking the accepted daily intake of aspartame, as approved by the FDA into account, this study planned to distinguish the effects of aspartame at three doses, 34mg/kg, 100mg/kg and 150mg/kg aspartame, of which the first are accepted to be safe (FDA and World Health Organization) and the last two abuse doses for humans.

It is a well known and definite fact that hemostasis of the blood coagulation system plays an integral part in the normal functioning of any human being. A wide variety of different animal models (mouse, rat, guinea pig, rabbits, pigs and monkeys) have been tested. These animal models have been used to determine whether the factors influencing coagulation are comparable between animals and humans (Pretorius *et al*., 2007). Pretorius *et al*. (2007) determined that the fibrin fibres and platelets of humans and rabbits are comparable, while the same cannot be said for the mouse.

In the past, primate models have been utilized during drug development as models of studying thrombosis, seeing that they are regarded as appropriate predicting parameters of human efficacy and safety (Harker, Hanson and Kelly, 1995). However, due to an increase in costs

and ethical concerns, smaller and more widely available experimental animals have been used for this purpose. 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). 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. The preferred species are now rabbits and rats since statistically significant comparisons between control and drug-treated animals can be done relatively easily (Chi *et al*., 1998).

Over the last few years, research has focused on using animal models in the studying of disease or action of different products on different systems of these models. Recently, a new approach has also been successfully combined with animal models, namely systems biology. According to Verpoorte *et al*. (2005) the following points are important in the systems biology approach:

- By measuring the activity in a living organism (which can be anything from a cell culture, animals to patients) for extracts with different composition, possibly one may identify a compound or a combination of compounds that correlate with the activity;
- Pro-drugs or synergism can be recognized;
- This may also lead to identification of new modes of action, including new targets; may replace the single compound, single target approach which for many years had been the paradigm of drug development.

Systems biology is one of the most important novel scientific concepts in natural sciences (Seger and Sturm, 2007), particularly because of great achievements in methods that allow the researcher to study genomics, RNAomics, and proteomics, successful genome-wide sequencing, achievements in studying comprehensive expression level profiles at the transcript levels and 2D gel and chromatography-based methodologies. These methods allow researchers to identify and quantify expressed proteins, and from this knowledge, a better

understanding of the complexity of the interactions within a living organism emerged. In this systems biology approach it is fundamental that the reductionist approach seen in traditional medicine development is not followed, but rather a holistic *in vivo* approach, of which perhaps the easiest is through animal experiments (Verpoorte *et al*., 2005). Such an *in vivo* approach allows researchers to study, using the above methods, gene expression, transcriptomes, proteomes and metabolomes.

Metabolomics aims at measuring all metabolites in an organism qualitatively and quantitatively; in studies of pathogenesis analyzing materials such as urine and serum, this is referred to as metabolomics (Verpoorte *et al*., 2005). Metabolomics is currently best described as an approach which "seeks to identify and quantify the complete set of metabolites in a cell or tissue type and to do so as quickly as possible and without bias" (Krishnan *et al*., 2005; Seger and Sturm; 2007). Analytical tools within metabolomics including mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy that can profile the impact of time, stress, nutritional status, and environmental perturbation on hundreds of metabolites simultaneously resulting in massive, complex data sets (Dixon *et al*., 2006). In the current protocol, techniques whereby coagulation factors are studied by measuring the change of coagulation factors due to the intake of aspartame can also be categorized as metabolomics.

1.2 PURPOSE OF THE STUDY

In the light of the controversies relating to the effects of the dipeptide aspartame, the purpose of this study was to focus on the effects of aspartame on the blood coagulation system of the New Zealand white rabbit.

Thus, the attention of this study will centre on determining whether the rabbit can be used and implemented successfully as an experimental animal model in obtaining blood samples to determine the effects of aspartame, and if so, how aspartame will influence the ultra-structural morphology of the fibrin networks, platelet aggregates and the endothelial lining. Will the coagulation profile of the rabbit be altered due to treatment with aspartame and can an immune response be activated by change in the morphology and counts of the different leukocytes present in blood? And if all of the above were affected by the intake of aspartame, will it cause changes in the normal histological morphology of the liver and kidney?

1.3 HYPOTHESIS

The hypothesis of this study is therefore that long term exposure to aspartame at different concentrations (34mg/kg, 100mg/kg and 150mg/kg aspartame) will alter the morphology of both the platelets and the fibrin fibres and that the concentration of the different coagulation factors will be changed. It is also thought that the morphology of the endothelial cells lining the blood vessels will be modified and if all of the above mentioned is true, the liver and kidneys which filter and detoxify the blood, will most certainly also be affected.

Thus the aim of this thesis was therefore to answer the following research questions:

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?
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?
3. Is there a difference between the blood clotting time, coagulation profile and different coagulation factors of the control and aspartame treated groups and how does these values compare to that of humans?
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?
5. Has the morphology and number of the leukocytes (light microscopy) changed after treatment with aspartame and how is the endothelial lining (SEM and TEM) of the blood vessels affected when treated with aspartame?
6. How does aspartame affect the normal histological morphology of the liver and kidney?

1.4 ETHICAL CLEARANCE

Table 1.1: Ethical clearance numbers obtained for the different test subjects utilized for this study

Test subject	Ethical clearance nr.	Institution that granted ethical clearance
Humans	151/2006	
Balb-c mice	H 2905	Animal Use and Care Committee of the University of Pretoria
New Zealand white rabbits	H 2205 AEC 4/2006	Animal Use and Care Committee of the University of Pretoria Animal Ethics Committee of the University of Limpopo, Medunsa campus

Two clearances were obtained for the rabbits, as the animals were kept and all experimental procedures performed at the University of Limpopo (Medunsa campus), but the project was formally registered as a PhD at the University of Pretoria.

Chapter 2 - Literature review

LITERATURE REVIEW

The literature describing the effects of aspartame can be divided into two clear groups, those opposed to aspartame consumption and those approving its utilisation.

2.1 Aspartame consumption with adverse effects

Aspartame has been shown to cause negative effects on specific human functions, including brain tumours, memory loss, seizures, headaches (Newman and Lipton, 2001) confusion, personality disorders, visual difficulty, and dizziness are some of the negative effects that have been reported (Tollefson and Barnard, 2001). Aspartame consumption as part of a number of chronic studies has also been implicated in learning or memory. Potts *et al*. in 1980 showed that learning behaviour in male rats changed after administration of aspartame as 9% of their diet for 13 weeks. Dow-Edwards *et al*. in 1989 treated pregnant guinea pigs throughout gestation with a much lower concentration of aspartame, which resulted in pups with a disruption of odour-associative learning. Lewis, Lyon and Elliot (1985) performed a study to determine what the outcome will be in the pregnancy of rats with mild hyperphenylalaninaninaemia and hypertyrosinaemia and what the implications would be for the management of human maternal phenylketonuria (PKU). In an attempt to determine the effect of mildly elevated maternal phenylalanine blood levels on the developing foetal rat brain, a dietary supplement of phenylalanine was given, under taste cover of aspartame. Phenylalanine and tyrosine levels were mildly elevated throughout pregnancy without evidence of malnutrition. Mild hyperphenylalaninaninaemia with concurrent hypertyrosinaemia induced in rats prior conception resulted in microcephaly and lasting behavioural problems in the offspring, specifically hyperactivity and learning difficulties. Dams fed tyrosine to produce tyrosine levels equivalent to the phenylalanine-fed animals showed only learning difficulties among the offspring. Alpha-Methyl phenylalanine, phenylalanine derivative hydroxylase inhibitor, fed in conjunction with phenylalanine, at the level relevant to these experiments, resulted in raised tyrosine levels and did not provide a better method for determining whether mildly elevated maternal phenylalanine levels alone, or phenylalanine and tyrosine in combination, cause the abnormality found in the offspring of phenylalanine-supplemented dams. Therapeutic addition of tyrosine to diets of mother with even mild hyperphenylalaninaninaemia should be approached with caution as mild co-elevation of phenylalanine and tyrosine in the foetus may be harmful. In the face of such a

possible therapeutic dilemma alternatives, such as dietary additions or other essential amino acids to limit foetal brain damage, need to be explored.

Aspartame consumption has led to various reported neurochemical effects (Coulombe and Sharma, 1986; Goerrs, Wagner and Hill, 2000; Pan-Hou *et al*., 1990). Rats treated with aspartame for 14 weeks showed a decrease in the concentration of neuropeptide Y in their arcuate nuclei (Beck *et al*., 2002). After aspartame consumption certain brain amino acid levels were reported to have increased (Dailey *et al*., 1991; Diomede *et al*., 1991; Yokogoshi *et al*., 1984). Neurochemical changes were observed after the simultaneous consumption of high-dose aspartame and dietary carbohydrates (Wurtman, 1983). These studies thus generally indicate aspartame has a negative influence on brain neurotransmitters and receptors, becoming more conspicuous with long-term consumption.

Muscarinic cholinergic receptors have been implicated in numerous studies on learning and memory (Bartus *et al*., 1982; Granon *et al*., 1995; Kadar *et al*., 1990; Mezey *et al*., 1999; Rose, Gibbs and Hambley, 1980; Russell, 1996; Uchida *et al*., 1991; van der Zee and Luiten, 1999; Vogt *et al*., 1991). Elevated muscarinic binding has been shown in the anterodorsal nucleus of the rabbit, during early stages of the learning process, which was maintained all through succeeding training (Vogt *et al*., 1991). A correlation exists between the density of muscarinic receptors in the CNS and cognitive performance in ageing Wistar rats (Kadar *et al*., 1990). Age-related memory deficits in laboratory animals have been associated with two or more muscarinic receptors states (Lippa *et al*., 1985). Results have also shown that there were alterations in the muscarinic receptor binding in the forebrain and midbrain regions of chicks during passive avoidance learning (Longstaff and Rose, 1981). Muscarinic systems may play an integral part in the general working memory process, while nicotinic transmission may be important in delayed response tasks (Granon *et al*., 1995). The studies lead Christian *et al*. in 2004 to hypothesize that an alteration might be eminent in brain muscarinic cholinergic receptor densities if memory impairment were seen with chronic aspartame consumption in the rat.

The Na^+, K^+-ATPase enzyme is crucial for maintaining ionic gradients in neurons and tissues and ions are also involved in memory formation (Conrad and Roy, 1993; Ng *et al*., 1992). Studies performed by Christian *et al*. in 2004 indicated that chronic aspartame consumption

extended the time period it took rats to find the reward in a T-maze and that muscarinic receptor numbers increased in specific brain areas. The inability to locate the reward was seen only after 90 days of aspartame consumption, and their ability to locate the reward only decreased with longer exposure periods of up to 120-days. At this final endpoint, in addition to having problems finding the reward, two of the treated rats ended in the wrong side of the T-maze, totally forgetting where the reward was. Thus, the aspartame treated animals had a decreased retention of the learned behaviour when compared to the control rats. A decrease in smell to locate the reward or a decrease in the desire to obtain the chocolate reward could count as possible explanations for the above-mentioned results; however, once the reward was located by the aspartame-treated rats, they devoured it immediately. Learning was not affected by aspartame consumption early in the experiment when the animals were being trained in the maze (Christian *et al*., 2004).

Christian *et al*. (2004) also found that aspartame-treated rats learned at the same rate as the control rats. The second major finding of the study done by Christian *et al*. (2004) demonstrated that muscarinic receptor densities were higher in numerous brain areas after four months of aspartame treatment. If the increases were related to decreased memory retention, then their data are contradictory to the results of others. These studies showed a correlation between impaired memory and muscarinic blockers or a decreased number of brain muscarinic receptors (Granon *et al*., 1995; Uchida *et al*., 1991; Okuma *et al*., 2000; Power, Roozendaal and McGaugh, 2000). A direct correlation exists between learning and memory together with an increase in cholinergic receptor binding. The results from the study by Christian *et al*. (2004) indicates that chronic consumption of aspartame may be partially responsible for memory-retention problems associated with increases in muscarinic receptors. The only area of the brain where Christian *et al*. (2004) found altered Na^+, K^+-ATPase activity was in the midbrain. This may be an effect unrelated to memory retention, but may be specific for chronic aspartame consumption. Christian *et al*. (2004) thus supports the idea that an increase in the density of brain muscarinic receptors could lead to the inability to remember where the reward is in the T-maze. Thus other receptors, enzymes or transmitters could also be altered with long-term aspartame treatment and so contribute to a decreased maze performance.

Conflicting data exist concerning aspartame's effect on various receptors and transmitters. Pan-Hou *et al*. in 1990 demonstrated significant changes in affinity of L-[^{3}H]glutamate binding due to aspartame consumption, whereas Reilly *et al*. in 1989 found that after 30 days of aspartame treatment no changes were visible in receptor binding for six different amine neurotransmitter receptors. Various neurochemical alterations have also been reported due to aspartame consumption (Goerrs, Wagner and Hill, 2000; Beck *et al*., 2002; Beck *et al*., 2002; Fernstrom, Fernstrom and Grubb, 1986; Melchior *et al*., 1991). Thus, the possibility exists that other receptors or transmitters can be altered by chronic aspartame treatment in addition to the increased density of muscarinic receptors that Christian *et al*. (2004) has shown if the above-mentioned data are taken collectively.

2.2 Aspartame consumption with no negative effects

An increased number of reports concerning adverse reactions related to aspartame were received by the FDA shortly after aspartame was marketed (Garriga and Metcalfe, 1988). However conclusive evidence was not found to link any adverse reactions to the consumption of aspartame (Council of Scientific affairs, 1985; Butchko and Stargel, 2001; Butchko *et al*., 2002; Stegink, 1987; Stegink, Filer and Baker, 1981; Yost, 1989). Moser, in 1994 conducted numerous short-term studies and none of these studies suggest any relationship between aspartame consumption and memory loss. Very few long-term studies have been done. One long-term study, done by Ishii in 1981, determined the incidence of brain tumours in rats fed aspartame. The brain tumourigenicity of aspartame (APM) and of its metabolite diketopiperazine (DKP) was studied in 860 Wistar rats. Aspartame at dietary levels of 1g/kg, 2g/kg, 4g/kg or aspartame in combination with diketopiperazine (3:1) 4g/kg were fed for 104 weeks. One atypical astrocytoma was found in a control rat and two astrocytomas, two oligodendrogliomas and one ependymoma were scattered among the four test groups. There was no significant difference in the incidence of brain tumours between control and test groups. It was concluded that neither aspartame nor diketopiperazine caused brain tumours in rats.

Upon ingestion of aspartame, this artificial sweetener produces methanol as a metabolite. In order to examine the possibility of aspartame toxicity, the effects of methanol and its metabolites (formaldehyde and formate) on dissociated rat thymocytes were studied by flow cytometry (Oyama *et al*., 2002). Oyama and co-workers found that methanol and formate did not affect cell viability in the physiological pH range, formaldehyde at 1-3 mmol/L started to induce cell

death. Further increase in formaldehyde concentration produced a dose-dependant decrease in cell viability. Formaldehyde at 1 mmol/L or more greatly reduced cellular content of glutathione, possibly increasing cell vulnerability to oxidative stress. Furthermore, formaldehyde at 3 mmol/L or more significantly increased intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$) in a dose-dependant manner. It was suggested that aspartame at abuse doses is harmless to humans.

Short-term studies are characterised by administering a single large dose of aspartame or treatment with aspartame for a short period (a few days or weeks). The outcome is then assessed to determine the effect aspartame has on learning or memory. Studies, whether done on humans or animals, have shown no adverse effects of aspartame on memory (Lapierre *et al*., 1990; Mullenix *et al*., 1991; Saravis *et al*., 1990; Shaywitz *et al*., 1994; Spiers *et al*., 1998; Stokes *et al.*, 1994; Tilson, Hong and Sobotka, 1991; Wolraich *et al*., 1994). In one of the long-term studies, Holder in 1989 demonstrated that a treatment period of fifty days with NutraSweet had no effect on reflex or spatial memory development. Leon *et al*. in 1989 demonstrated that a treatment period of twenty-four weeks showed no persistent changes in vital signs, body weight or standard laboratory tests, but extensive memory testing was never performed.

The effects of excessive doses of phenylalanine on seizure susceptibility were examined in animal models in the past, primarily because of their relevance to phenylketonuria (PKU). It was thought that such effects might involve brain monoaminergic mechanisms. Recently, this issue has been pursued with a renewed interest but for a different reason. The dipeptide sweetener, aspartame, contains a phenylalanine residue. Over a long period, a number of studies involving as many as nine animal models of seizures have re-examined the effects of phenylalanine and aspartame on seizure thresholds. Data from these studies are in general agreement that aspartame at doses below 1,000mg/kg, or phenylalanine at equimolar doses, is without an effect on seizure susceptibility in animals. When the dosage level of aspartame reached 1,000mg/kg, the findings between various laboratories and from different animal models of seizures were inconsistent, showing either no effect or a proconvulsant effect. The Acceptable Daily Intake of aspartame in humans set by the Food and Drug Administration is 50mg/kg/day (Sze, 1989). A study performed by Tilson *et al*. in 1989 determined that oral administration of aspartame was not a proconvulsant in rats. The researches also found that prior exposure to aspartame had no significant effect on the rate of kindling at ninety days of age. A single dose

of 1,000 mg/kg aspartame had no effect on the number of animals developing tonic seizures after electroconvulsive shock, nor did aspartame affect the frequency or duration of seizure activity after treatment with pentylenetetrazol.

Fifty-eight Sprague-Dawley rats were treated with aspartame (25, 50, 100mg/kg) or saline for six days. An additional group of animals received daily injections of saline and on the sixth treatment day received a 150mg/kg dose of aspirin 30 minutes prior to behavioural testing. On day six, animals received in intra-articular injection of 2% lambda carrageenan (CARR) or an equal volume of saline and were tested four hours later on threshold to mechanical and thermal stimuli, open field activity and knee joint diameter. Aspirin-treated arthritic animals exhibited significantly less mechanical hyperalgesia and knee joint inflammation compared with vehicle treated arthritic animals. However, aspirin did not reverse thermal hyperalgesia or increase motor activity to control levels. Aspartame did not reduce inflammation, increase motor activity, or attenuate thermal allodynia, but at 50mg/kg did attenuate mechanical allodynia compared with vehicle treated arthritic animals. The anti-hyperalgesic effects on mechanical hyperalgesia were not seen at 25mg/kg or 100mg/kg aspartame. These results suggest that a certain amount of aspartame may provide relief of arthritic pain to a similar degree as aspirin in some individuals. The specific effect of aspartame and aspirin on mechanical hyperalgesia should however be considered when these agents are used for the therapeutic treatment of arthritic conditions (LaBuda and Fuchs, 2001).

The effects of aspartame on plasma glucose and insulin levels were investigated in diabetic rats and patients with non-insulin-dependant diabetes mellitus. The oral administration of 0.45mg aspartame per 100g body weight, which is equivalent to 150mg of glucose in sweetness, to streptozotocin-induced diabetic rats had no effect on the plasma glucose or insulin levels. Also, 225mg oral aspartame loading, which is equivalent to 75g of glucose in sweetness, to patients, with non-insulin-dependant diabetes mellitus did not increase plasma glucose or insulin levels, although 75g of oral glucose loading increased plasma glucose and insulin levels in diabetic patients as expected. Aspartame ingestion for three days at a dose of 24-48 mg per day and the intake of snacks flavoured with 240mg of aspartame also did not increase fasting plasma glucose levels. These results suggested that acute administration of aspartame has no influence on plasma glucose or insulin levels in diabetic rats or patients with non-insulin-dependant diabetes mellitus (Yoshida *et al*., 1985).

Aspartame was also investigated in several pharmacological tests to delineate any effects on the gastrointestinal system (Bianchi *et al*., 1980). Bianchi and co-workers determined that the compound did not affect food consumption at one hour following a single intragastric dose of 200mg/kg in rats. There was no evidence of inhibition or stimulation of the gastric juice secretion rate, the concentration of gastric acid, acid output or proteolytic activity following an intragastric dose of 250mg/kg in five-hour pylorus-ligated rats. Likewise, aspartame at the same dosage did not significantly affect gastric ulceration induced by nineteen hours of pylorus-ligation. In several *in vitro* tests it was demonstrated that aspartame did not affect the proteolytic activity of pepsin or the lipolytic activity of pancreatic lipase at concentrations of 143 µg and 1.25mg/ml, respectively. Its anticholinergic activity was found to be insignificant, less than 0.001 times the potency of atropine sulphate, when measured against acetylcholine-induced concentration of isolated rabbit ileum. These data indicate that aspartame may be devoid of undesirable side effects on the gastrointestinal tract when used as a food sweetening agent.

Saunders, Pautsch and Nutting (1980) ran a series of studies with aspartame on mice, rats and rabbits using standard procedures to characterize possible estrogenic, androgenic, progestational and glucocorticoid activities. Aspartame was administered orally at levels (300mg/kg/day) substantially in excess of expected maximal human intake when used as a sweetening agent. No significant hormone-mimetic response was observed in the endocrine target organs evaluated. Thus it was concluded that ingestion of aspartame should not produce any estrogenic, androgenic, progestational or glucocorticoid-like effects. Further that it should not alter the actions of the endogenous steroid hormones.

The study done by Scheffler and Berliner (2004) on the effects of aspartame and its derivatives on the thrombin catalytic activity, dismiss any concerns about the consumption of aspartame and homeostasis. They showed that the levels of aspartame required for inhibition of α-thrombin far exceeds that ingested, and thus have little physiological consequence, even in overdose amounts. Aspartame, when entering the blood, is hydrolysed by esterases (Stegink *et al*., 1983; Filer, Baker and Stegink, 1983). Additionally, aspartame's inhibition of fibrinogen clotting, which has an I50 of 9 mM would be much stronger than that of free aspartate.

Aspartame is the chemical chosen in this study for the determination of adverse effects on the blood coagulation system of the New Zealand white rabbit.

In the light of the aforementioned discussion a more detailed literature review focusing on the blood coagulation system and tests involved in determining homeostasis was considered in the following section.

2.3 Blood coagulation system

The co-ordination of fibrin formation and fibrinolysis are now well defined. Research of the structure and function of all major fibrinolytic proteins (including serine proteases, their inhibitors, activators and receptors) have been characterised.

2.3.1 Basic concepts of fibrinolysis

Coagulation and fibrinolysis are precisely regulated by the measured participation of substrates, activators, inhibitors, cofactors and receptors (Esmom *et al*., 1999; Degen, 2001; Hajjar, 2003a; Kolev and Machovich, 2003) under certain physiological conditions. Thrombin is generated by the activation of coagulation, resulting in thrombus formation, when fibrinogen is converted to fibrin after platelet activation. Plasmin is a major fibrinolytic protease. Tissue plasminogen activator (tPA) as well as urokinase plasminogen activator (uPA) can convert plasminogen, a circulating plasma zymogen, to plasmin. Plasmin cleaves both tPA and uPA through a positive feedback mechanism, resulting in the transformation of a single chain to a more active two-chain polypeptide. Degradation of fibrin, the major plasmin substrate, is regulated by the binding of both plasminogen and tPA on its surface, thus localising and amplifying plasmin generation. Plasminogen is activated by tPA in the presence of fibrin by at least two orders of magnitude. When fibrin is absent, tPA is a weak activator of plasminogen activity. The presence of fibrin significantly increases the affinity between tPA and plasminogen, and the affinity is therefore low in the absence of fibrin. Soluble degradation products are generated, where after plasmin cleaves fibrin, exposing carboxy-terminal lysine residues (Cesarman-Maus and Hajjar, 2005).

Agents regulating tPA gene expression independently of plasminogen activator inhibitor-1 (PAI-1) include histamine, butyrate, retinoids, arterial levels of shear stress and dexamethasone. Intracellular cyclic adenosine 3,5-monophosphate (cAMP) levels which are increased by

forskolin, has been shown to diminish synthesis of both tPA and PAI-1 (Hajjar, 2003b). Endothelial cells synthesise and primarily secrete tPA. A variety of stimuli, such as thrombin, histamine, bradykinin, adrenaline, acetylcholine, arginine, vasopressin, gonadotrophins, exercise, venous occlusion and shear stress mediate the release of tPA. tPA has an exceptionally short half-life of 5 min. The major intravascular activator of plasminogen seems to be tPA, although it is only expressed by extra-vascular cells (Hajjar, 2003a). tPA is an effective plasminogen activator in both the presence and absence of fibrin and has a higher affinity for fibrin than uPA (Gurewich *et al*., 1984; Lijnen *et al*., 1986). tPA appears to mediate excitatory neuronal cell death and may play a role in neuronal plasticity and protection against demyelination following nerve crush injury (Strickland, 2000).

Leukocyte recruitment to sites of inflammation is fundamental to plasminogen activity. But plasminogen may also be co-opted by bacteria from a host organism during the invasive phase of infection (Plow and Hoover-Plow, 2004). Vascular remodelling and angiogenesis are controlled by the crucial presence of plasmin and its parent molecules (Lijnen, 2001; Pepper, 2001), while the model system employed will determine the impact of PAI-1 on experimental intimal hyperplasia (Fay, 2004). Plasminogen is not strictly required for normal development, but is essential for maintenance of postnatal fibrin homeostasis in both intra- and extra-vascular settings (Cesarman-Maus and Hajjar, 2005).

Lysine-binding sites are found on ‘Kringles’ 2 of tPA and 1 and 4 of plasminogen (homologous triple loop structures). These lysine-binding sites lead to enhanced plasmin generation and fibrin removal after further mediated binding to fibrin. Lysine analogues, such as epsilon aminocaproic acid and tranexamic acid, as well as the recently characterised, thrombin-activatible fibrinolysis inhibitor (TAFI) can block the binding process. TAFI removes carboxy-terminal lysine residues when activated by thrombin and so attenuates plasmin generation, stabilisation of fibrin thrombi, and establishment of a regulatory connection between coagulation and fibrinolysis. Inhibitors of plasminogen activation, such as plasminogen activator inhibitor-1 (PAI-1), and by inhibitors of plasmin itself, such as α_2-plasmin inhibitor (α_2-PI) also regulate fibrin dissolution. Occupancy of the lysine-binding sites protects plasmin bound to fibrin from α_2-PI (Cesarman-Maus and Hajjar, 2005). Deleting plasmin-binding lysine residues on fibrin decreases this protection of TAFI. Platelets contain TAFI, which is expressed by the liver

(Nesheim, 2003). The occurrence of thrombomodulin accelerates the activation of thrombins by about 1250-fold. High concentrations of plasmin can also lead to the activation of TAFI. Plasminogen and tPA are bound to fibrin at sites containing arginine and lysine and on annexin 2 (Redlitz *et al*., 1995), while activated TAFI functions as a potent attenuator of fibrinolysis (Nesheim, 2003).

Plasmin generation is promoted through the expression of cell surface receptors on diverse cell types (Hajjar, 2003b). Plasminogen, as well as tPA and/or uPA are bounded by endothelial cells, monocytes, macrophages, neutrophils and some tumour cells. Cell surface fibrinolytic activity is localised by their receptors, which serve as co-factors in acute or ongoing plasmin generation and provide specialised environments. These environments are protected from circulating inhibitors (Cesarman-Maus and Hajjar, 2005). Monocytoid cells, platelets, renal epithelial cells, neuroblastoma cells, leukocytes and endothelial cells contain plasminogen receptor proteins (Hajjar, 1995). The kringle structures of plasminogen, through their carboxyl-terminal lysine residues, commonly interact with these binding proteins (Miles and Plow, 1991). Distinct annexin 2 domains bind both plasminogen and tPA (Cesarman-Maus and Hajjar, 2005). Under certain circumstances proteases have been revealed to activate plasminogen directly. These proteases are traditionally classified within the intrinsic part of the coagulation pathway. These factors include kallikrein, factor Xia and factor XIIa (Colman , 1986; Goldsmith, Saito and Ratnoff, 1978), accounting for less than 15% of total plasmin-generating activity in plasma (Hajjar, 2003b). The focal contacts and the leading edge of migrating cells contain uPA receptors co-localised with integrins (Xue *et al*., 1994). Caveolin, a key component of caveolae (abundant structures in endothelial cells) is associated with uPA and is contemplated to partake in signalling events (Anderson, 1993; Stahl and Mueller, 1995; Okamoto *et al*., 1998). Thus, cellular adhesion is integrated with proteolysis via action of the uPA receptor (Bugge *et al*., 1995b).

A family of serine protease inhibitors known as serpins (Travis and Salvesan, 1983) negatively modulates the action of plasmin. After the proteolytic cleavage of the inhibitor by the target protease, an irreversible complex is formed between the serpins and the active site serine of the target proteases. Both the protease and inhibitor lose activity. Endothelial cells, monocytes, macrophages, hepatocytes, adipocytes and platelets (Ny *et al*., 1986; Samad, Yamamoto and

Loskutoff, 1996), release PAI-1. Many cytokines, growth factors and lipoproteins common to the global inflammatory response (Hajjar, 2003b) stimulate release of PAI-1. PAI-2 is secreted by leucocytes and fibrosarcoma cells. Significant levels of PAI-2 are found in human plasma during pregnancy (Cesarman-Maus and Hajjar, 2005).

Plasmin has been indicated as playing a large role in tissue remodelling, arthritis and toxic neuronal death by a great number of *in vitro* studies (Hajjar, 2003b). Plasmin is degraded *in vitro* by a number of basement membrane proteins: thrombospondin, laminin, fibronectin and fibrinogen. This suggests possible roles of above mentioned in inflammation, tumour cell invasion, embryogenesis, ovulation, neurodevelopment and prohormone activation *in vivo*. Coagulation factors V and IX can be activated and inactivated by plasmin (Cesarman-Maus and Hajjar, 2005).

The structurally diverse cell surface fibrinolytic receptors responsible for the homeostatic control of plasmin activity can be classified into two groups (Hajjar, 1995). Plasminogen activation are localised and potentiated by 'activation' receptors, while plasmin and plasminogen activators are eliminated by 'clearance' receptors from the blood or focal microenvironments.

2.3.2 Thrombus formation

Platelet activation and thrombus formation is dependant on the plasma serine protease, thrombin. The prothrombinase complex is the sole site of thrombin formation in the vasculature. It comprises the factor Xa (fXa) assembled with cofactor Va in the presence of calcium (Ca^{2+}) on the surface of activated platelets (Mann *et al*., 1990). Thrombin is highly specific in its interaction with fibrinogen, its primary polypeptide substrate. This site of action has only two arginine-glycine (Arg-Gly) linkages that are sensitive to thrombin's action. Strong evidence exists that phenylalanine is present at position P_9 and aspartate at position P_{10} of the Aα-chain of human fibrinogen. This plays an important role in the conversion of fibrinogen to fibrin by the catalyst α-thrombin (van Nispen, Hageman and Scheraga, 1977; Meinwald *et al*., 1980; Marsh *et al*., 1982; Marsh *et al*., 1983).

2.3.3 Intrinsic and extrinsic coagulation pathways

Various tests have been developed to assay the entirety of the coagulation process. Factors from the intrinsic pathway this project will focus on is factor 5 (V, Proaccelerin), factor 8 (VIII, Antihemofillic factor), factor 9 (IX, Christmas factor) and from the extrinsic pathway factor 7 (VII, Proconvertin). Fibrinogen will also be tested but this factor forms part of both the intrinsic and extrinsic pathways (NovoSeven® US Hematologic testing).

2.3.4 Clotting time

Clotting time is used for measuring the activity of the intrinsic pathway. 2 types are distinguished, which are the whole blood clotting time and the more sensitive plasma clotting time. More standardized test, the partial prothrombin time (PTT) and activated partial thromboplastin time (aPTT), where artificial tissue lipoproteins initiate the clotting process after addition to the plasma. Langdell and colleagues in 1953 devised the PTT test, which is a kinetic test measuring the intrinsic coagulation activity of plasma. The test utilizes a partial thromboplastin such as cephallin or soya phosphatide. The clotting mechanism is activated and so reduces/eliminates inaccuracies caused by natural variations in platelet number. Plasma is prepared by centrifugation from whole blood collected into trisodium citrate. The clotting time is determined at 37^{o}C after addition of the partial thromboplastin and calcium chloride. Although the PTT test eliminates inaccuracies caused by differences in platelet number, it is unable to distinguish between differences in activation occurring when blood is in contact with different surfaces for different periods of time (NovoSeven® US Hematologic testing).

The activated partial thromboplastin test (aPTT) overcomes this limitation, and it now mainly replaces the PTT in clinical diagnoses (Owen, 1990; Bowie and Owen; 1996). Proctor and Rapaport in 1961 developed the modified PTT-test, dispensing with the variable of contact activation by activating the plasma to a maximum level before clotting could occur. Thus, this test is a kinetic test, measuring the recalcification time of plasma. This test screen for deficiencies in coagulation factors involved in the intrinsic pathway (factor VII and factor VIII excluded), and to determine the presence of a non-specific inhibitor, such as lupus-like inhibitor. The aPTT is also utilised to evaluate the effect of therapy and to monitor and regulate heparin therapy. In this test, laolin (powdered glass, Celite or ellagic acid) is added to the plasma for approximately 3 minutes (depending on the activator) at 37^{o}C to activate it, after which partial thromboplastin (cephalin or soya phosphatide) and calcium chloride are added to introduce

clotting. Spectrophotometry or electromechanical methods measure the time to fibrin clot formation. Results are expresses in absolute time – in normal blood of humans, the aPTT is approximately 35-42 seconds, but this reference range is very dependant on the reagent and the method used. A deficiency in one of the clotting factors involved in the intrinsic pathway, especially factors XII, XI, X, IX, VIII, V, II, and I can lead to a prolonged aPTT. Prolonged aPTT is observed after a massive blood transfusion, heparin therapy, hemophilia A, acquired FVIII inhibitor, Lupus anticoagulant, over-anticoagulation with coumarins, or an error in specimen collection. More specific single factor assays and mixing studies should be performed when a prolonged aPTT is observed, as to identify its exact cause and decide on a possible course of action for treatment (NovoSeven® US Hematologic testing).

2.3.5 Prothrombin time (PT)

This test is utilized for determining the integrity of the extrinsic coagulation pathway. This test determines the amount of thrombin that develops after anti-thrombin activity has been decreased by dilution of the plasma and was developed by Quick *et al*. in 1935. Blood that is being tested is diluted with 3.13 or 3.2% trisodium citrate anticoagulant in a ratio of 1:9, respectively. A plasma rich sample containing few platelets is then obtained from centrifuging the mixture. Coagulation is initiated by addition of complete thromboplastin reagent (recombinant tissue factor now available) and calcium chloride. A photo-optical or an electrochemical device is utilized to determine the time to fibrin strand formation. Results are usually expressed as an absolute time compared to the control plasma or as a prothrombin index (control plasma time / patient plasma time) or as the International Normalized Ratio (INR). The INR is calculated from the patient's prothrombin time divided by a laboratory control prothrombin time and the ISI (International Sensitivity Index, which controls for differences between different thromboplastins). A prolonged PT may indicate a deficiency of or the presence of inhibitors of FI (fibrinogen), factor II (FII), FV, FVII or FX. PT is most sensitive to decreases in FVII, which may be caused by coumarin anticoagulant therapy, vitamin K deficiency, severe liver disease, massive blood transfusions, disseminated intravascular coagulation, the earlier administration of high-dose heparin, as well as congenital deficiency in factor VII (NovoSeven® US Hematologic testing).

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

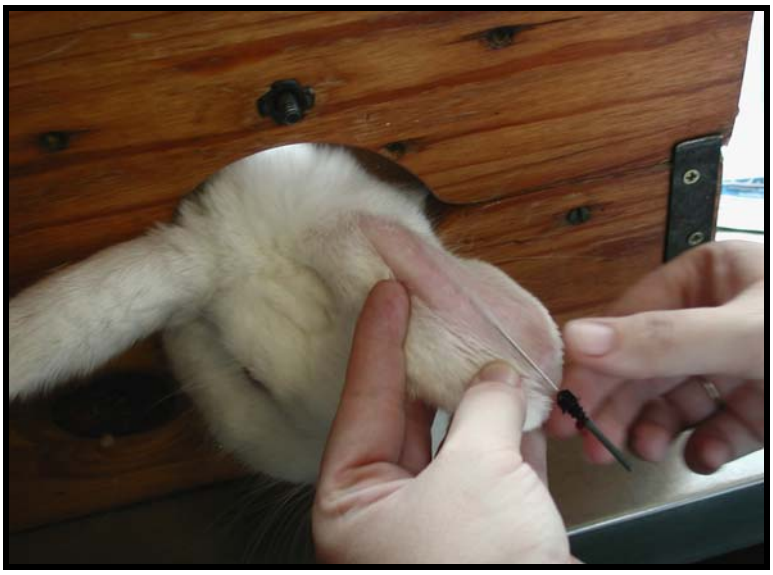


Figure 3.2: Collecting the blood sample into prepared vacutainer tubes by utilizing vacutainer needles

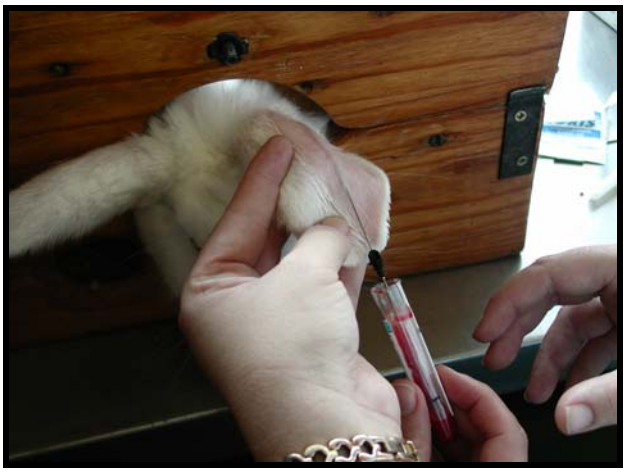


Figure 3.3: Weighing of rabbits for calculation of aspartame concentrations

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

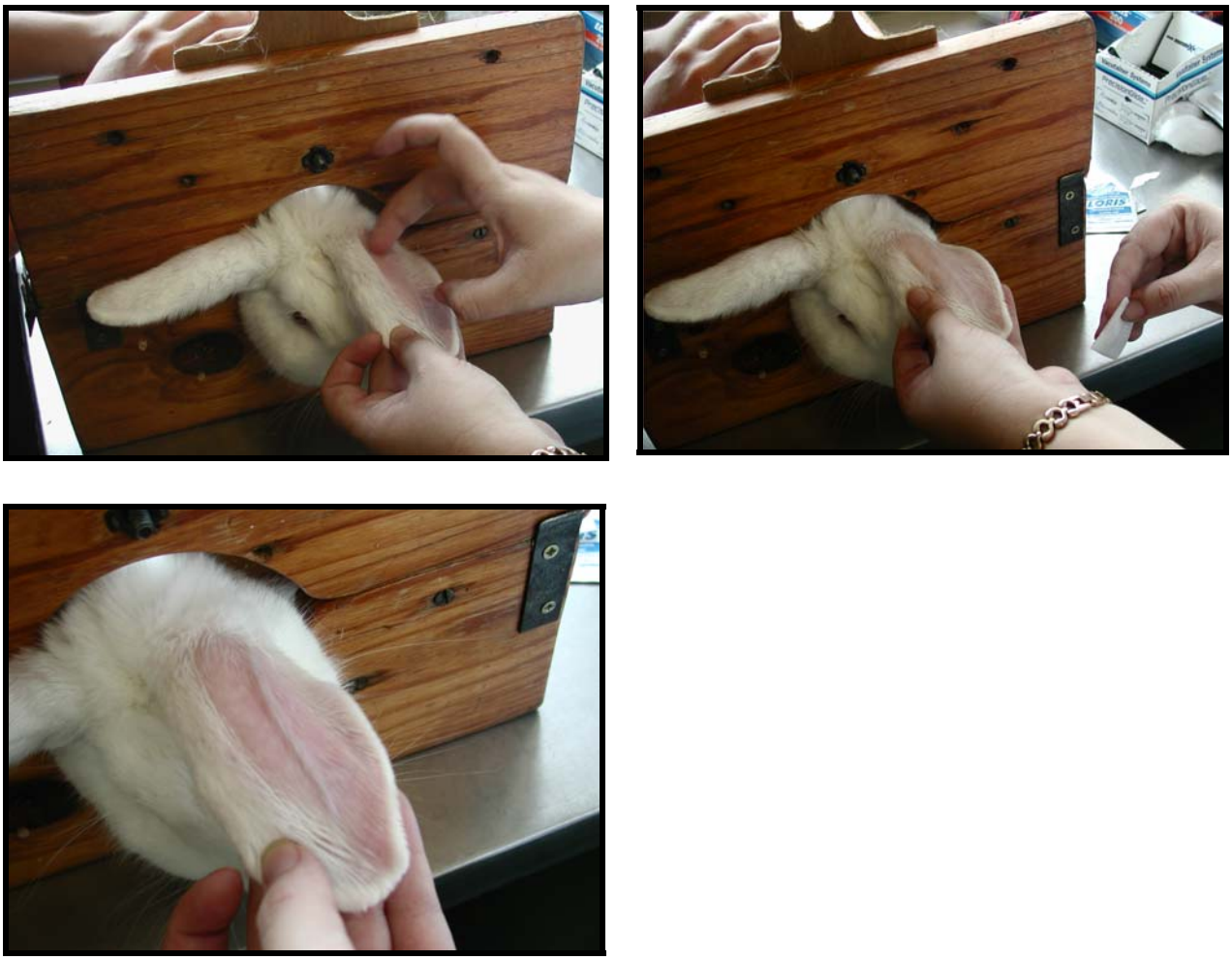


Figure 3.5: Rabbit inside modified constraint before blood is drawn

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

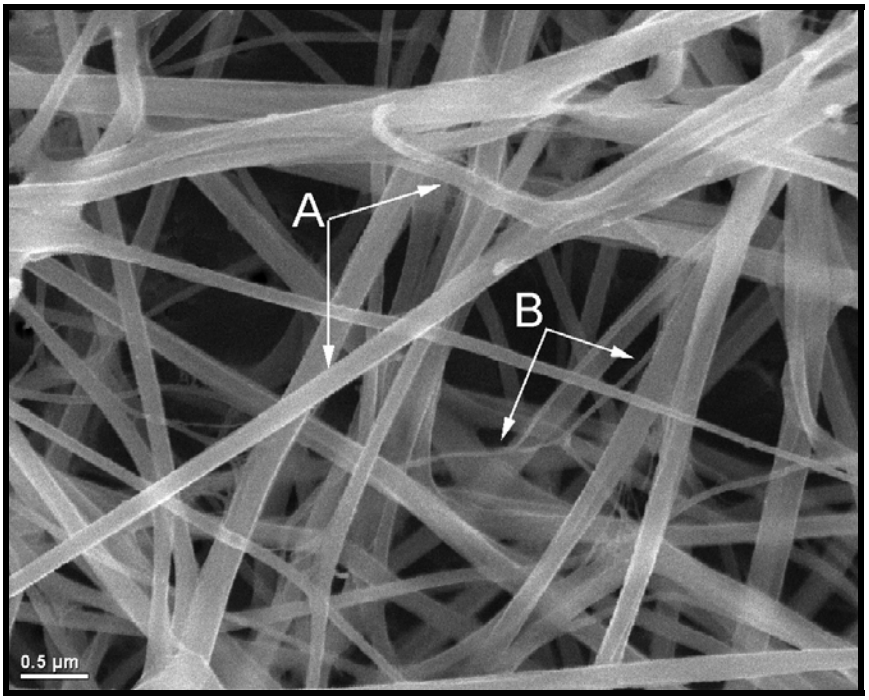


Figure 4.1b: Human platelet aggregate showing globular, pseudopodia-like morphology

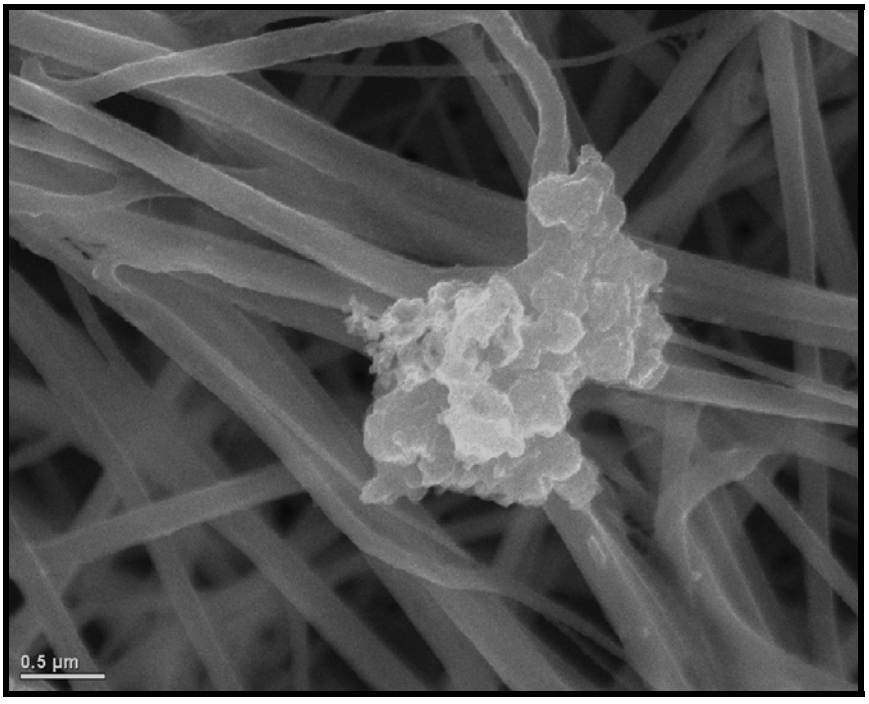


Figure 4.2a and 4.2b show the fibrin network and clot of rabbit blood.

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

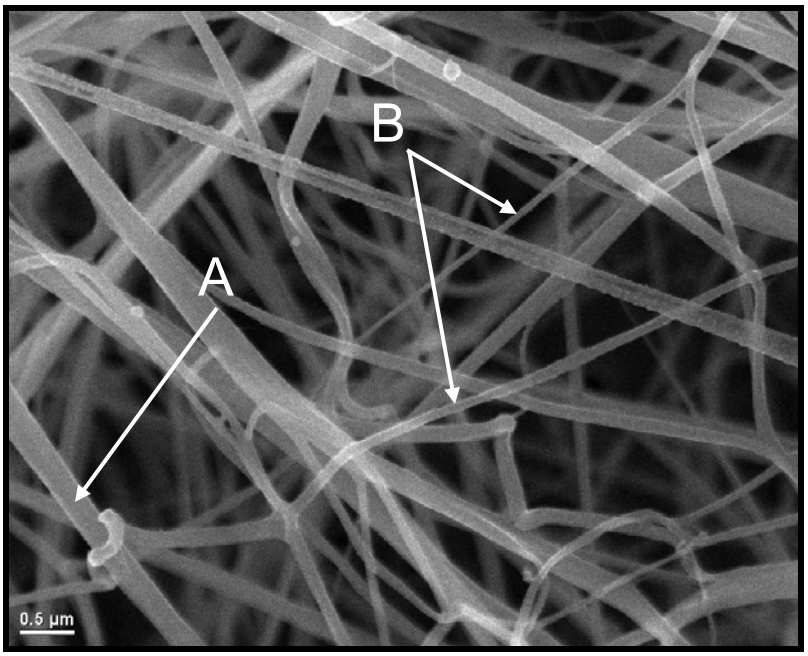


Figure 4.2b: Rabbit platelet aggregate showing globular, pseudopodia-like morphology

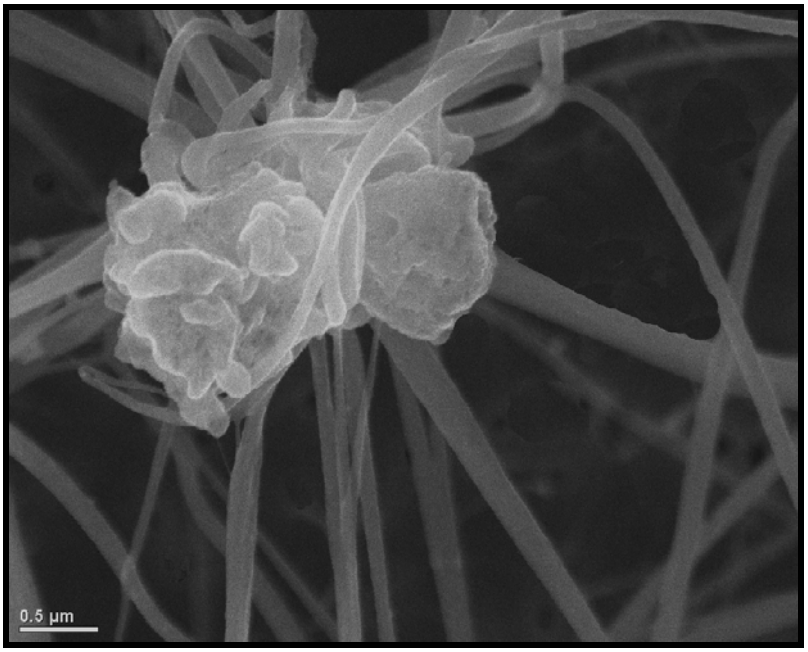


Figure 4.3a and 4.3b show the fibrin network and platelet aggregate of the mouse.

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

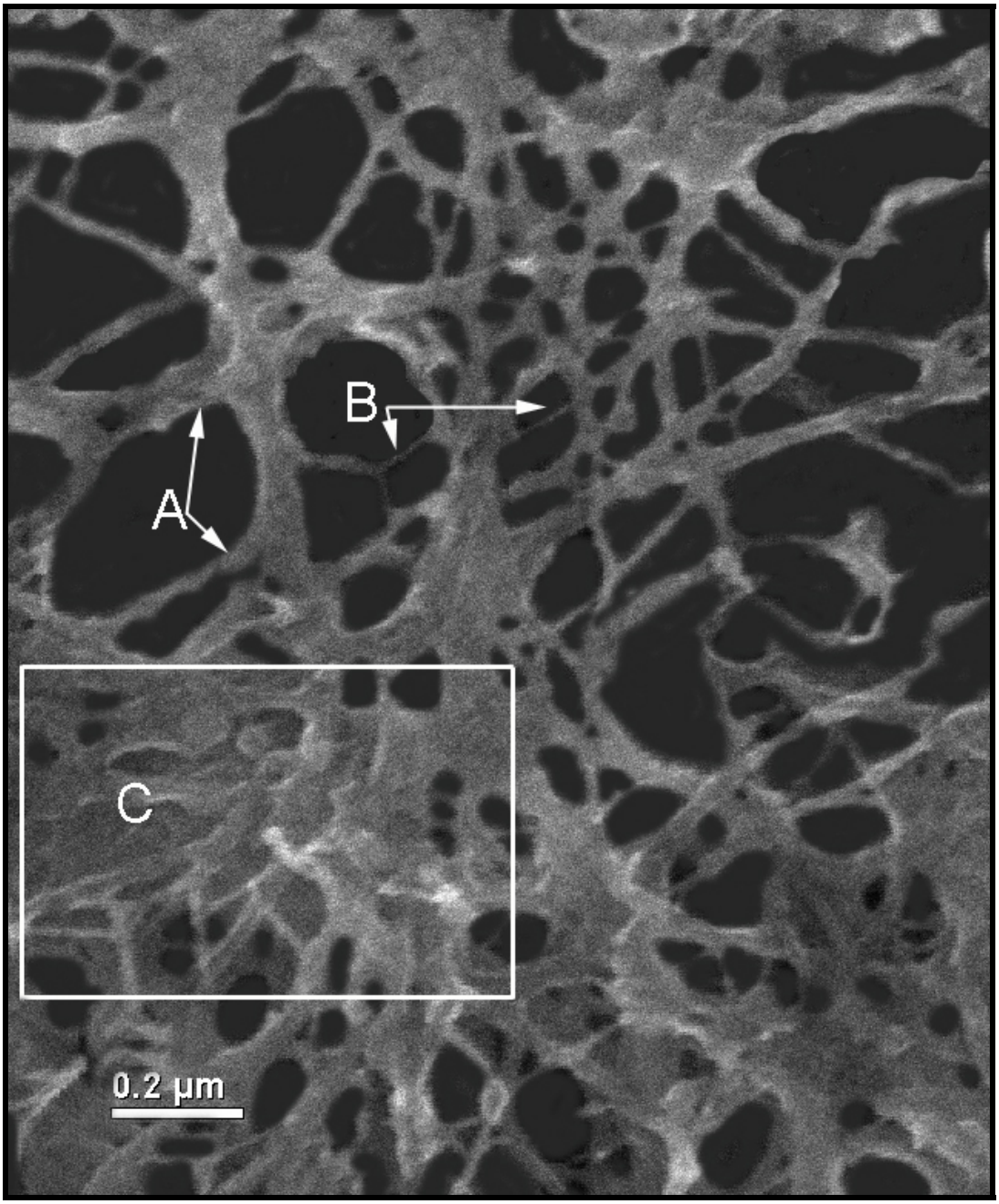
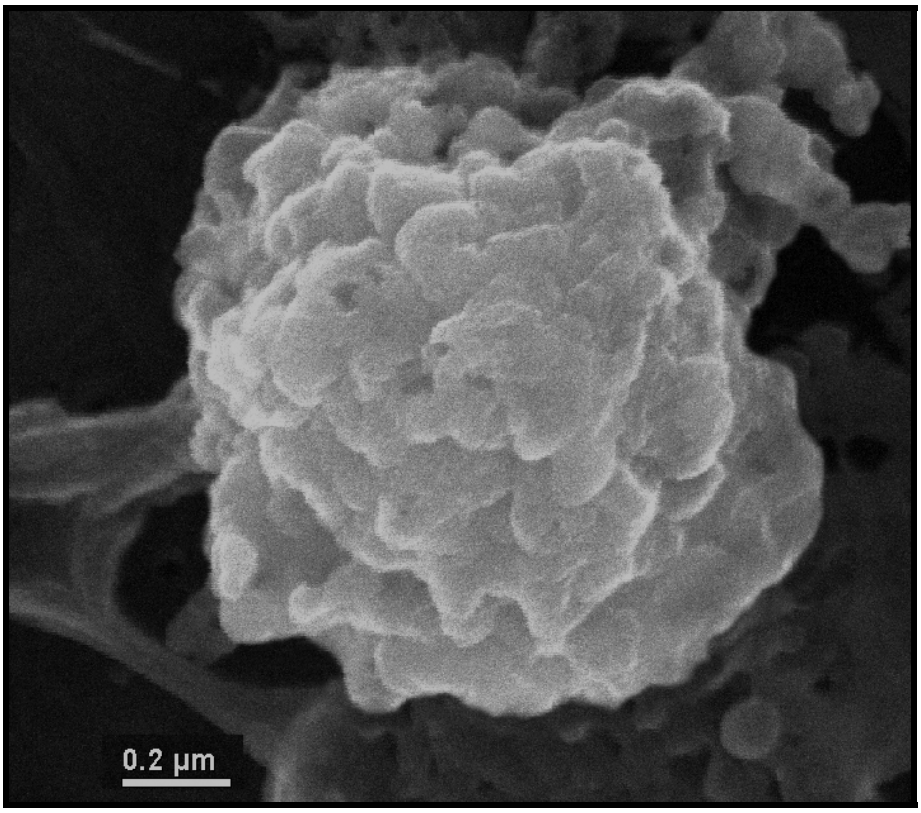


Figure 4.3b: Mouse platelet aggregate showing globular, pseudopodia-like morphology



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.

Chapter 5 -

Comparative analysis of the concentration of certain coagulation factors between controls, aspartame treated rabbits and humans

RESEARCH QUESTION 3:

Is there a difference between the blood clotting time, coagulation profile and different coagulation factors of the control and aspartame treated groups and how does these values compare to that of humans?

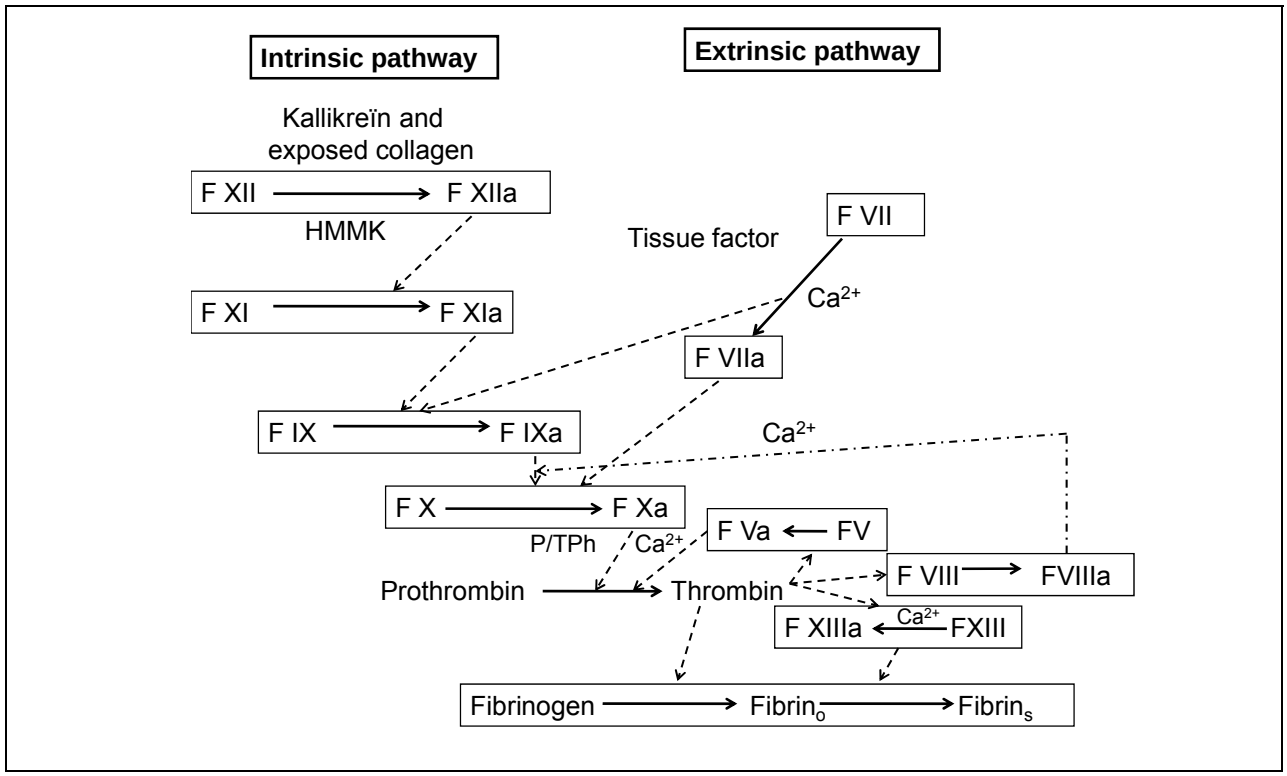
It was determined previously that the rabbit could indeed be used as a successful experimental animal model. This chapter will focus on techniques whereby coagulation factors were studied by measuring the change of coagulation factors due to the intake of aspartame, also defined as metabolomics. Metabolomics aims at measuring all metabolites present in an organism qualitatively and quantitatively; in studies of pathogenesis analysing materials such as urine and serum, this is referred to as metabonomics (Verpoorte *et al*., 2005). However, to obtain statistically sound results when testing a pharmaceutical product, it is compulsory to establish normal ranges and determine whether these results compare to that of the rabbits in the treatment groups, leading us into this chapter.

5.1 INTRODUCTION

Coagulation and haemostasis are important processes in the human body and the haemostatic system involves primary haemostasis (platelet aggregation), secondary haemostasis (coagulation) and tertiary haemostasis platelet (fibrinolysis) (Stassen *et al*., 2004). During the coagulation process, activation cascades of various factors and proteins in the intrinsic and extrinsic pathway play and important role (Troy, 1988; Kalafatis *et al*., 1997) and different proteins are involved, including von Willebrand's factor, fibrinogen, fibronectin, and specific glycoprotein receptors on platelet surfaces (Troy, 1988; Hawiger, 1987). Fibrinogen and calcium are also required for successful aggregation, while thrombin is important for formation of fibrin and for exposure of platelet receptors for adhesive molecules and for "activation of factors V and VIII" (Ofosu *et al*., 1996; Torbet, 1995). The contact of factor XII with a negatively charged surface, e.g. collagen, activates the intrinsic system. The common pathway is propagated through a series of reactions with prekallikrein, high-molecular weight kininogen (HMWK), and factors XI, IX, VIII. The common pathway and factor IX in the intrinsic pathway are stimulated by the extrinsic, or tissue factor system (Troy, 1988; Lammle and Griffin, 1985). The fact that the intrinsic pathway is stimulated by factor VII in the extrinsic pathway has inspired reassessment of the biological importance of the extrinsic system. The common pathway includes factor X and V and causes thrombin to convert fibrinogen to fibrin. Calcium and

platelet phospholipids are substances that have vital roles in steps in the coagulation scheme. Once fibrin is formed, factor XIII interacts with the substance, providing a stabilizing effect (Troy 1988; Mcvey, 1999)(Diagram 5.1).

Diagram 5.1: Coagulation pathway in the human



HMMK – High molecular mass kininogen; ⟶ - conversion of inactive protein to active enzyme; - - -> Active enzyme as cofactor for activation of inactive protein; ·····> - active factor VIII working as cofactor on active factor IX to activate factor X; P/TPh – platelet or tissue phospholipids; $Fibrin_o$ – unstable fibrin; $Fibrin_s$ – stable fibrin. Diagram adapted from Meyer and Meij, 1996.

Because the various proteins and factors involved in the coagulation process in humans is such a complex process and sometimes difficult to study, we investigate here the suitability to use an animal model. Traditionally, mice are used as animal model. However, a few studies have used rabbits, and from these results it seems as if results from rabbits compare well with those of humans. However, studies of rabbit values compared to that of humans are limited. Clarke and co-workers studied factor VII antigen and factor VIIa in the plasma of 36 normal rabbits Clarke *et al*. in 2003. The concentrations for the factor VII antigen ranged between 38 -198% in

the 36 normal rabbits, with a mean value of 766ng/ml plasma and these values correlate with those found in humans (Howards *et al.* 1994). The quantitation of activated FVII has not been described previously in rabbit plasma. On average, 1.1% of rabbit plasma FVII exists as FVIIa and its plasma concentration varied over 15-fold range in normal rabbits (Clarke *et al.* 2003). These values also correspond with data published for FVIIa concentration in human plasma (Morrissey *et al.,* 1993).

Noguchi *et al*. in 1989 performed a comparative study for measuring condition of activated partial thromboplastin time and contact factors in experimental animals. For establishing the optimal incubation time (OIT) for measurement of activated partial thromboplastin time (aPTT), they determined the shortest clotting time of the plasma from dogs, rabbits, guinea pigs, rats and mice. These findings were compared with that of human plasma. The OIT for aPTT determination was 15 – 30 seconds in guinea pigs, rats and mice and 5 – 10 minutes in dogs and rabbits. The mouse aPTT (about 30 seconds) with the OIT thus determined was similar to human aPTT, and relatively longer than aPTT in other animal species (10-20 seconds). The plasma of each animal species was tested for clarification of the mechanism for species differences in OIT, Noguchi et al. examined for the activity of the contact factors XII, XI, high molecular weight kininogen (HMWK) and prekallikrein (PK) and their effect on the coagulation of contact factor-deficient plasma. The total activity of contact factors was higher in dogs and guinea pigs and lower in rabbits and mice than that in humans. Species difference was noted in clotting time but not in OIT for factor XII, XI and HMWK. Due to the difference in activity of the plasma contact factors and the mode of coagulation for each contact factor there is a probable difference in OIT for aPTT in the different species.

Thus, the aim of this chapter was therefore to obtain a possible answer for the research question leading this part of the study: *Is there a difference between the blood clotting time, coagulation profile and different coagulation factors of the control and aspartame treated groups and how does these values compare to that of humans?*

Thus, this chapter focused on a comparison between the control and aspartame treated groups and to compare concentrations of different coagulation factors between the human and rabbit.

Specific attention was given to factors associated with the common pathway, namely factors II, V, X and fibrinogen; factors in the intrinsic pathway, namely Factors VIII, IX as well as factor VII, found in the extrinsic pathway. The *prothrombin time* (PT; measures how long blood takes to form a clot) and activated *partial thromboplastin time* (aPTT; measures recalcification time of plasma) was also measured.

5.2 MATERIALS AND METHODS

5.2.1 Test animals and collection of samples

Eight adult New Zealand white rabbits (2 male and 7 female, ±4 kg), obtained from the University of Witwatersrand, Johannesburg, were used for testing of aspartame. The rabbits were single housed by means of cage housing, without bedding at the University of Limpopo, Medunsa campus. Rabbits were weighed on a weekly basis to ensure that they were not losing any weight. Normal blood clotting factor ranges for rabbits was established by testing blood from the seven rabbits as there is no reference to these values in literature. It is also necessary to calibrate the instrument being used for testing of the coagulation factors found in rabbit blood. In this way, the normal ranges for the different coagulation factors can be determined.

1.5ml blood was drawn once weekly for four weeks, where after the rabbits were allowed to recuperate for two weeks (time needed to replace drawn blood). This procedure was repeated once more (blood drawn once weekly for four weeks, followed by a two week recuperation period). The data obtained from the samples from the above mentioned eight weeks was utilised to establish the normal ranges before treatment with the aspartame commenced.

5.2.2 Preparation of animals for drawing of blood samples:

Rabbits were placed in the modified restraint (as shown in chapter 3) before any blood was drawn. To increase visibility of the marginal ear vein of the rabbit, the area surrounding the vein was wiped with an alcohol swab. The ears were also flicked with the middle finger to improve the visibility of the vein.

5.2.3 Handling of the rabbits:

The person handling the rabbits used his/her thumb and little finger of one hand/ left hand/ non-dominant hand to grip the skin of the back of the neck and the head of the rabbit to lift the rabbit applying minimal pressure. The two hind paws were grabbed with the right hand/ dominant hand/ other hand and the rabbit turned so that its legs faced away from the handler. A firm grip on the paws were maintained and none on the back of the rabbit as it is fragile and could easily be injured.

5.2.4 Obtaining the blood samples:

1.5ml blood was drawn from the marginal ear vein of the rabbits (without sedation) at the Biology section of the Production unit (University of Limpopo, Medunsa campus). After the normal ranges were established, the rabbits were divided into 2 groups: 3 rabbits (1 male and 2 female) in the control group and 5 rabbits (1 male and 4 females) in the aspartame-treated group. The minimum amount of rabbits was chosen to decrease the number of animals that was treated with aspartame. The control group was utilized to ensure that normal ranges were maintained. 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, as described in chapter 3. Aspartame was utilised 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 abuse concentrations (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.

After exposure to the aspartame/double distilled water, 1.5ml blood was drawn from the marginal ear vein of ears of the rabbits (without sedation) in both the control and aspartame treated groups. Blood was drawn with a vacutainer needle and transferred into a 5ml vacutainer tube with a blue top, containing anti-coagulant (0.109M or 3.2% trisodium citrate) for testing. Two weeks of recovery time was allowed between consecutive concentrations. Blood was drawn on three set intervals - after 3 exposures, 13 exposures and 25 exposures to aspartame.

5.2.5 Preparation of sample for Start 4 instrument

The 1.5ml blood was centrifuged at 2500rpm for 10 minutes. The plasma was pippeted into small Eppendorf tubes and frozen at -80^{o}C till needed for use on the Start 4. The plasma could only be stored for 1 month.

All coagulation experiments were performed on the Start 4 Diagnostica Stago (Paris, France). This machine is the smaller, manual version of the larger machines routinely used in all haematology laboratories to determine the concentration of specific coagulation factors and profiles. The programming of the parameters into the Start4 was completed before any of the assays were attempted, and these parameters remained in the memory of the Start4. These parameters could only change when altered by the researcher. Examples of these parameters are maximum incubation time, precision (5%) and whether the experiment is run singularly or in duplicate. These parameters were dictated by the assays that were being performed.

5.2.5.1 Assays kits that were utilized:

- Neoplastine® Cl Plus (determination of Prothrombin time [PT])
- STA-Deficient II (substrate plasma for Factor II assay; Prothrombin)
- STA-Deficient V (substrate plasma for Factor V assay)
- STA-Deficient VII (Immuno-depleted plasma for Factor VII assay)
- STA-Deficient X (Immuno-depleted plasma for Factor X assay)
- STA-PTT Automate with silica activator (Sensitivities: Factors +++; Heparin: +++; Lupus ++; determination of Activated Partial Thromboplastin Time [aPTT])
- STA-Deficient VIII (Immuno-depleted plasma for Factor VIII assay)
- STA-Deficient IX (Immuno-depleted plasma for Factor IX assay)
- STA-Fibri-prest Automate (Clauss method; Quantitative determination of Fibrinogen)

All procedures were done according to the standardized operating procedures for determining human values.

The PT value may vary according to the origin of the thromboplastin reagent and to the instrument used to measure it. A solution for standardization adopted by the World Health Organization is a 'system of international reference standards for thromboplastins permitting the definition of an international scale for the intensity of anticoagulant therapy'. In this system the PT ratio is converted into the International Normalized Ratio (INR). The INR value corresponds to the value of the ratio of the patient's PT to that of the standard PT raised to the ISI (International Sensitivity Index) power of the thromboplastin used:

$$INR = \left(\frac{\text{Patient's PT}}{\text{Mean normal PT}} \right)^{ISI}$$

The ISI value of a given thromboplastin is determined by testing normal plasmas and Coumadin-treated patient plasmas with that thromboplastin and with the International Reference Preparation for thromboplastin. The PT values obtained with the two thromboplastins are plotted on log-log graph paper, and the orthogonal regression line is drawn. The slope of this line multiplied by the ISI value of the reference thromboplastin represents the ISI value of the thromboplastin of interest. The use of INR is recommended for the assessment of the PT in patients under oral anticoagulant therapy (Package insert - Neoplastine® CI Plus 2; Ref 00376).

5.2.5.2 Preparation of standards, patients' samples and controls

The unicalibrator (standard) was used for establishing the calibration curve for all of the assays performed. The dilutions that were to be used for each assay were determined after the normal ranges were set. The package insert of the unicalibrator supplies the researcher with information regarding the amount of active ingredient present for each specific assay. This value may vary from package to package, thus care was taken to use the correct package insert at any given time. From here, the concentration of active ingredient was calculated, which in turn was used for setting of the calibration curve.

For example:

Unicalibrator package insert values for factors II and V
1 : 10 = 100%
Dilutions utilized: 1:2, 1:3, 1:5, 1:10 and 1:20
1 : 20 = 50%
1 : 10 = 100%
1 : 5 = 200%
1 : 3 = 333.33%
1 : 2 = 500%

The plasma samples obtained were defrosted and the necessary dilutions were made for the different assays. For determining the concentration of factors II, V, VII, X, VIII and IX present in rabbit blood, 50µl rabbit plasma was added to 500µl Owren-Köller solution (1:10 dilution). For determining the concentration of fibrinogen present in the plasma, a 1:20 dilution was made with 25µl rabbit plasma and 475µl Owren-Köller solution. For determining the PT and aPTT, the plasma samples were used undiluted. A normal and pathological control was tested consecutively with all the assays performed, and their dilutions were the same as that of the plasma being tested for that assay.

Standards (utilizing unicalibrator) were diluted as follows:

a.) PT – 1:2, 1:3 and 1:4

b.) aPTT – no calibration necessary

c.) Factors II, V, VII, X, VIII, IX – 1:2, 1:3, 1:5, 1:10 and 1:20

d.) Fibrinogen – 1:7, 1:10; 1:20 and 1:40

The values obtained from the Start 4, together with the percentages calculated (as described above) were then used to draw the standard curve.

5.2.5.3 Assays

The reagents from the different assay kits were prepared according to their package inserts. The indicated amount of distilled water was added to each reagent, left for 5 minutes to homogenize and then swirled to improve optimal mixture of reagent. Reagents were then left to stand for 30 minutes to stabilize. Care was taken with the timing of preparation of factors VIII and IX, as they were only stable for four hours. Thus, they were prepared just before they were needed.

Table 5.1: Assays performed with different solutions and activators for each assay

Assay	**Dispensed into cuvette**	**Incubation period**	**Activator (pre-warmed at 37 ^{o} C)**
PT	50µl plasma	60 seconds	100µl Neoplastine CI plus
Deficient II	50µl plasma + 50µl Deficient II	60 seconds	100µl Neoplastine CI plus
Deficient V	50µl plasma + 50µl Deficient V	60 seconds	100µl Neoplastine CI plus
Deficient VII	50µl plasma + 50µl Deficient VII	60 seconds	100µl Neoplastine CI plus
Deficient X	50µl plasma + 50µl Deficient X	60 seconds	100µl Neoplastine CI plus
aPTT	50µl plasma + 50µl PTT automate	180 seconds	50µl $CaCl_2$
Deficient VIII	50µl plasma + 50µl Deficient VIII	180 seconds	50µl $CaCl_2$
Deficient IX	50µl plasma + 50µl Deficient IX	180 seconds	50µl $CaCl_2$
Fibrinogen	100µl plasma	60 seconds	50µl FibriPrest

5.3 RESULTS

The prothrombin time (PT; measures how long blood takes to form a clot) and activated partial thromboplastin time (aPTT) was determined. aPTT (measures recalcification time of plasma) is used to screen for deficiencies in coagulation factors involved in the intrinsic pathway (factor VII and factor VIII excluded). The aPTT is also utilized to evaluate the effect of therapy and to monitor and regulate heparin therapy (Proctor and Rapaport, 1961). PT measures the presence and activity of factors I, II, V, VII and X. A Normal PT time, for a healthy human is 10 – 13 seconds with the International Normalized Ratio (INR) being 1.0 – 1.4 (WebMD Medical Tests, 2006). Partial thromboplastin time in a normal healthy human is 30 – 45 seconds, while the activated partial thromboplastin time is 25 – 39 seconds (WebMD Medical Tests, 2006).

Literature does not describe normal values for the different coagulation factors in rabbits, thus the normal ranges for rabbits for the different factors were established.

Analysis indicated that rabbits have higher concentration levels for the factors tested. Therefore, the standard curve was extrapolated to determine the values for rabbits. The instrument automatically extrapolates the curve to 200%, but the values for rabbits were more than 200%. Statistically, extrapolation was done from the standard curve obtained, to determine the true percentage from the actual seconds (raw data) measured.

5.3.1 Determining normal ranges

The normal ranges for the different coagulation factors within the group of rabbits were obtained and are represented in Table 5.2.

Table 5.2: Normal ranges for the different coagulation factors for the group of rabbits

Assay performed	***Lower spectrum***	***Higher spectrum***
PT (Prothrombin time) %	93.15	107.13
INR	1.19	1.39
Deficient factor II	170.72	264.35
Deficient factor V	609.11	670.06
Deficient factor VII	319.89	422.06
Deficient factor X	240.37	351.76
APTT (activated partial prothrombin time)	45.98	69.89
Deficient factor II	488.49	713.3
Deficient factor II	76.44	355.33
Fibrinogen	1.81	3.58

These values was used during the study do make certain that the controls remained within the normal range limits, demonstrating that the results obtained for the rabbits in the treatment group was indeed due to the aspartame and not due to continues drawing of blood. The values were also used to determine whether the aspartame had any adverse effects on the concentration of the factors discussed and the time to form a blood clot (prothrombin time and activated partial thromboplastin time).

The values obtained for the rabbits were placed in a table (Table 5.3) and compared to the normal values of human beings.

Table 5.3: Comparison between normal ranges determined for rabbit plasma and human plasma

Assay Performed	**Values obtained for determining normal ranges for rabbits**	**Expected values for normal human adults**
PT **%** (n = 41) **INR** (n= 42)	93.15 – 107.13 % 1.19 – 1.39 INR 7.16 ± 0.55 sec	Greater than 70% 1.0 – 1.4 INR 10 – 13 sec
Factor II (n = 33)	170.72 – 264.35 %	70 – 120 % (Bezeaud *et al.* 1979)
Factor V (n = 39)	496.69 – 556.55 %	70 – 120 %
Factor VII (n = 37)	319.89 – 422.06 %	55 – 170 %
Factor X (n = 40)	240.37 – 351.76 %	70 – 120 % (Marian *et al.* 1999)
aPTT (n = 35)	45.98– 69.89 sec	25 – 39 sec (Levin and Lusher, 1982)
Factor VIII (n = 41)	488.49 – 713.3 %	60 – 150 %
Factor IX (n = 42)	76.44 - 355.33 %	60 – 150 %
Fibrinogen (n = 37)	1.81 – 3.58 g/l	2 – 4 g/l (Bezeaud *et al*. 1979)

Values for humans obtained from package inserts of the assays performed; Values for rabbits were obtained statistically by utilizing mean ± 2 standard deviations. n indicates the number of values used when obtaining the means.

When the results obtained for the rabbits were compared with those of humans, it was clear that the PT (in seconds) is shorter than that of the human although the percentage and INR values

correspond with that of the human. The percentage of factor II found in rabbits was found to be twice as much as that of a human. The amount of factor V and VIII is four to six times greater for rabbits. The rabbit contains two to three times more factor VII and X. The lower end of the range for factor IX in rabbits fall inside the lower spectrum set for humans and the higher end of the spectrum is two times more than that of a human. The rabbit also has a much longer activated partial thromboplastin time when compared to a human. And finally, and certainly most importantly, the amount of fibrinogen is comparable between humans and rabbits.

5.3.2 Blood clotting time and coagulation profile after treatment with aspartame

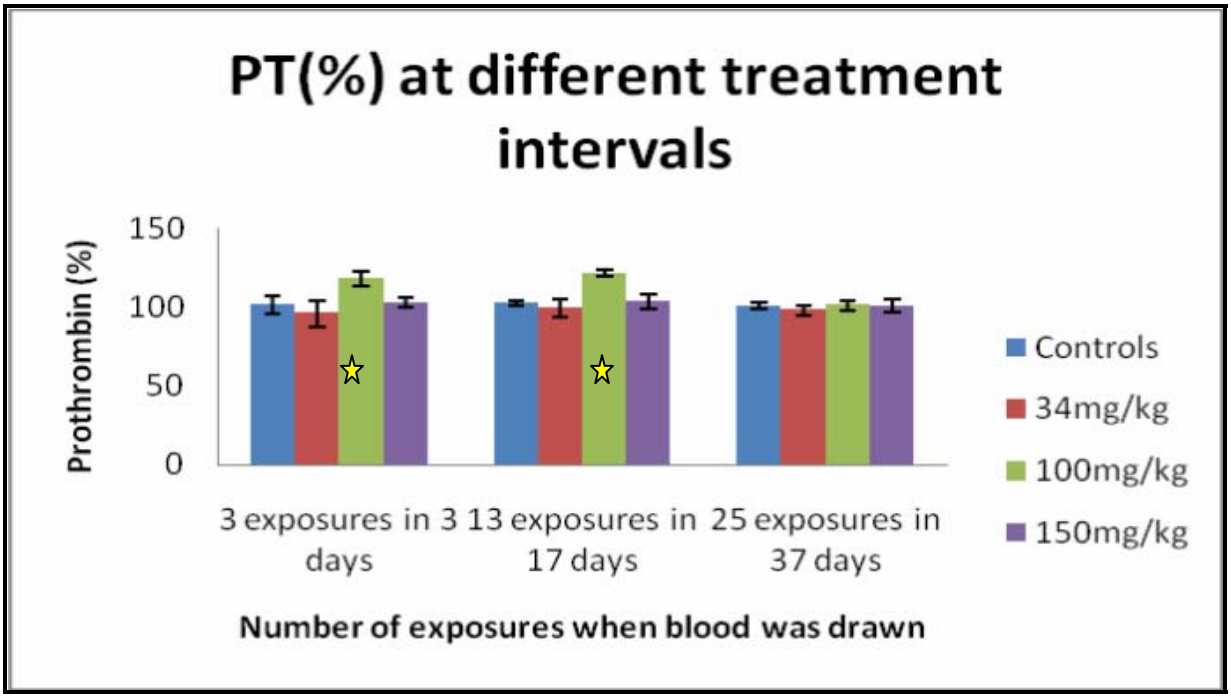
Table 5.4 and graph 5.1 indicate the values for PT (%) after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.4: Effects of aspartame on the PT (%) for the three different concentrations at the set intervals for drawing blood

PT (%)	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	101.06	102.49	100.41	93.15 - 107.13
34mg/kg	95.78	99.35	97.85	
100mg/kg	117.94	121.29	100.99	
150mg/kg	102.55	103.81	100.59	

Values highlighted in yellow fall outside the normal set ranges

Graph 5.1: Effects of three different concentrations of aspartame on the PT (%) of the rabbit



Yellow star indicates the values that fall outside the normal set ranges

The results indicate that the prothrombin time (PT) never exceeded the values set for normal ranges during the 34mg/kg aspartame treatment. However, the PT was prolonged after the 100mg/kg aspartame treatment. The two-way ANOVA was performed on the data highlighted in yellow, and a statistically significant (P=0.04) difference was found between the rows of data, but not the columns. This indicates that the different intervals for drawing blood after a certain number of exposures were not significant, but the effects of the different concentrations were significant. These results were confirmed by performing the t-Test: Paired Two Sample for Means. There was a significant difference (P=0.034 two-tailed) between the control and the first two values obtained for the 100mg/kg aspartame treatment group. It would seem that the rabbits were mostly affected during the earlier stages of treatment (3 and 13 exposures) with the 100mg/kg aspartame. The values obtained for the 150mg/kg aspartame treatment never exceeded the values set as normal ranges.

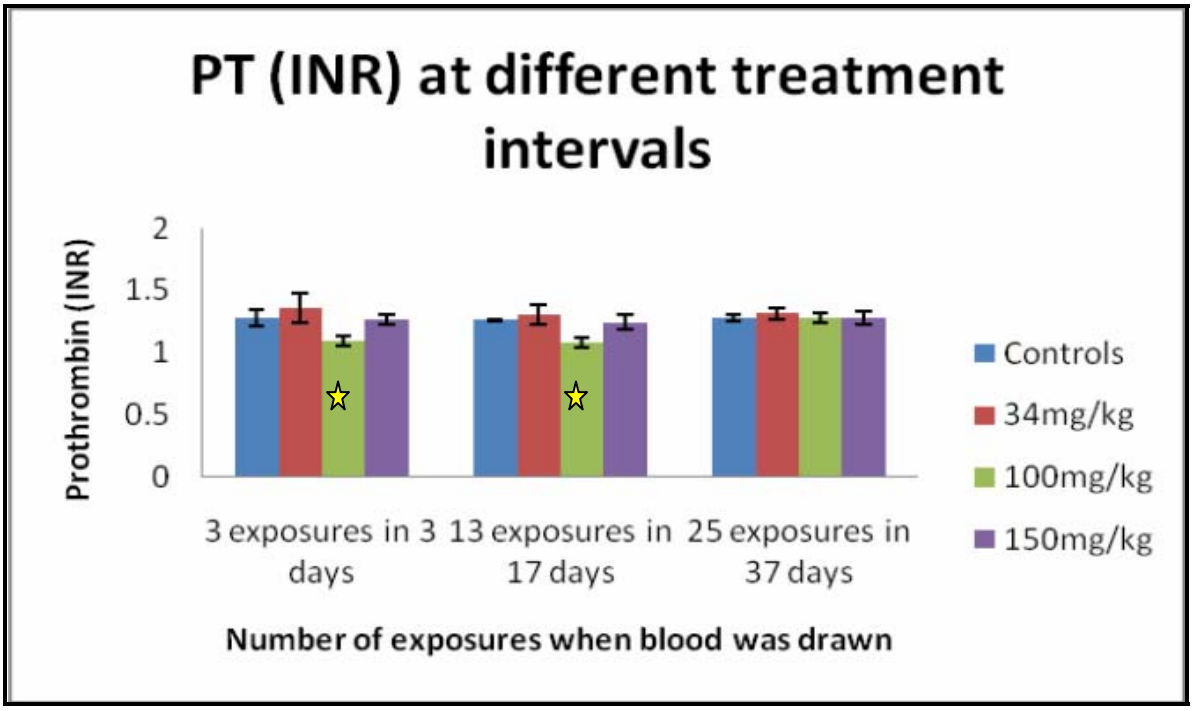
Table 5.5 and graph 5.2 indicate the values for PT (INR) after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.5: Effects of aspartame on the PT (INR) for the three different concentrations at the set intervals for drawing blood

PT (INR)	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	1.28	1.26	1.28	1.19 - 1.39
34mg/kg	1.36	1.3	1.31	
100mg/kg	1.09	1.08	1.28	
150mg/kg	1.26	1.24	1.28	

Values highlighted in yellow fall outside the normal range

Graph 5.2: Effects of aspartame on the PT (INR) of the rabbit after treatment with the three concentrations of aspartame



Yellow stars indicate values that fall outside the normal range

The results for PT (INR) were the same as for PT (%), seeing that only the measurement units were different. A significant difference (*P*=0.017 two-tailed) were seen between the control and 100mg/kg aspartame.

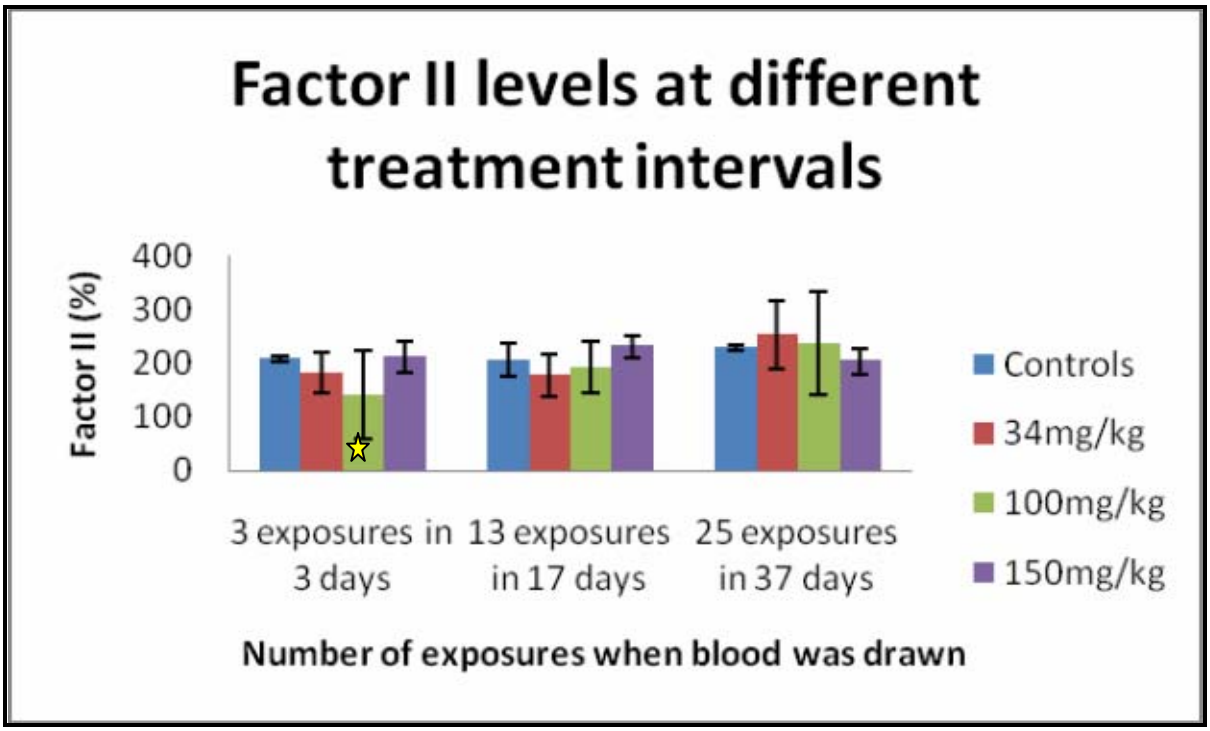
Table 5.6 and graph 5.3 indicate the values for Factor II after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.6: Effects of aspartame on the concentration of F II (%) after treatment with three concentrations aspartame at the three intervals set for drawing blood

Factor II	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	208.35	206.38	229.3	170.72 - 264.35
34mg/kg	181.92	178.55	252.5	
100mg/kg	140.78	192.47	236.34	
150mg/kg	210.4	231.24	203.94	

Values highlighted in yellow fall outside the normal range

Graph 5.3: Effects of aspartame on the concentration of F II (%) after treatment with the three concentrations of aspartame



Yellow stars indicate values that fall outside normal range

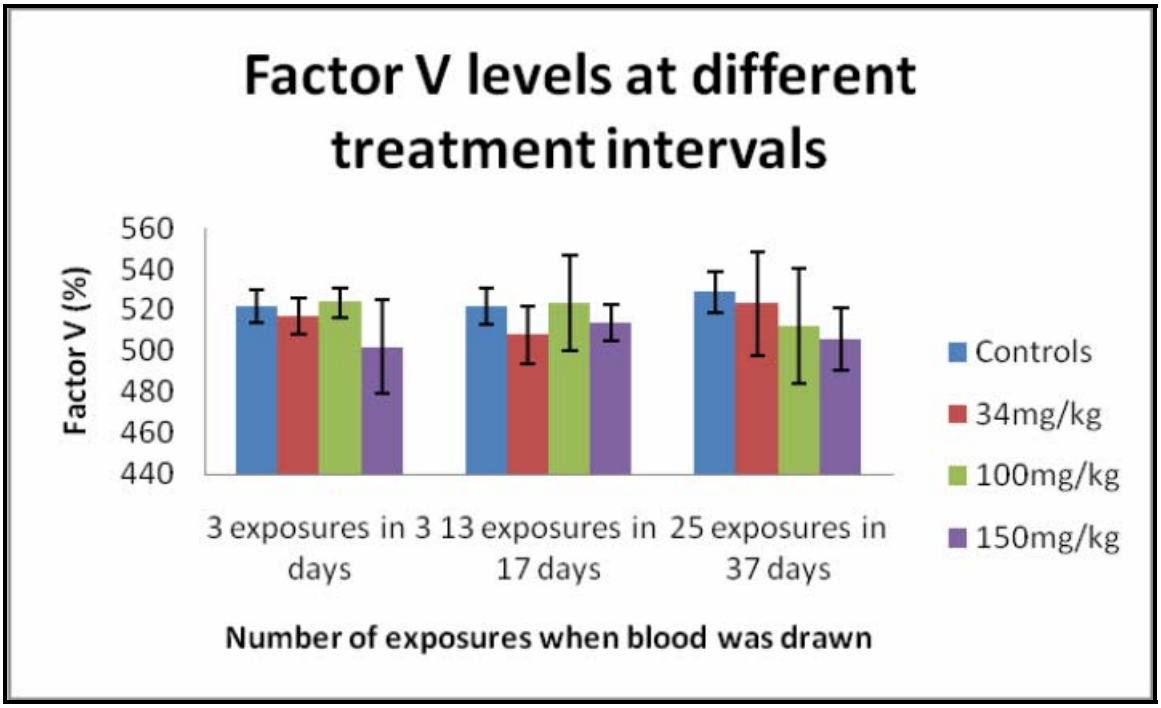
The results indicate that the concentration of factor II were not adversely affected by any of the concentrations of aspartame tested. The value highlighted in yellow does not differ significantly from the control, as proven by the two-way ANOVA and t-Test: Paired Two Sample for Means.

Table 5.7 and graph 5.4 indicate the values for Factor V after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.7: Effects of aspartame on the concentration of factor V (%) for the three different concentrations at set intervals for drawing blood

Factor V	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	521.67	521.94	528.67	496.69 - 556.55
34mg/kg	517.04	507.91	523.05	
100mg/kg	523.64	523.23	512.28	
150mg/kg	501.96	513.77	505.69	

Graph 5.4: Effects of aspartame on the concentration of factor V (%) at different concentrations



All the values obtained for factor V fell within the normal set ranges, thus it would appear as if factor V was not adversely affected by the aspartame.

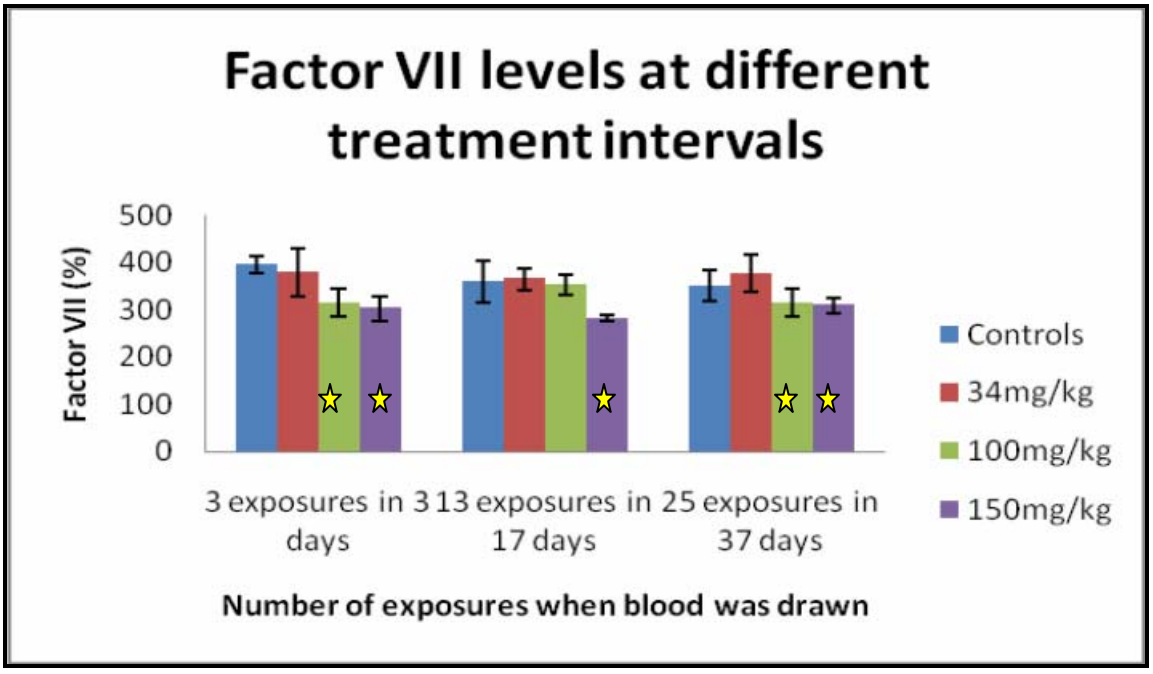
Table 5.8 and graph 5.5 indicate the values for Factor VII after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.8: Effects of aspartame on the concentration of factor VII (%) after treatment with the three concentrations at set intervals for drawing blood

Factor VII	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	395.42	358.78	350.46	319.89 - 422.06
34mg/kg	379.26	365.26	376.76	
100mg/kg	315.16	353.39	314.56	
150mg/kg	302.86	282.05	309.05	

Values highlighted in yellow fall outside the normal range

Graph 5.5: Effects of aspartame on the concentration of factor VII (%) after treatment with different concentrations



Yellow stars indicate values that fall outside the normal range

The obtained results indicate that the concentration of factor VII decreased at 34mg/kg aspartame, but the values still remained within the normal range of values. The values obtained for 100mg/kg aspartame were even lower, with two of the values falling outside the normal range. A two-way ANOVA was performed on this data and it was found that there was a statistically significant (*P*=0.010) difference between rows, but not between columns. Thus, the aspartame treated groups differed significantly from the controls, but there was no significant difference between the values for the different exposure time intervals for drawing blood. The effect of the aspartame remained the same throughout the exposure period. Furthermore, a t-Test was performed on the values obtained for the 100mg/kg aspartame, and no significant difference were found between the controls and affected values. When comparing the values obtained for the 150mg/kg aspartame-treated group with the controls (t-Test), there was a statistically significant (*P*=0.043 two-tailed) decrease in the concentration of factor VII. Factor VII, which forms part of the extrinsic pathway, is necessary for activation of factor IX *in vivo*. Factor VII in turn is activated by tissue factor in the presence of Ca^{2+}. Tissue factor is an integral protein of cell membranes (Meyer and Meij, 1996). Thus, it could be stated that a decreased concentration of factor VII could induce a decreased concentration of factor IX, leading to bleeding disorders. If there is deficient amounts of tissue factor or Ca^{2+} in the test animals, factor VII formation will also be decreased.

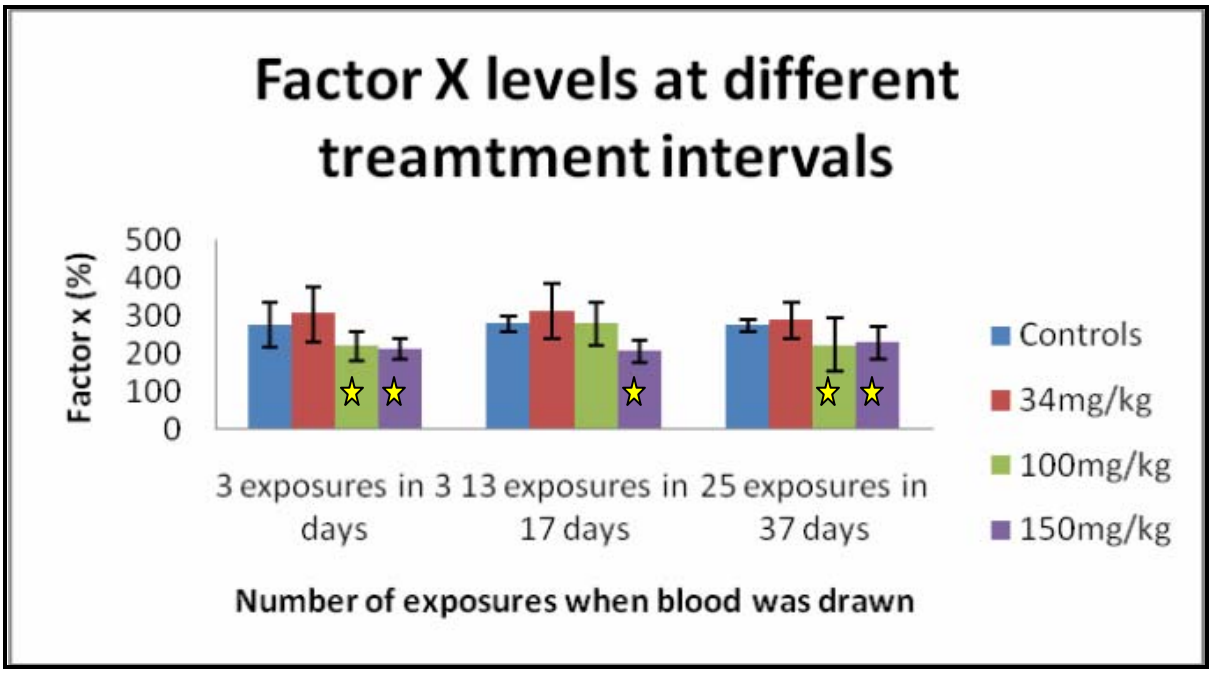
Table 5.9 and graph 5.6 indicate the values for Factor X after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.9: Effects of aspartame on the concentration of factor X (%) after treatment with the three different concentrations at set intervals for drawing blood

Factor X	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	275.92	278.82	274.54	240.37 - 351.76
34mg/kg	304.34	312.11	288.46	
100mg/kg	219.91	278.33	222.94	
150mg/kg	213.03	205.54	228	

Values highlighted in yellow fall outside the normal ranges

Graph 5.6: Effects of aspartame on the concentration of factor X (%) after treatment with the different concentrations



Yellow stars indicate values that fall outside the normal range

From the results obtained it is clear that the concentration of factor X was not adversely affected by the 34mg/kg aspartame treatment. However, values from the 100mg/kg and 150mg/kg aspartame treatment groups fall outside the normal rage. A two-way ANOVA test was performed on the data, and the values in the rows were found to differ significantly (P=0.005) from the controls. There was no significant difference between the values in the columns, thus the aspartame had no different effect on the concentration of factor X at the different treatment intervals. The values that fall outside the normal range for the 100mg/kg and 150mg/kg aspartame treatments were subjected to further testing (t-Test). No significant difference were found between the control and 100mg/kg aspartame, but a statistically significant (P=0.016 two-tailed) decrease were found between the control and 150mg/kg aspartame. Thus, the decrease of the concentration of factor X was significant. Factor IX (in its active form) works in on factor X and split this protein to form an active enzyme, F Xa. The active form of factor X can be produced by either the intrinsic or extrinsic pathways. The active form of factor X, in combination with factor V, platelet or tissue phospholipids and calcium ions, convert prothrombin to thrombin (Meyer and Meij, 1996). Thus, a decreased amount of factor X could lead to lower concentrations of thrombin, needed for conversion of fibrinogen to fibrin.

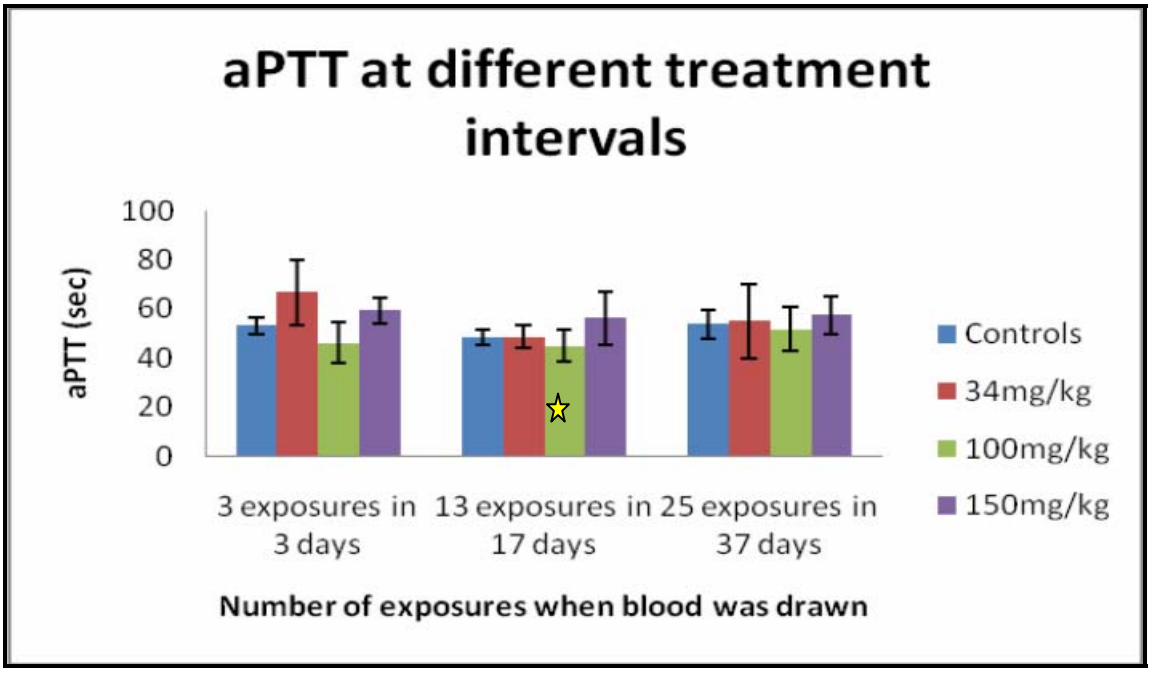
Table 5.10 and graph 5.7 indicate the values for aPTT after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.10: Effects of aspartame on the aPTT (sec) after treatment with the three different concentrations at the set intervals for drawing blood

aPTT	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	53.16	48.28	53.73	45.98 - 69.89
34mg/kg	66.84	48.72	54.95	
100mg/kg	46.18	44.87	51.64	
150mg/kg	59.48	56.14	57.44	

Values highlighted in yellow fall outside the normal range

Graph 5.7: Effects of aspartame on the aPTT (sec) after treatment with the different concentrations



Yellow stars indicate values that fall outside normal range

From the results shown in table 10, it is clear that the time for recalcification of the plasma (aPTT) was not affected by the treatment with aspartame as the values all fell within the normal range. The one value for the 100mg/kg aspartame after 13 exposures that did fall outside the

normal range was subjected to a two-way ANOVA test and it was found that the value did not differ significantly from the control values.

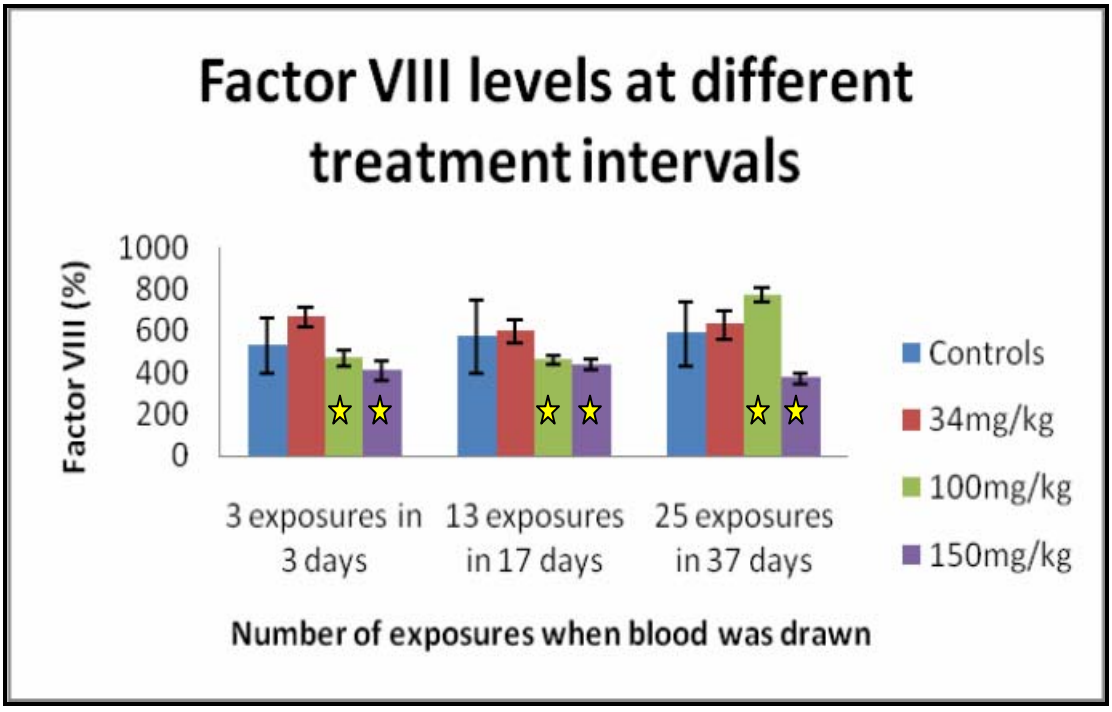
Table 5.11 and graph 5.8 indicate the values for Factor VIII after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.11: Effects of aspartame on the concentration of factor VIII (%) after treatment with the three different concentrations at the set intervals for drawing blood

Factor VIII	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	528.72	569.75	585.54	488.49 - 713.3
34mg/kg	664.72	599.25	630.36	
100mg/kg	471.07	461.16	770.8	
150mg/kg	407.36	437.82	373.95	

Values highlighted in yellow fall outside the normal range

Graph 5.8: Effects of aspartame on the concentration of factor VIII (%) after treatment with the different concentrations



Yellow stars indicate values that fall outside the normal range

From the obtained results it appears as though 34mg/kg aspartame increased the concentration of factor VIII present in the tested rabbit plasma, but the values remained within the set normal ranges. It was noted that the values for both the 100mg/kg and 150mg/kg aspartame treatments fell outside the normal range. The data was analyzed by means of the two-way ANOVA, but no statistical significance was found between the rows and columns of data. The data was further subjected to analysis by means of the t-Test, to ensure that data was not significant. It was found that the values obtained for the 100mg/kg aspartame did indeed not differ significantly from that of the control, but it was not the same scenario for the 150mg/kg aspartame treatment. There was a statistically significant (P=0.032 two-tailed) decrease between the controls and 150mg/kg aspartame treatment groups. Thus, the concentration of factor VIII decreased significantly. Factor VIII is known as an antihemofillic globulin and is activated by thrombin, thus a decrease in the concentration of any of these factors can lead to bleeding disorders (Meyer and Meij,1996).

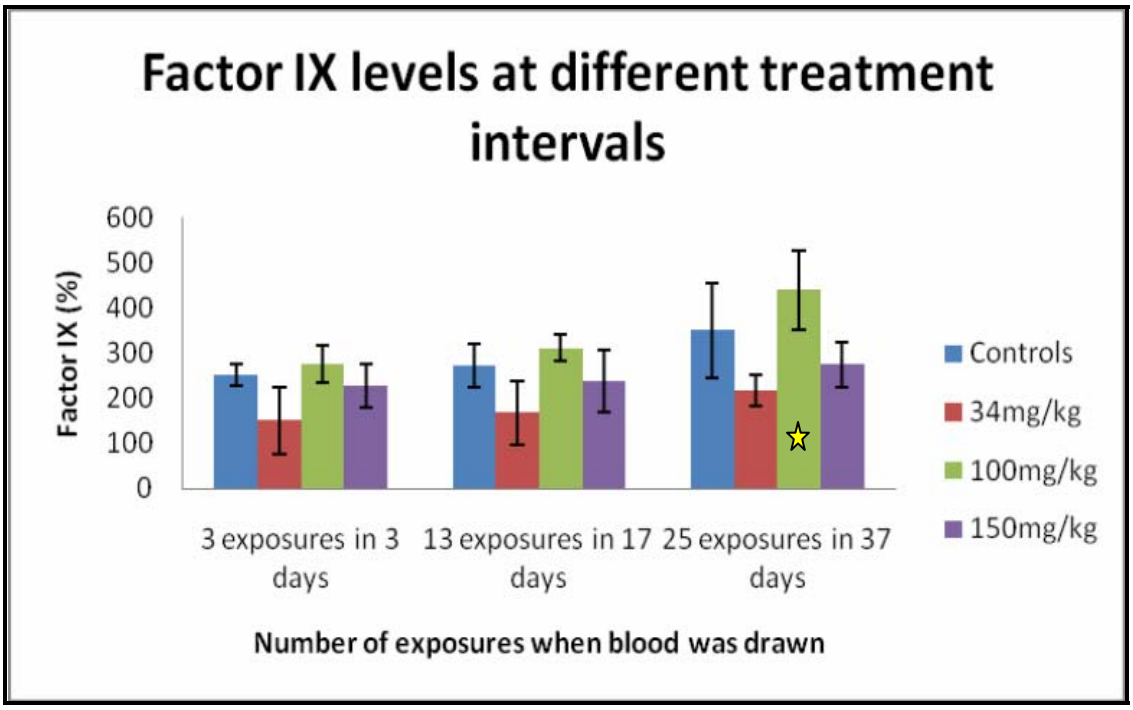
Table 5.12 and graph 5.9 indicate the values for Factor IX after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.12: Effects of aspartame on the concentration of factor IX (%) after treatment with the three different concentrations at the set intervals for drawing blood

Factor IX	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	251.41	271	349.32	76.44 - 355.33
34mg/kg	150.42	167.5	216.97	
100mg/kg	274.22	310.13	438.3	
150mg/kg	226.5	237.3	274.06	

Values highlighted in yellow fall outside normal range

Graph 5.9: Effects of aspartame on the concentration of factor IX (%) after treatment with the different concentrations



Yellow star indicate values that fall outside the normal range

After analyzing the results obtained for factor IX, it was clear that aspartame did not adversely affect the concentration of factor IX in the plasma of the rabbit. The amount of factor IX decreased after treatment with 34mg/kg aspartame, but not to a degree that the values fall outside the normal range. The amount of factor IX increased readily from exposure interval to exposure interval for the 100mg/kg aspartame treatment, but the values did not fall outside the normal ranges. The value obtained for 100mg/kg aspartame after 25 exposures that did fall outside the normal range were tested with the t-Test, and the results indicated that there was no significant difference between the tested value and the values of the control.

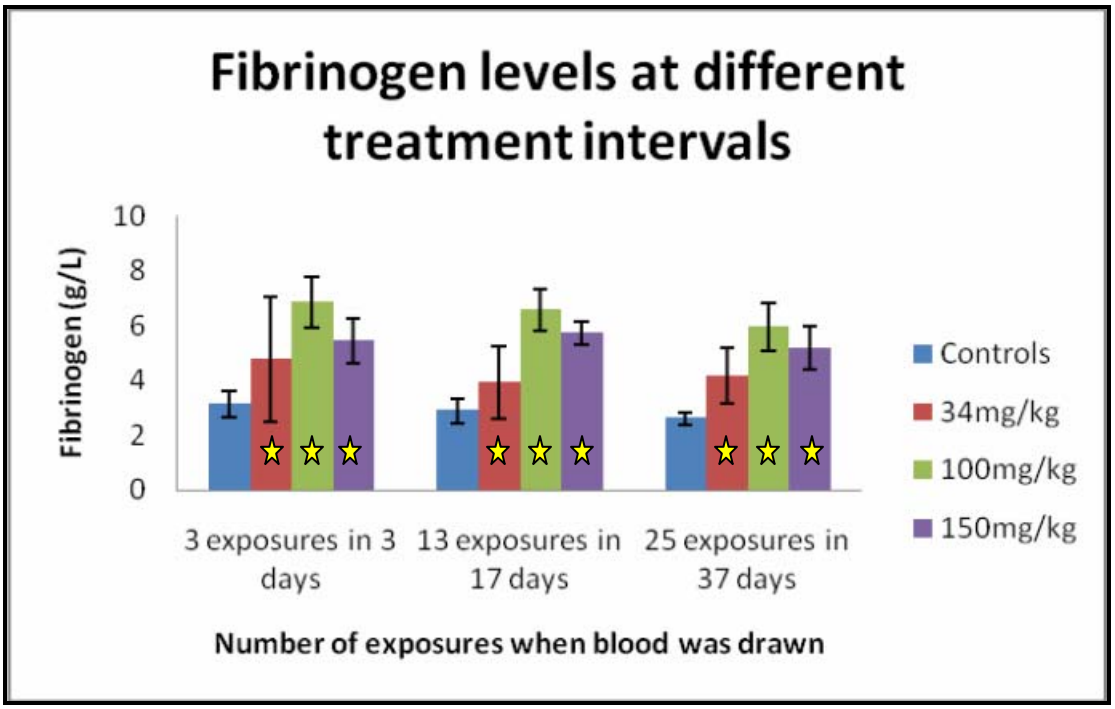
Table 5.13 and graph 5.10 indicate the values for Fibrinogen after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.13: Effects of aspartame on the concentration of fibrinogen (g/L) after the three different concentrations at set intervals for drawing blood

Fibrinogen	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	3.15	2.9	2.62	1.81 - 3.58
34mg/kg	4.75	3.91	4.17	
100mg/kg	6.85	6.56	5.96	
150mg/kg	5.45	5.74	5.17	

Values highlighted in yellow fall outside the normal range

Graph 5.10: Effects of aspartame on the concentration of fibrinogen (g/L) after treatment with the different concentrations



Yellow stars indicate values that fall outside the normal range.

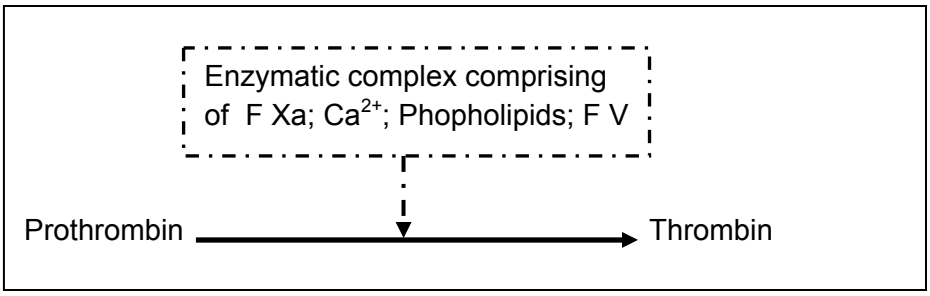
When looking at the effects of aspartame on the concentration of fibrinogen, it was clear that the amount of fibrinogen increased from the first treatment with aspartame at the lowest concentration (34mg/kg). A two-way ANOVA was performed on the results, indicating that there was a statistical significant (P=1.75x10^5) difference between the different concentrations and the controls. However there was no difference between the different numbers of exposures. The data was further subjected to analysis (t-Test) to determine whether there was any difference

between the different concentrations. The t-Test indicated that there was a statistically significant (*P*=0.018 two-tailed) increase between the concentration of fibrinogen of the control and 34mg/kg aspartame treated group. The t-Test was performed on the values of the control and 100mg/kg aspartame treated groups, and a statistically significant (*P*=0.001 two-tailed) increase were found between the control and aspartame treated groups. A statistically significant (*P*=0.0036 two-tailed) increase was also found between the control and 150mg/kg aspartame. The values of the 34mg/kg aspartame treatment group was compared to the values of the 100mg/kg aspartame treatment group (t-Test), and a significant increase (*P*=0.013 two-tailed) were found between the two concentrations. When comparing (t-Test) the 34mg/kg aspartame results with the 150mg/kg aspartame results, a one-tailed significance (*P*=0.037) were found between the results. There was a significant increase in the concentration of fibrinogen between the above mentioned two concentrations. The last comparison (t-Test) was made between the 100mg/kg values and the 150mg/kg values. A two-tailed significant (*P*=0.037) decrease were found between the values of the 150mg/kg aspartame and the 100mg/kg aspartame. However, the concentration of fibrinogen for the 150mg/kg aspartame was still higher after the decrease than the values for the 34mg/kg aspartame. Thus, an overall increase of the concentration of fibrinogen was noted after treatment with the aspartame.

5.4 SUMMARY AND EXPLANATION

The ***prothrombin time*** (PT) is a basic coagulation screening test, useful in the assessment of congenital and/or acquired deficiencies of the extrinsic coagulation pathway (factors II, V, VII and X). A prolonged PT has been observed in the following clinical states like treatment with vitamin K antagonists, hemorrhagic disease of the newborn, intestinal reabsorption disorders, liver failure (cirrhosis; hepatitis; jaundice), fibrinolysis and distributed intravascular coagulation (DIC) (Package insert – Neoplastine® CI Plus 2; Ref 00376). The PT was prolonged after treatment with aspartame with deficiencies in factors VII and X.

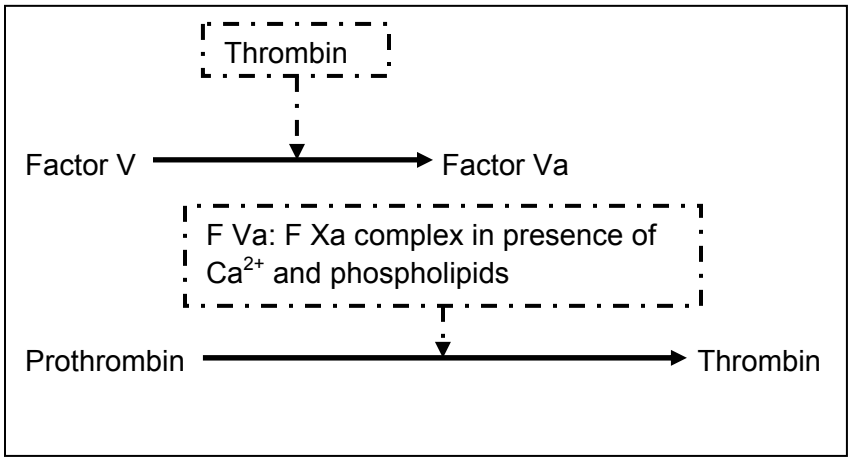
Factor II, also known as prothrombin, is synthesized in the liver. The complete synthesis of the prothrombin molecule requires the presence of vitamin K.



Solid arrow indicates activation of the inactive protein (F II; Prothrombin) to its active form (F IIa; Thrombin); dashed arrow and box indicate the enzymatic complex needed for the activation of prothrombin to thrombin.

Factor V must undergo a molecular modification under the influence of traces of thrombin to aid in the activation of thrombin. Acquired F II deficiencies can be found in several clinical states; during oral anticoagulation therapy (which depresses the vitamin-K dependant factors II, VII, IX and X), intake or absorption deficiencies of vitamin K, liver damage (cirrhosis; hepatitis), during fibrinolysis, disturbed intravascular coagulation (DIC) and the presence of factor II inhibitors (Package insert – STA Deficient II; Ref 00745). Aspartame did not affect the concentration of circulating factor II.

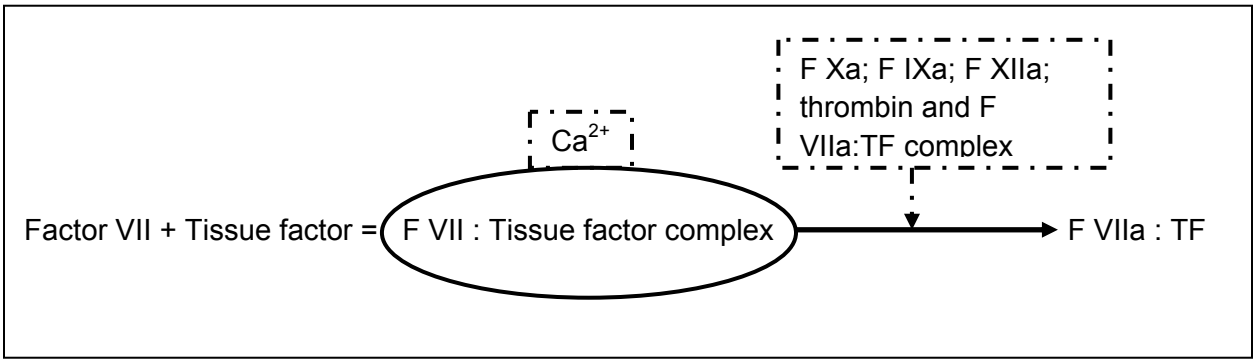
Factor V is synthesized by the reticuloendothelial system. The structure of the platelet factor V is closely related if not identical with that of the plasma factor V.



Solid arrow indicates activation of the inactive protein (F V) to its active form (F Va) with the dashed arrow and box indicating that thrombin is needed for activation; dashed arrow and box indicate the enzymatic complex needed for the activation of prothrombin to thrombin. Note that F Va is needed for activation.

Factor Va is inactivated by factor Xa, activated protein C and plasmin. Liver cells synthesize various coagulation factors, some of which require the presence of vitamin K for their elaboration (factors II, VII, IX and X). Any hepatic disorder can lead to a more or less important decrease in the level of these factors. Liver damage, therefore, can result in hemorrhagic disorders. Specific testing for the factors is of major interest for reasons of prognosis and diagnosis with respect to liver damage. If both factors VII and X are decreased prolonged hepatitis can be diagnosed (Package insert – STA Deficient V; Ref 00744). Factor X concentrations were decreased after treatment with aspartame. The enzyme complex between F Va and F Xa were thus compromised and so prothrombin could not be converted to thrombin.

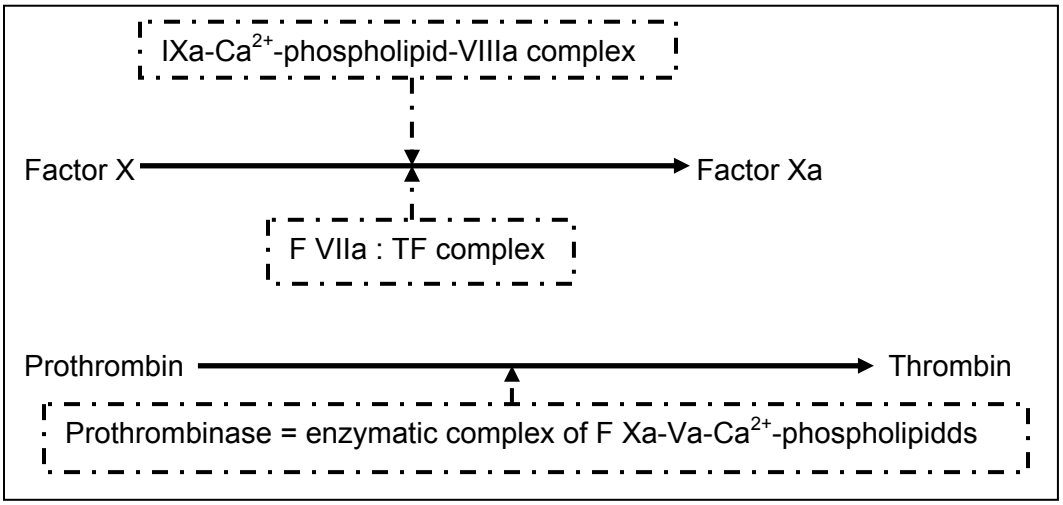
Factor VII is a vitamin K dependant glycoprotein synthesized in the liver.



F VII can only be activated if in complex with Tissue Factor (TF); Oval indicate F VII:TF complex; Solid arrow indicate activation of inactive protein (F VII) to is active counterpart (F VIIa); dashed arrow and box indicate factors/enzymatic complexes/co-factors needed for activation of protein.

F VIIa : TF complex can activate either factor X or factor IX and is inhibited by tissue factor pathway inhibitor (TFPI). TFPI forms a complex with factor Xa then with F VIIa : TF complex. This results in the formation of an inactive tetramolecular complex. F VIIa : TF complex could be inhibited by antithrombin in the presence of heparin. Acquired deficiency of factor VII associated with deficiencies of other coagulation factors: intake or absorption deficiency of vitamin K (hemorrhagic disease of newborn, obstructive icterus, antibiotic therapy); oral anticoagulant therapy; hepatic disorders; fibrinolysis; disturbed intravascular coagulation (DIC) (Package insert – STA Deficient VII; Ref 00743). Factor VII concentrations were decreased after exposure to aspartame. Decreased concentrations of factor VII, leads to decreased amounts of active enzyme of this factor, which in turn can lead to a decrease in conversion of factors X and IX to their active forms. F Xa is necessary for conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin. Thus a deficiency of F Xa could indirectly lead to bleeding disorders as fibrin cannot be formed (lack of thrombin).

Factor X is a vitamin K dependant glycoprotein which is synthesized in the liver.

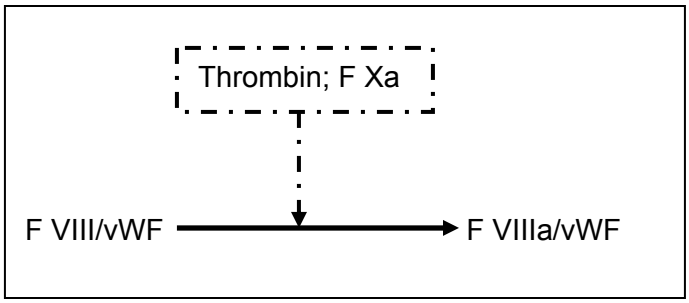


Solid arrows indicate activation of inactive protein (F X) to its active counterpart (F Xa); F X can be activated via 2 pathways, with 2 different enzymatic complexes indicated by dashed arrows and boxes; activated F Xa is needed for the conversion of prothrombin to thrombin as indicated by the dashed arrow and box.

Factor Xa can activate factor VII to F VIIa. Factor Xa is inhibited by antithrombin III, associated or not with heparin. Acquired deficiencies of factor X associated with deficiencies of the factors II, VII and IX: oral anticoagulant therapy; intake or absorption deficiency (hemorrhagic disease of the newborn; obstructive icterus; antibiotic therapy). Acquired deficiencies of factor X associated with deficiencies of the factors II, V and VII: hepatic disorders (cirrhosis; hepatitis); fibrinolysis; disrupted intravascular coagulation (Package insert – STA Deficient X; Ref 00738). Factor X was decreased after treatment with aspartame. Factor X can be activated either via IXa-Ca^{2+}-phospholipid-VIIIa complex or the F VIIa : TF complex. The amount of circulating F VII decreased after exposure to aspartame, thus activation via this route could be hindered due to inability of complex formation between F VII and Tissue factor occurring, which further prevents activation of F VII. The other pathway of activation involves F IXa, F VIIIa, Ca^{2+} and phospholipids. As was indicated, the amount of circulating F VIII was decreased after treatment with aspartame. Although the amount of F IX was not affected, there will be complications in the formation of the enzymatic complex for activation of F Xa. Factor X is needed in its activated form for conversion of prothrombin to thrombin, thus a deficiency of F X indirectly leads to a deficiency of thrombin.

Activated partial thromboplastin time (aPTT) is a general coagulation screening test of the intrinsic coagulation pathway (factors XII, XI, VIII, X, V, II and I). Acquired deficiencies and abnormal conditions are caused by liver diseases, consumptive coagulopathy, circulation anticoagulants (antiprothrombinase or circulating anticoagulant against a factor), during heparin or oral anticoagulant therapy, treatment with thrombin inhibitors (Package insert - PTT Automate 5; Ref 00482). The aPTT was not affected by the treatment with aspartame.

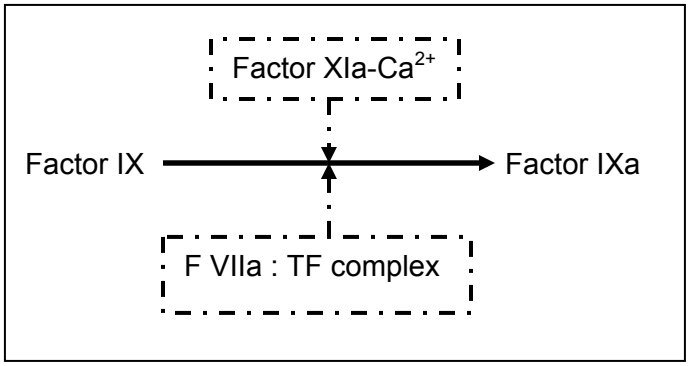
Factor VIII is a glycoprotein which is synthesized and released into the blood stream by the vascular endothelium (Rubin and Leopold, 1998). In the plasma, factor VIII circulates in a complexed form with the von Willebrand factor as F. VIII/vWF by means of a covalent linkage.



Solid arrow indicates the activation of the inactive protein (F VIII) to its enzymatic counterpart (F VIIIa). Note that F VIII can only be activated if in complex wit von Willebrand factor; dashed arrow and box indicate factors needed for activation of protein.

F VIIIa increases the activation of factor X by the F IXa in the presence of phospholipids and calcium (Package insert – STA Deficient VIII; Ref 00725). As seen from description for F X, the amount of factor VIII was decreased by treatment with the aspartame, thus activation of F X by the pathway including both factors VIIIa and IXa are indirectly inhibited. It was also noted that the amount of thrombin would be decreased due to the inability of F X to convert prothrombin to thrombin. It could therefore be stated that due to the decreased concentrations of both thrombin (indirectly caused by aspartame) and F X (caused directly by aspartame), a diminished capacity would exist for activation of factor VIII.

Factor IX is a glycoprotein synthesized by the liver and its synthesis is vitamin K dependant. This vitamin is necessary for the carboxylation of glutamic acid residues which are essential for the fixation of factor IX on platelet or tissue phospholipids in the presence of calcium ions.



Solid arrow indicates activation of the inactive protein (F IX) to its enzymatic counterpart (F IXa); Note that F IX can be activated via two pathways, either F XIa-Ca^{2+} complex or F VIIa:TF complex as indicated by the dashed arrows and boxes.

Factor IX can be activated via two pathways, either via a F XIa-Ca^{2+} complex or the F VIIa : TF complex. Factor IXa forms an enzymatic complex with phospholipids, Ca^{2+} and factor VIIIa which then activates factor X (Package insert – STA Deficient IX; Ref 00724). It was noted earlier in the summary that F VII concentrations were decreased by aspartame, thus it is hypothesized that factor IX was activated via the other pathway. Aspartame did not affect the concentration of circulating factor IX.

Fibrinogen is a glycoprotein that is synthesized in both the liver and by megakaryocytes. Fibrinogen is composed of six chains: two Aα, two Bβ and two γ. Thrombin (F IIa) breaks up the fibrinogen molecule to split out two fibrinopeptide A (FPA) fragments from the Aα chains and two fibrinopeptide B (FPB) fragments from the Bβ chains. The fibrin monomers that are produced from these reactions then aggregate to form fibrin, which is subsequently stabilized by factor XIIIa. The first step of stabilization consists of the binding of the two γ chains of the two fibrin monomers. This binding is the origin of the D-dimer, the degeneration product that is specific of fibrin. Fibrinogen can be degraded by plasmin. An increase in fibrinogen levels is usually found in cases of diabetes, inflammatory syndromes and obesity. A decrease of the fibrinogen level is observed in DIC and fibrinogenolysis. Furthermore, fibrinogen seems to be involved in the pathogenicity of thrombotic cardiovascular events (Package insert – Fibri-Prest

Automate 2; Ref 00854). Aspartame increased the concentration of circulating fibrinogen. This could be due to the fact that F VII levels and F X levels were decreased, and they are two key factors necessary for the conversion of prothrombin to thrombin. Decreased levels of thrombin leads to higher concentrations of circulation fibrinogen, which was the case after treatment with aspartame.

5.5 CONCLUSION

Blood coagulation involves a biological reinforcement system where a number of circulating proteins form part of a cascade of reactions to for a fibrin clot. The coagulation pathway is activated and coagulation occurs through two pathways known as the intrinsic and extrinsic pathways, made up of twelve different coagulation factors as shown in Diagram 5.1 in the introduction.

For the first part of the study which compares the concentration of the different factors tested between humans and rabbits, it was concluded that there were a number of differences in the quantities of the factors between humans and rabbits, when taking the results obtained in this study into account (Table 5.2). Literature indicates that the rabbit is a good candidate for testing pharmaceutical products. This study established normal ranges for rabbits and therefore rabbits can be used successfully as a model in testing the effect of pharmaceutical products on the different coagulation factors.

The second part of the study focused on the effects of aspartame on the concentration of different coagulation factors. Different factors in the different pathways were studied, and the following can be summarized:

- F II, F V, F IX and the recalcification time of the plasma (aPTT) was not adversely affected by treatment with aspartame.
- The first concentration of aspartame, 34mg/kg, did not adversely affect the tested factors, except for fibrinogen, where a significant increase (P=0.018 two-tailed) was noted. Thus it could be stated that if aspartame were to be taken in small doses, like

the above mentioned concentration, it would not adversely affect blood coagulation except for higher concentrations of circulation fibrinogen being present.

- The 100mg/kg aspartame treatment affected a number of the tested factors. The PT was prolonged, which could be an indication that a deficiency of/or the presence of inhibitors of FI (fibrinogen), factor II (FII), FV, FVII or FX were present. It was found during this study that the concentration of F VII and F X indeed decreased, but not significantly for this concentration. It also did not significantly adversely affect the concentration of F VIII and F IX. However, the concentration of fibrinogen was significantly higher (*P*=0.001 two-tailed) than the values obtained for the first and last concentration of aspartame.

- The values obtained for the PT for the last concentration of aspartame, 150mg/kg, never exceeded the normal set ranges. There was a significant decrease ($p<0.05$) of circulating amounts of F VII, FX and F VIII after treatment with this concentration. F IX was not adversely affected by the last concentration of aspartame. There was a significant increase (*P*=0.0036 two-tailed) in the amount of circulating fibrinogen after treatment with this concentration. A significantly higher concentration of fibrinogen was present than after treatment with 34mg/kg aspartame, but the amount of fibrinogen was significantly less than after treatment with 100mg/kg aspartame. However, an overall increase of fibrinogen was noted.

Factor VII is needed for activation of F IX. Thus lower concentrations of F VII could indicate an inability to activate F IX. Although F VII was decreased, F IX was not adversely affected by the treatment with aspartame. F VII is activated by tissue factor and circulating Ca^{2+} ions, thus a deficiency of any of these 2 cofactors and treatment with the aspartame could have lead to the decrease in circulating F VII. Activated F IX is needed for activation of F X, together with activated F VII, activated F VIII and Ca^{2+} ions. Lowered concentrations of activated F X leads to the inability of prothrombin to be converted to thrombin, which in turn is needed for activation of F VIII. Thus, lower concentrations of activated cofactors F VII and FVIII will indirectly lead to decreased concentrations of F X, which in turn will lead to decreased amounts of thrombin. These findings were supported by the results obtained on the START 4, where decreased concentrations of F VII, F X and F VIII were noted. Thus, it could be said that lower amounts of

prothrombin is converted to thrombin after treatment with aspartame, due to the decrease in concentrations of F VII, F X and F VIII. A decrease in the amount of thrombin could lead to the inability to convert fibrinogen to fibrin and activation of FXIII (needed to convert unstable fibrin to stable fibrin), which explains the high amounts of circulating fibrinogen found in the results.

The results obtained on the START 4 were also corroborated with results obtained in other chapters of this thesis. When the plasma, obtained from the aspartame treated rabbits, was utilized for creating fibrin clots (Chapter 5), the high amount of circulating fibrinogen was converted to fibrin by addition of human thrombin to the plasma. The clots obtained were studied with SEM and it was evident that the change in the amount of fibrin fibres present when compared to the controls and the change in the morphology of the major fibres and mat-like minor fibres could be contributed to the high concentrations of circulating fibrinogen.

Thus, it can be concluded that overuse of aspartame (100mg/kg aspartame and higher per day) may cause bleeding disorders as a number of the important factors (F VII, F X and F VIII) needed for production of thrombin were significantly decreased. Deficiency of thrombin leads to the inability of conversion of fibrinogen to fibrin, and activating of F XIII to stabilize fibrin fibres were also compromised. Thus, seeing that the rabbit and humans have similar fibrin network and platelet aggregate morphology (Chapter 4) and similar amounts of circulating fibrinogen (Table 5.3) it could be postulated that humans with bleeding tendencies should minimize their consumption of aspartame.

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 al 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 if 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 and150mg/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 166.67μl of 0.109M trisodium citrate. The blood was used to from 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 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 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 (o.15M PO_4) for 5 minutes before being fixed for 1 hour with Osmium tetraoxide (OsO_4) (50% PO_4 buffer at pH=7.4 and 50% OsO_4 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)

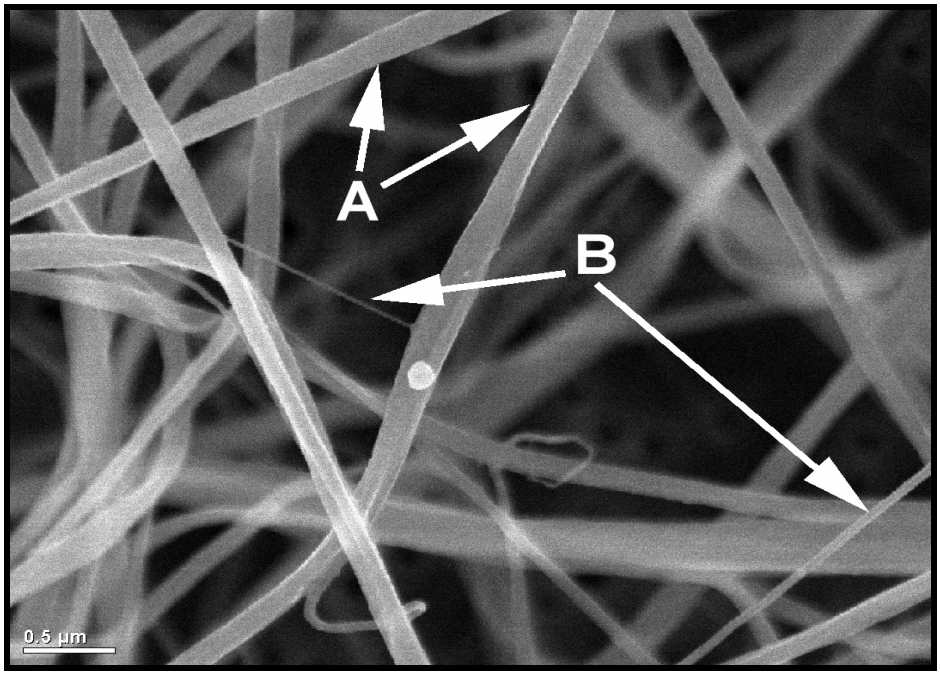


Figure 6.1b: Higher magnification of the fibrin network of the control rabbit, illustrating major fibers (Label A) and minor fibers(Label B) (x10 000 magnification)

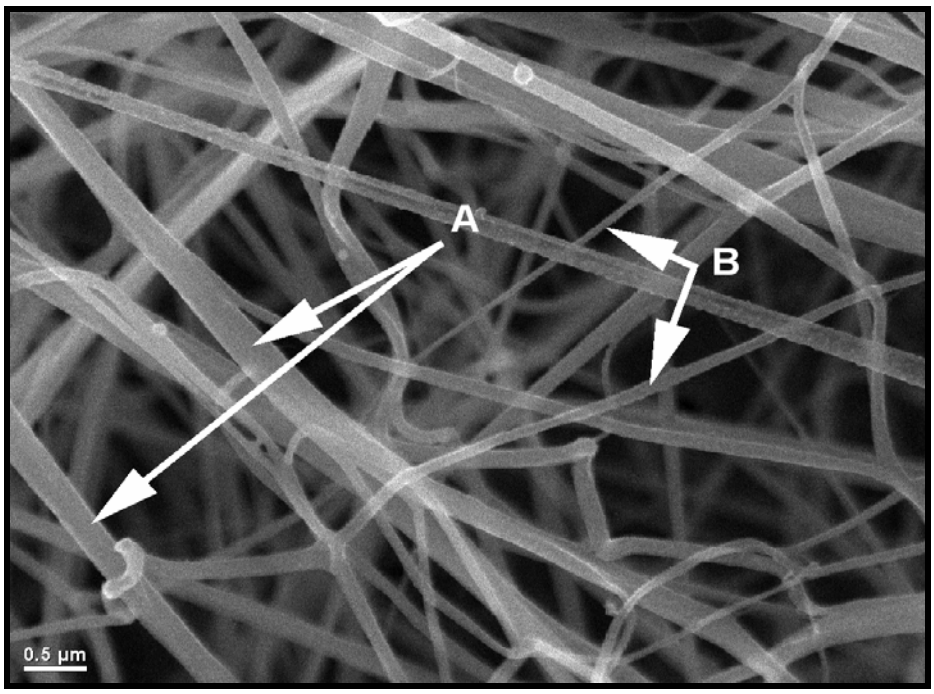
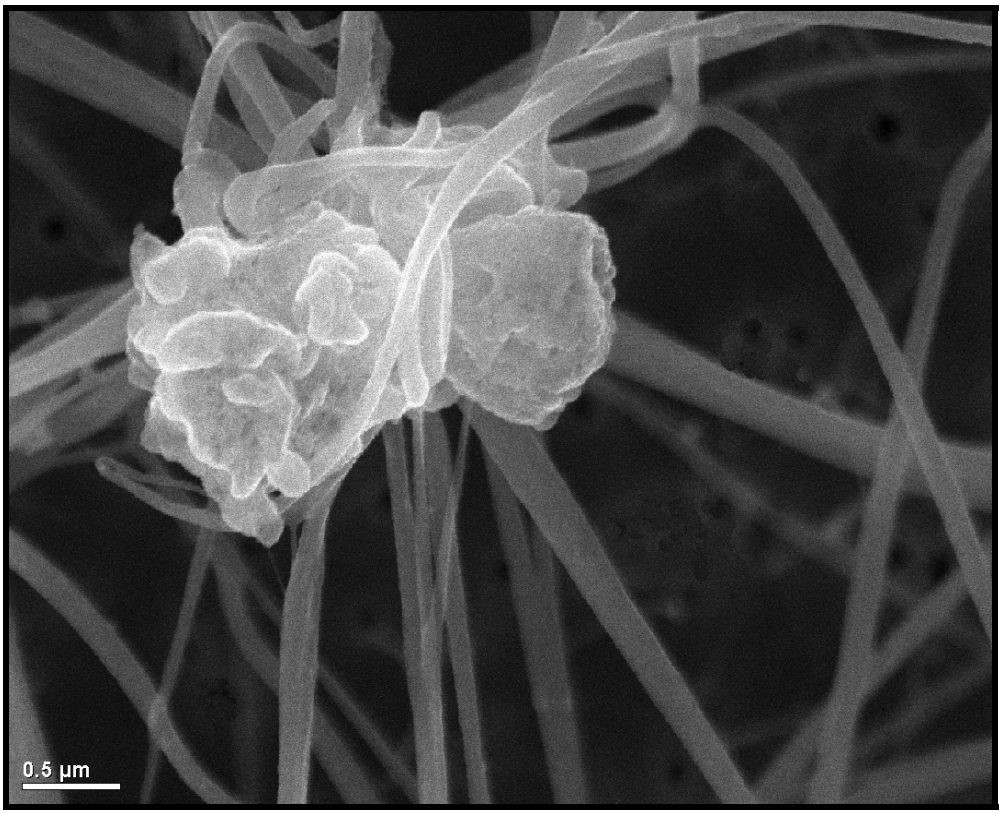


Figure 6.2: Rabbit platelet aggregate illustrating globular, pseudopodia-like morphology (x10 000 magnification)



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)

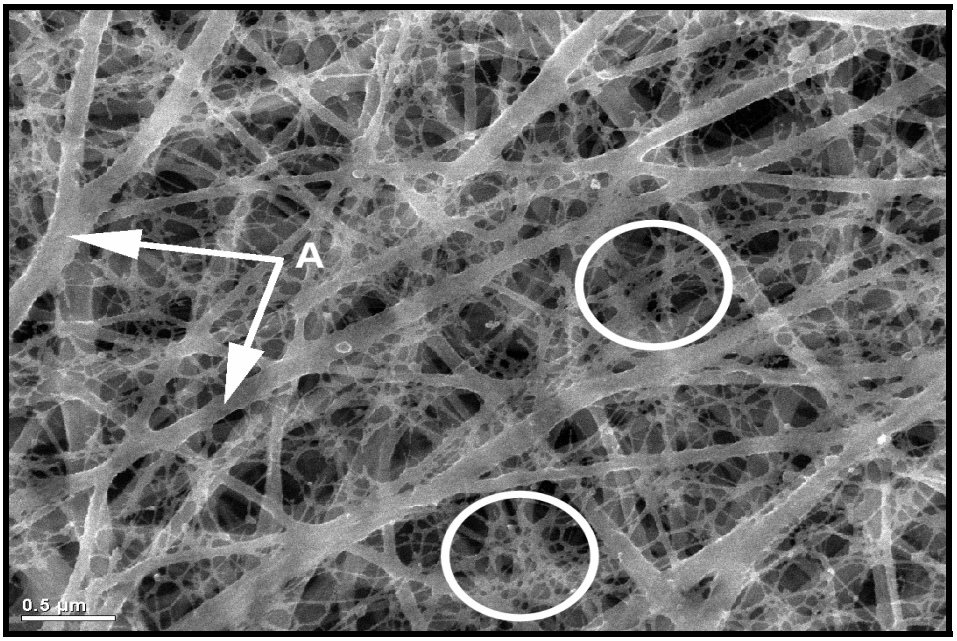


Figure 6.4: Platelet aggregate of a rabbit exposed to the 34mg/kg aspartame. Platelet aggregate more granular appearance, but globular pseudopodia-like morphology still present. (x10 000 magnification)

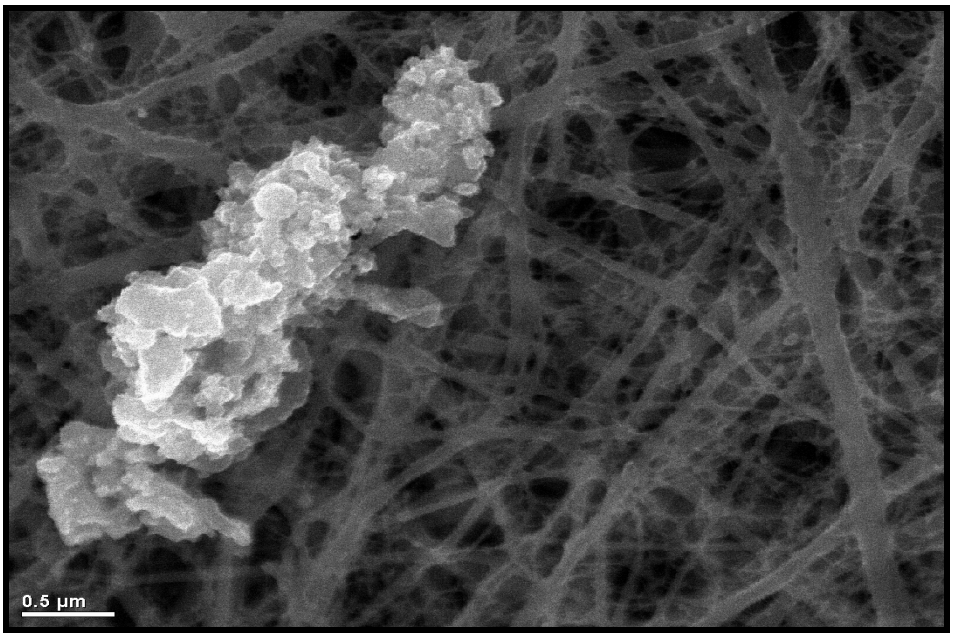


Figure 6.5 illustrates the fibrin network and figure 6.6 the morphology of the platelet aggregate of the rabbit after treatment with 100mg/kg aspartame.

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)

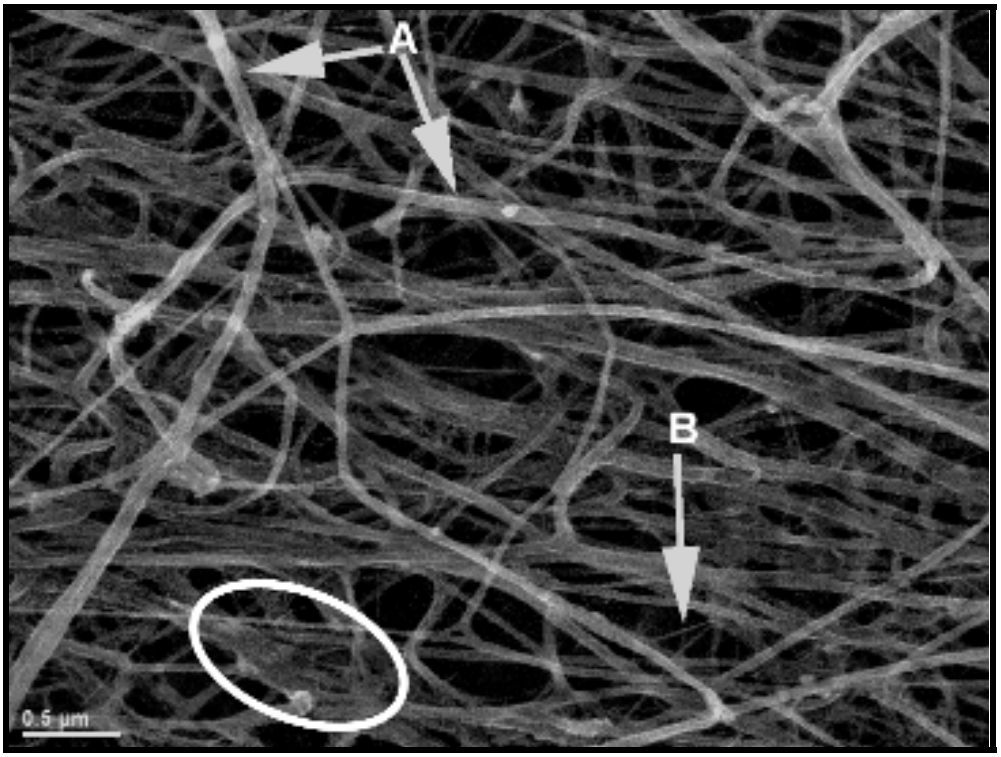


Figure 6.6: Platelet aggregate of a rabbit treated with 100mg/kg aspartame. Platelet aggregate show globular, pseudopodia-like morphology with disheveled membranes (x10 000 magnification)

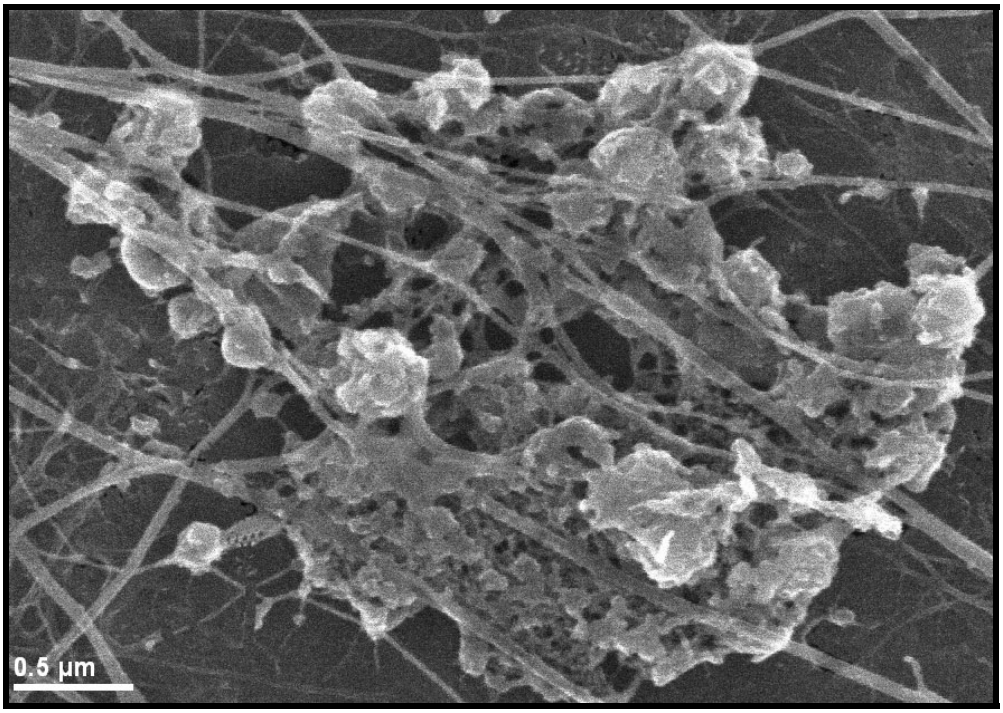


Figure 6.7 illustrates the rabbit fibrin network and figure 6.8 the platelet aggregate morphology after treatment with 150mg/kg aspartame.

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)

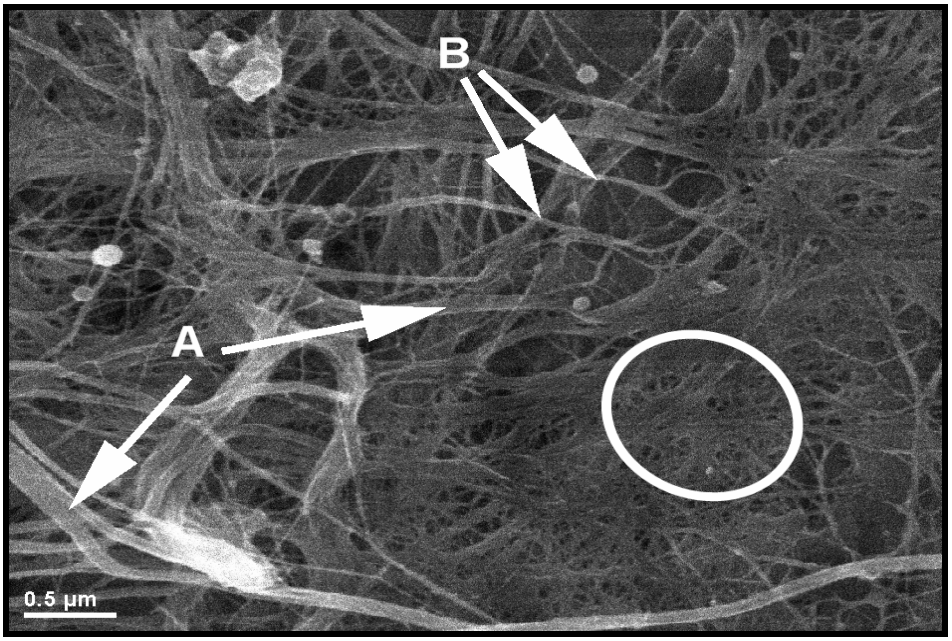
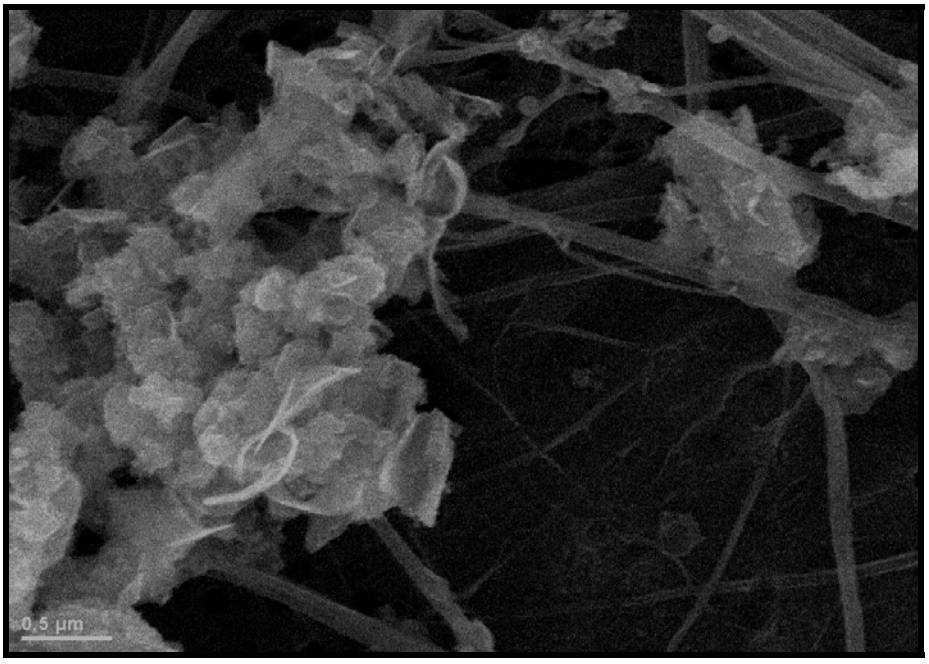


Figure 6.8: Platelet aggregate of a rabbit treated with 150mg/kg aspartame (x10 000 magnification)



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 staring 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, fibrinonectin, 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 is 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 to 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.

Chapter 7 –

Effects of aspartame on the morphology and count of the different leukocytes and the ultra-structure of the endothelial lining of the aorta of the rabbit

RESEARCH QUESTION 5:

Has the morphology and number of the leukocytes (light microscopy) changed after treatment with aspartame and how is the endothelial lining (SEM and TEM) of the blood vessels affected when treated with aspartame?

7.1 INTRODUCTION

The blood coagulation system does not only consist of coagulation factors, fibrin networks and platelet aggregates which is in constant equilibrium, but also cells and plasma. Blood itself consists out of plasma, erythrocytes and leukocytes. The plasma contains all the circulating coagulation factors and proteins needed to form fibrin networks and for release of platelet granules to form platelet aggregates. The erythrocytes are necessary for transport of oxygen, while the leukocytes play a key role in the immune system. The blood vessels through which the blood flows also forms an integral part of this whole system in equilibrium as it forms part of the intravascular anti-coagulation mechanism. Thus, having focussed on the effects of aspartame on the coagulation factors (Chapter 5), fibrin networks and platelet aggregates (Chapter 6), the aim of this chapter was to determine the effects of aspartame on the leukocytes (morphology and count) and the endothelial lining.

Most of the leukocytes in rabbits and guinea pigs appear very similar to those of other mammals. Novices may easily mistake heterophils (lapine and cavian equivalent of neutrophils) for eosinophils. Heterophils have the same function as other mammalian neutrophils, but they have acidic or eosinophilic granules in their cytoplasm. They are sometimes referred to as “pseudoeosinophils” in the literature. Heterophils are present in a number of animal species including birds, reptiles, amphibians, some fish, rabbits, guinea pigs and hamsters. The functions of the leukocytes of rabbits are similar to those of other mammals, including humans (Lester, Tarpley and Latimer, 2005).

Zhang and co-workers in 1995 used scanning and transmission electron microscopy to determine the influence of 5-fluorouracil on the endothelium in small arteries of rabbits. 5-Fluorouracil (5-FU) is a widely used antieoplastic agent. 5-FU induced cardiotoxcicity is still a relatively unknown side-effect of this drug. This phenomenon could be due to a direct cytotoxic effect on the endothelial cells. Zhang and co-workers tested this hypothesis in an experimental

study in rabbits, by scanning or transmission electron microscopic evaluation of endothelium in small arteries (the central artery of the ear) after *in vivo* treatment with 5-FU. Both local and systemic effects of 5-FU on endothelium were studied 15, 30, 60 and 120 minutes after intra-arterial or intraperitoneal treatment. Perfusion fixation at physiological pressure and temperature was used in order to minimize damage to the endothelium during the preparation procedure. Irreversible cell damage was observed with 5-FU treated animals with disruption of the endothelial sheet and patchy exposure of the subendothelium, sometimes as focus for thrombus formation.

A quantitative SEM analysis was performed do determine the degree of injury to the endothelium of the rabbit aorta and carotid artery during experimental arteriosclerosis (Zaikina *et al*., 1982). The luminal surface of the aorta and the carotid artery in normal and cholesterol-fed rabbits was studied by scanning electron microscopy. To study the endothelial injury the vessels were perfused and stained under physiological pressure. The frequency of large and small endothelial defects was determined per surface unit of endothelium in the normal and experimental groups of rabbits. Loss of endothelial cells was regarded as a large defect, argyrophilic cells, craters, and stomata were regarded as small ones. It was found that the percentage of regions without endothelial cells was similar in both control rabbits and in rabbits with experimental atherosclerosis. The frequency of small endothelial defects increased in rabbits after 3 weeks of hypercholesterolemia but decreased to the control level after 6 weeks of hypercholesterolemia. In rabbits with 8 months of hypercholesterolemia the frequency in defects outside the plaques did not differ from the control group. In the group with hypercholesterolemia for 8 months 39.2% of the plaque surface contained endothelial cells in which there were no distinct silver-stained cell borders. The data that was obtained do not support the assumption that morphological endothelial injury is the structural precursor of plaque formation.

7.2 MATERIALS AND METHODS:

7.2.1 Light microscopy study of the leukocytes

7.2.1.1 Obtaining blood smears

The rabbits utilized for the When the last concentration of aspartame was completed, blood smears were used to determine the accumulative effect of the aspartame on the leukocyte count and morphology. After 1.5ml blood was drawn from the different rabbits and before the samples were centrifuged for obtaining plasma for the Start 4 coagulation and SEM studies, droplets of blood was taken from each rabbit to obtain the blood smears. A droplet of blood was touched to the clean surface of the slide on the right hand side. Another slide was placed at a 20^{o} angle on the first slide and to the left of the droplet of blood. The second slide was pulled to the right, till the droplet of blood touched this slide. When the blood was spread across the whole line of contact, the slide was pulled rapidly towards the left. This was done till all the blood disappeared or the other side of the slide was reached. The blood smears were air dried and stored till staining.

7.2.1.2 Staining of blood smears:

A combined Wright-Giemsa stain, developed by Wilcox in 1943, was utilized for staining of the blood smears (Humason, 1967). Giemsa powder was dissolved in glycerol and placed in an oven set at between 55-60^{o}C for 2 hours, while stirring occasionally. The mouth of the flask was covered with a double layer of aluminium foil to prevent absorption of moisture. After the 2 hours were complete, aged Wright's staining solution (2 g per 1000ml Methanol) was added to the Giemsa solution. This solution was left to stand overnight and additional Wright's solution was added the following day. The Wright-Giemsa stock solution was then filtered. A buffer solution was obtained from two different solutions in distilled water. Solution A was dibasic sodium phosphate dissolved in distilled water and solution B monobasic potassium phosphate dissolved in distilled water. The buffer was then prepared by diluting 61.1ml of solution A and 38.9ml of solution B in 900ml distilled water. A working stain was then prepared from this Wright-Giemsa stock solution by using 1 part Wright-Giemsa stock solution in 9 parts buffer solution.

This staining method was further combined with another staining method for old smears (Humason, 1967). Before the blood smears were stained with the working Wright-Giemsa stain, the smears were treated for 7 minutes in an alcohol-acetic acid solution (10 drops glacial acetic acid to 60ml absolute alcohol). This causes the old smears to stain more brilliantly.

The slides were then stained with the working Wright-Giemsa stain for 10 minutes. The stain was flushed from the slide with the prepared buffer which prevents precipitate on slides. The slides were then submerged in buffer solution for 1 minute. The slides were air dried by standing them on end and mounted.

7.2.1.3 Counting of blood smears

All blood smears were placed on white tissue paper to accentuate the appearance of the smears. The smears that had the same appearance and had the same degree of staining were then used for leukocyte counting on a Nikon Optiphod transmitted light microscope (Nikon Instech Co., Kanagawa, Japan). Smears were counted till a hundred leukocytes were distinguished. The number of zones that were counted to obtain the hundred cells was noted. These two methods were combined due to the difference in the appearance of the individual blood smears.

7.2.2 SEM and TEM studies of the endothelial cells of the aorta

7.2.2.1 Termination of the study to obtain tissue

After the treatment periods were completed, all the rabbits were euthanized by means of intra-peritoneal injection with sodium pentobarbitonehydrochloride (2ml) by a skilled person experienced in this technique (Mrs Annette de Freitas, animal technologist at the Medunsa campus of the University of Limpopo). Five rabbits, 3 controls and 2 treated with aspartame (with negative/adverse clotting profiles) were dissected for the soft tissue of their livers and kidneys and the arch of the aorta (for SEM and TEM). The organs were fixed in formaldehyde and reserved for light microscopy studies (discussed in following chapter) and the arch of the aorta fixed in 2.5% glutaraldehyde and reserved for SEM and TEM studies.

7.2.2.2 Preparation of the aorta for SEM and TEM

The dissected tissue was kept in the 2.5% gluteraldehyde and was kept at 8ºC till preparation for SEM and TEM. Care was taken that the fixative was in contact with the tissue at all times. The tissue was removed from the fixative and washed three times with Na/K phosphate buffer for 15 minute washes. This was followed by post-fixing with osmium tetraoxide (OsO_4) (50% PO_4 buffer at pH=7.4 and 50% OsO_4 from a 1% stock solution) for an hour. The osmium tetraoxide was removed from the tissue and three wash steps of 15 minutes each in Na/K phosphate buffer followed. The tissue was then dehydrated with different concentrations of ethanol and left in the last absolute ethanol wash. Tissue that was to be viewed with the SEM was then placed in the critical point dryer (Bio-Rad E3000; Watford, England).

Double-sided carbon tape was attached to bronze slides and the tissue was then placed onto the carbon tape. The slides were placed in a sputter coater (Polaron E5200C; Watford, England) and coated with Ruthenium (RuO4 vapour from a 0.5% solution for 30 minutes) and were then ready for viewing under the JSM-840 (JEOL, Tokyo, Japan).

The sections of aorta that were to be viewed with the TEM (Phillips EM301; Eindhoven, Netherlands) was placed in Quetol resin (Quetol 651, cured at 60^{o}C for 48 hours) and sectioned with an ultra-microtome (Reichert Ultracut E; Vienna, Austria). The sections were then coated with uranylacetate (to obtain contrast) for 30 minutes followed by a number of washing steps in 3 different washes of distilled water. The sections were then counter-stained with lead citrate for 3 minutes, followed with 3 washes of distilled water. The sections were then ready for viewing with the TEM (Phillips EM301; Eindhoven, Netherlands).

7.3 RESULTS AND DISCUSSION

7.3.1 Light microscopic study of the morphology of the leukocytes

Figure 7.1a and 7.1b compares the lymphocytes of the control and aspartame treated rabbits respectively.

Figure 7.2: Lymphocytes of a of a control rabbit (marked with circles) (Label a) compared to the lymphocyte of a rabbit treated with aspartame (Label b)

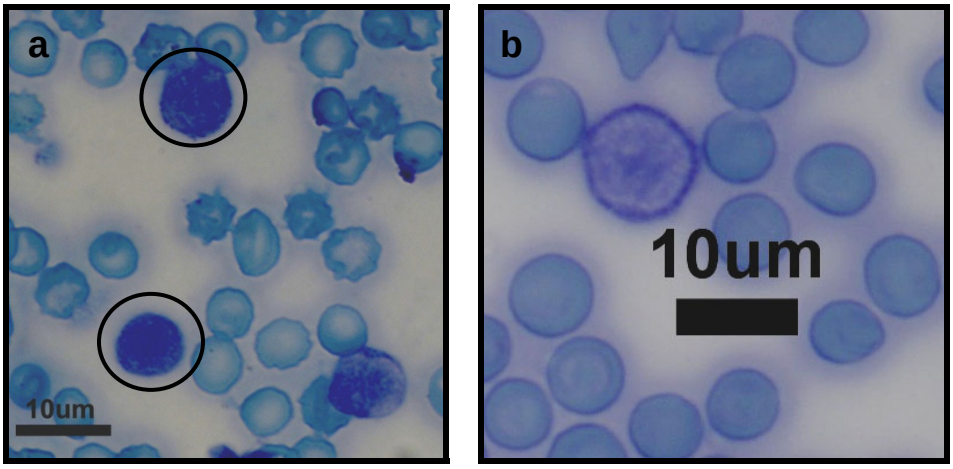


Figure 7.2a and 7.2b compares the monocytes of the control and aspartame treated rabbits.

Figure 7.2: Comparison between the monocytes of the control rabbit (marked with a solid circle; Label a) and the aspartame treated rabbits (Label b)

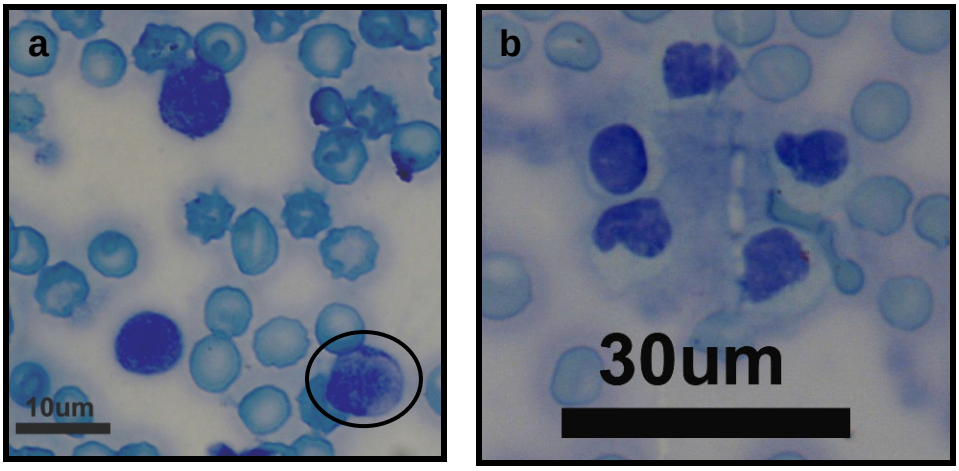


Figure 7.3a and 7.3b compares the basophils of the control and aspartame treated rabbits.

Figure 7.3: Comparison between the basophils of the control rabbit (Label a) and the aspartame treated rabbits (Label b)

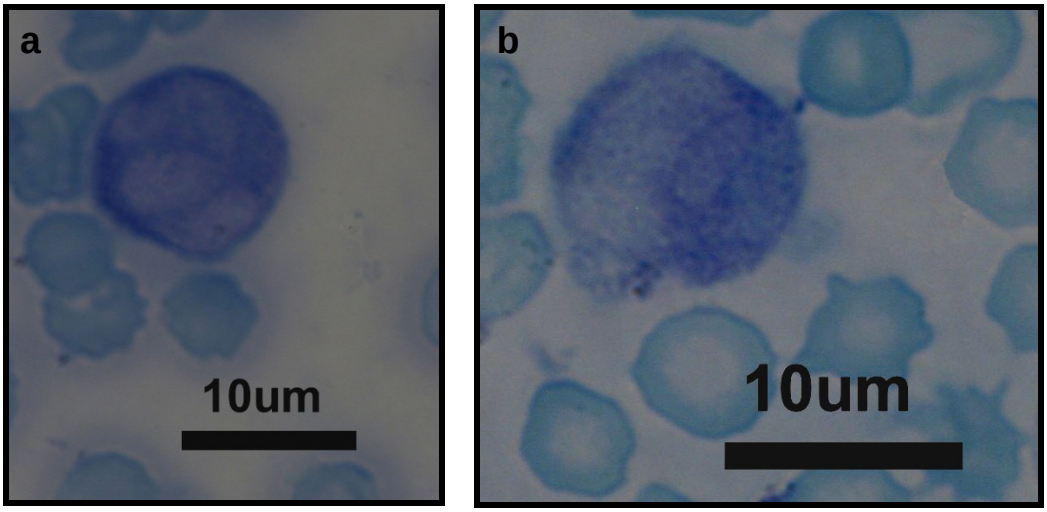


Figure 7.4a and 7.4b compares the eosinophils of the control rabbit and the aspartame treated rabbits respectively.

Figure 7.4: Comparison between the eosinophils of the control rabbit (inside the circle; Label a) and the rabbits after treatment with aspartame (Label b)

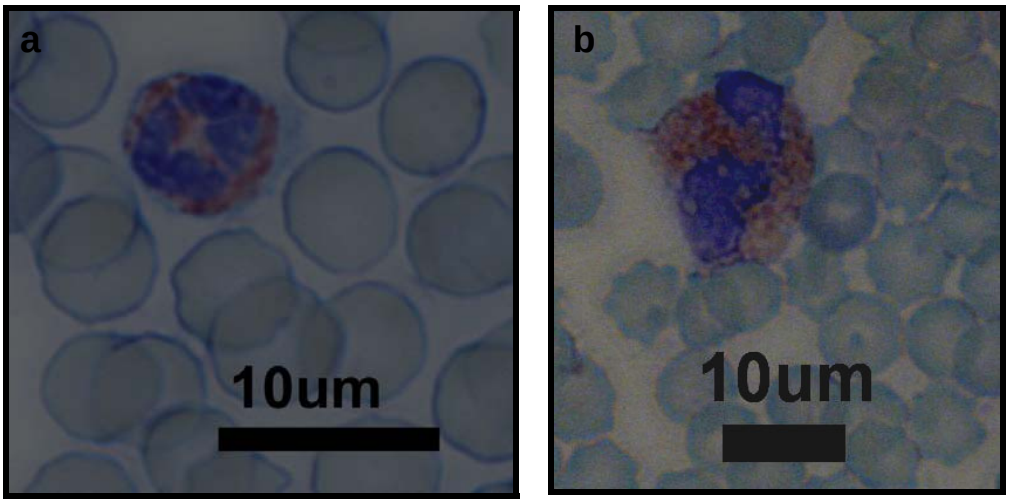
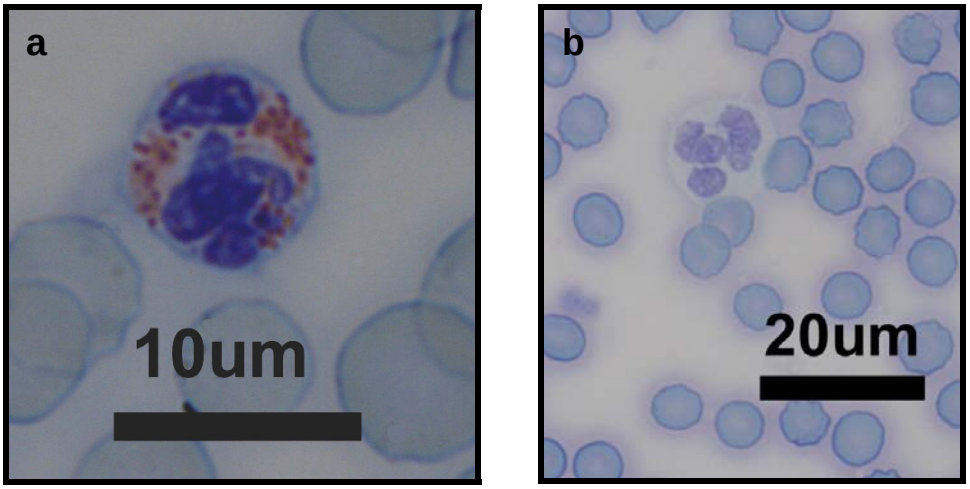


Figure 7.5a and 7.5b compares the heterophils of the control rabbit with that of the aspartame treated rabbits respectively.

Figure 7.5: Comparison between the heterophils of the control rabbit (Label a) and those of the rabbits after treatment with aspartame (Label b)



Morphology of the lymphocytes

The nuclei of the lymphocytes of the control rabbits stained dark blue with very little cytoplasm visible (Figure 7.1a). The lymphocytes for the rabbits treated with aspartame (Figure 7.1b) stained lighter blue, with the cytoplasm closely related to the cell membrane staining darker blue, almost forming a halo around the nucleus. It appeared as though the control rabbits had more densely packed chromatin throughout the nucleus. No difference was noted in the size of the lymphocytes between the control and aspartame treatment groups.

Morphology of the monocytes

The monocytes of the control rabbits possessed a dark staining nucleus with lighter areas of cytoplasm surrounding it. Densely packed chromatin was visible in the cytoplasm. A number of vacuoles could be seen as round white vesicles in the cytoplasm (Figure 7.2a). The size of the monocytes was not affected by the treatment with aspartame, but the morphology changed to a degree. The dark-blue stained nuclei could be distinguished quite easily with the characteristic bean-shape of the nucleus being prominent. The cytoplasm visible around the nucleus was larger and was almost transparent, not light blue like in the controls. No vacuoles were visible (Figure 7.2b). The cell membranes were clearly visible in both the control and aspartame treated rabbits.

Morphology of the basophils

No difference could be seen between the basophils of the controls and aspartame treated rabbits. Both exhibited bi-lobar nuclei with distinct blue staining granules in the cytoplasm. The cytoplasm of the control did stain a bit darker than the cytoplasm of the aspartame treated cells. The cell membrane of the control was also more clearly distinguishable than that of the basophils obtained from rabbits that were treated with aspartame. The size of the basophils was not adversely affected by the aspartame treatment.

Morphology of the eosinophils

The eosinophils of both the control and aspartame treated rabbit had the characteristic bi-lobar nuclei, with a distinct blue stain (Figure 7.4a and 7.4b). It appears as though the chromatin of the nucleus of the aspartame treated rabbits are more densely packed close to the nuclear envelope. The eosinophilic granules of the aspartame treated rabbits stained more brilliantly than that of the control (Figure 7.4b). There was no difference in the size of the cells after

treatment with aspartame. The cell membrane of the control rabbit was more clearly visible than that of the rabbits in the treatment group, staining light blue in colour.

Morphology of the heterophils

The nuclei of the heterophils of both the control and aspartame treated rabbits stained brilliantly blue and up to five lobes could be distinguished (Figure 7.5a and 7.5b). The chromatin of the nuclei of the aspartame treated rabbits appeared more densely packed (Figure 7.5b). The red granules in the cytoplasm stained more brilliantly in the control rabbit (Figure 7.5a) that that of the aspartame treated rabbits and individual granules could be distinguished. The granules of the heterophils of the aspartame treated rabbits (Figure 7.5b) seem to be less and individual granules could not be as clearly distinguished as in the control. The size of the cells was not affected by treatment with aspartame. The cell membrane of the control rabbit was more clearly visible than that of the rabbits in the treatment group, staining light blue in colour.

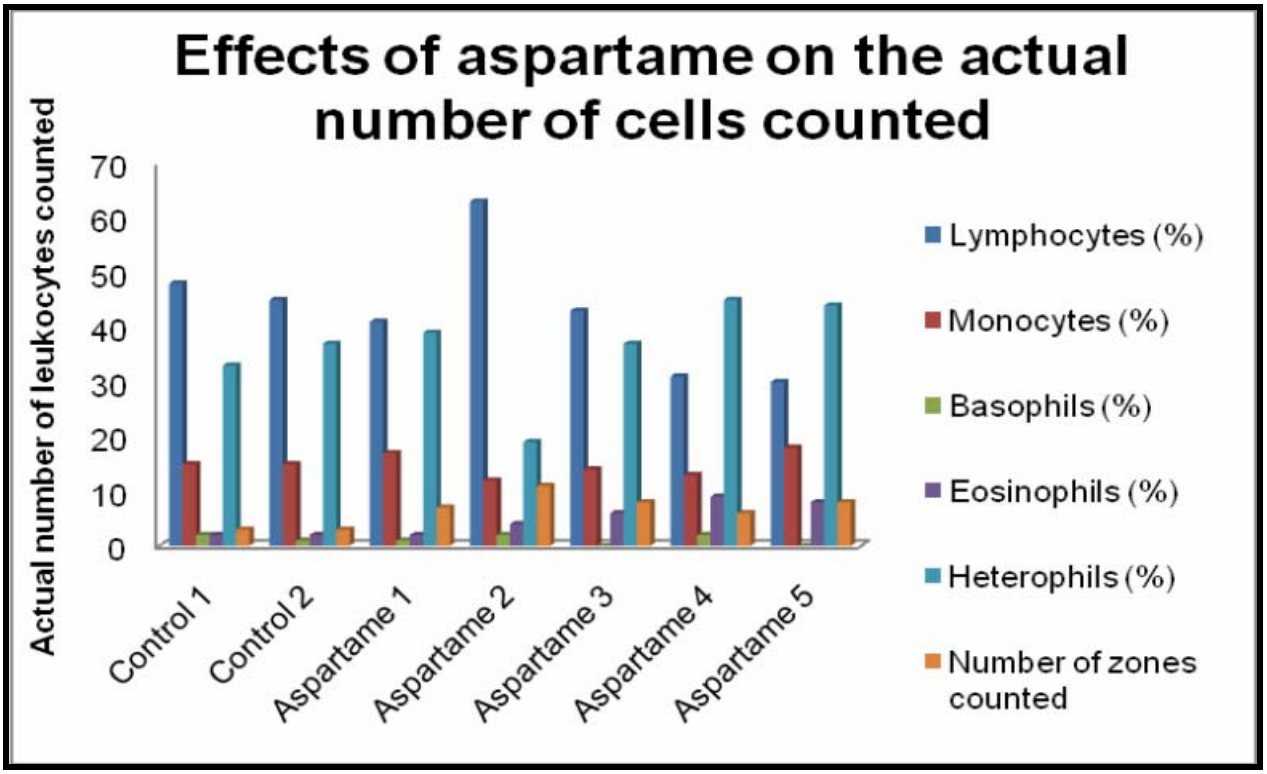
7.3.2 Light microscopic study of the number of leukocytes

After counting 100 leukocytes on each blood smear, the amount of each different leukocyte counted were expressed as a percentage of the 100 cells. These percentages are noted in Table 7.1. The table also indicates the number of zones that were counted to reach the 100 leukocytes.

Table 7.1: Number of leukocytes counted up to a 100 for each group, expressed as a percentage of that 100 leukocytes counted

Rabbits	Lympho-cytes (%)	Monocytes (%)	Baso-phils (%)	Eosino-phils (%)	Hetero-cytes (%)	Number of zones counted
Control 1	48	15	2	2	33	3
Control 2	45	15	1	2	37	3
Aspartame 1	41	17	1	2	39	7
Aspartame 2	63	12	2	4	19	11
Aspartame 3	43	14	0	6	37	8
Aspartame 4	31	13	2	9	45	6
Aspartame 5	30	18	0	8	44	8

Graph 7.1: The number of actual leukocytes counted for both the control and aspartame treated rabbits

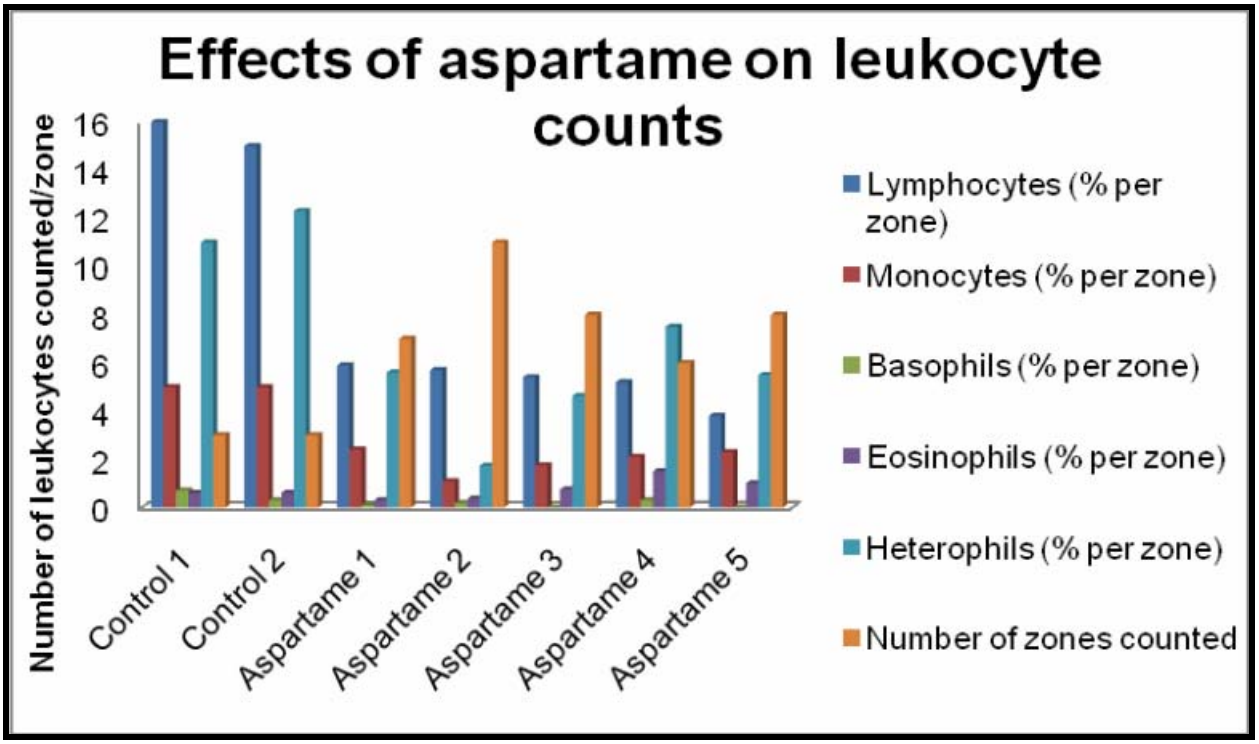


The number of zones was then used to determine the number of each specific leukocyte in each zone by dividing the total number of leukocytes counted through the number of zones counted (Table 7.2).

Table 7.3: Comparison between the number of leukocytes counted per zone for the control and aspartame treated rabbits

Rabbits	Lympho-cytes (% per zone)	Monocytes (% per zone)	Basophils (% per zone)	Eosinophils (% per zone)	Heterophils (% per zone)	Number of zones counted
Control 1	16	5	0.7	0.6	11	3
Control 2	15	5	0.3	0.6	12.3	3
Aspartame 1	5.9	2.4	0.1	0.3	5.6	7
Aspartame 2	5.7	1.09	0.18	0.36	1.73	11
Aspartame 3	5.4	1.75	0	0.75	4.63	8
Aspartame 4	5.2	2.1	0.3	1.5	7.5	6
Aspartame 5	3.8	2.3	0	1	5.5	8

Graph 7.2: Different leukocyte counts for the number of zones counted in both the control and aspartame treated groups



The number of zones counted in the controls was the same with small/no differences in the number of different leukocytes counted. The number of zones that was counted to obtain the 100 cells after treatment with aspartame increased from 3 to between 6-11 zones in the aspartame treated rabbits.

Table 7.4: Different leukocytes expressed as a percentage of the number of leukocytes in the control group

Rabbits	Lymphocytes	Monocytes	Basophils	Eosinophils	Heterophils
Mean of controls	15.5	5	0.5	0.6	11.65
Mean of aspartame treated rabbits	5.2	1.928	0.116	0.782	4.992
Percentage of cells after treatment	33.55	38.56	23.2	130.33	42.85

The number of lymphocytes obtained after treatment with aspartame decreased with 33.5% of that found in the control. A decrease in the number of cells were found and monocytes decreased by 38.52%, basophils by 23.2%, and heterophils by 42.85%. The eosinophils however increased by 30.33% if compared to the number of eosinophils counted in the control groups.

7.3.3 SEM study of the endothelial cells of the aorta

Figure 7.6a and 7.6b illustrate the endothelial lining of a control rabbit.

Figure 7.6a: Endothelial lining of the control rabbit. White arrows indicate some of the many microvilli of the aorta (x550 magnification)

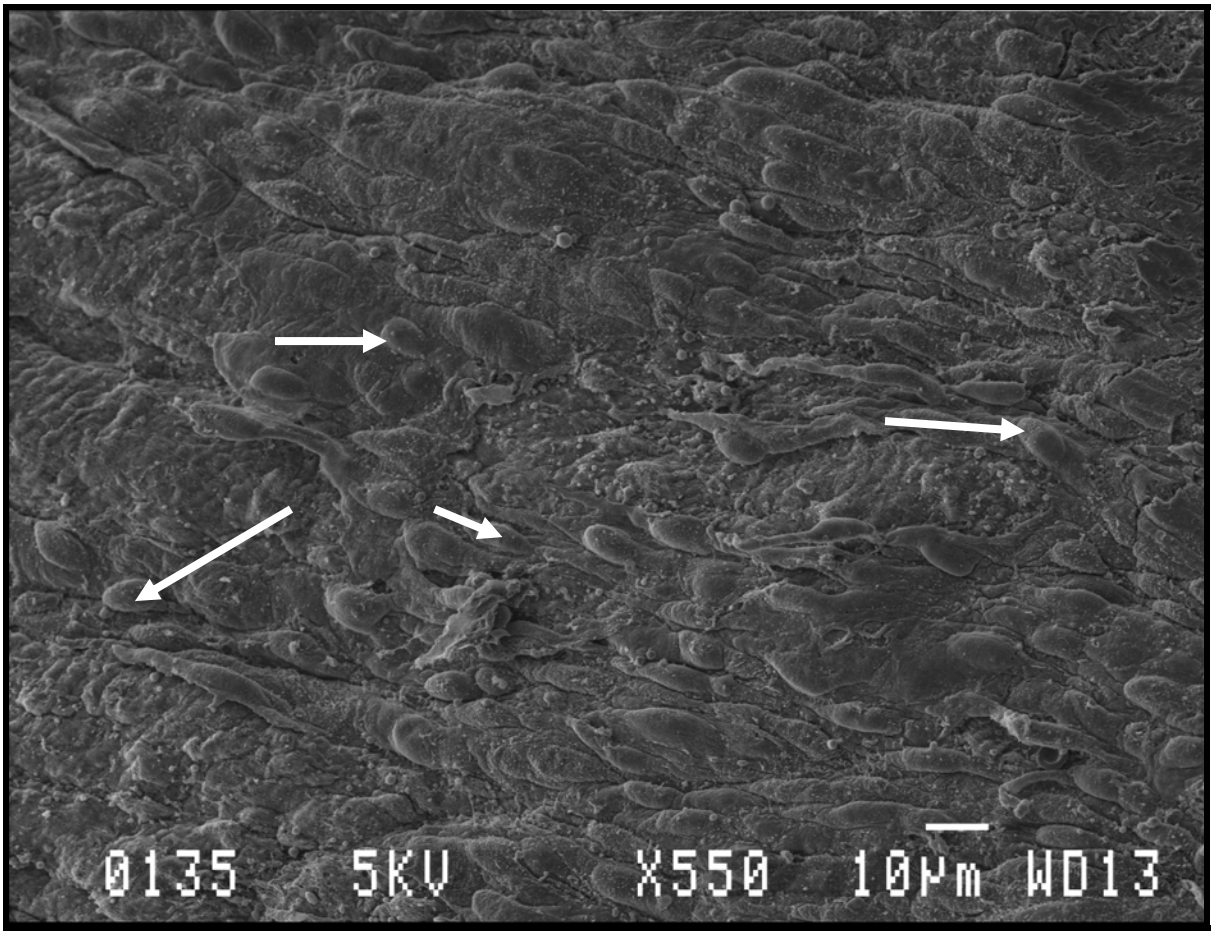


Figure 7.6b: Endothelial lining of the control rabbit (x500 magnification)

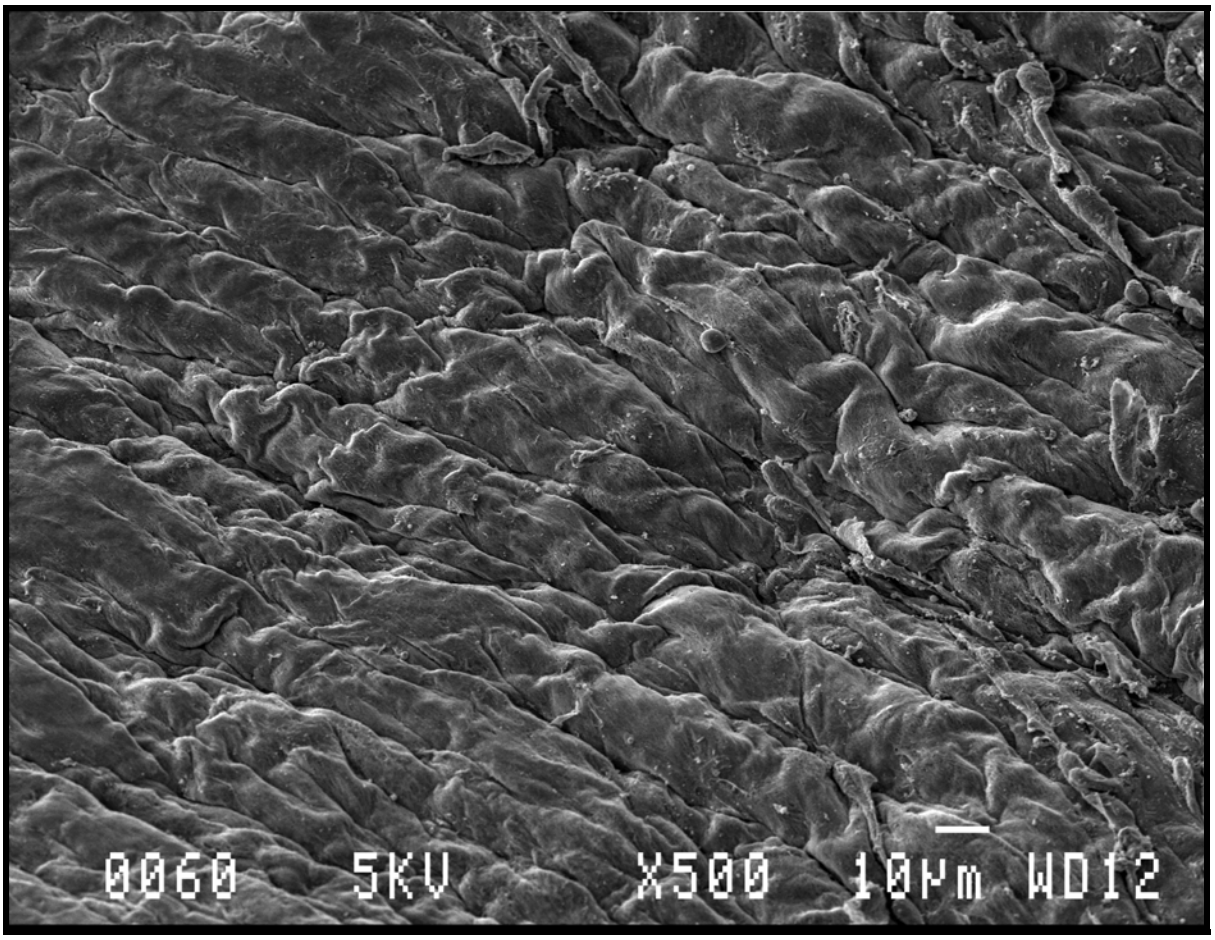


Figure 7.7 illustrates the endothelial lining of the control rabbit at an even higher magnification (x 3 500 magnification)

Figure 7.7: Smooth surface of the endothelial lining of a control rabbit. Circle indicate small microvilli of the aorta (x 3 500 magnification)

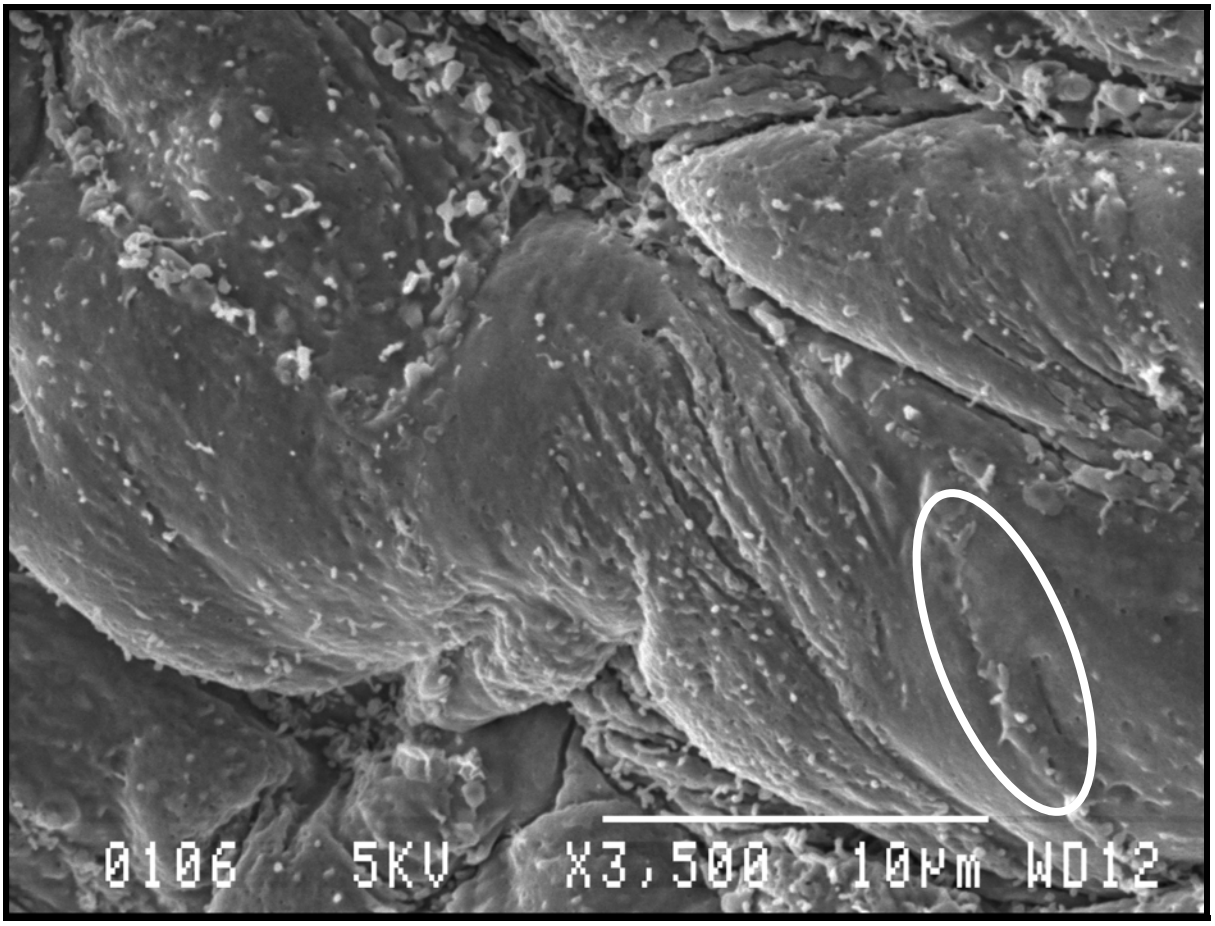


Figure 7.8 illustrates the endothelial lining of the rabbits after treatment with aspartame.

Figure 7.8: Endothelial lining of a rabbit after treatment with aspartame (x550 magnification)

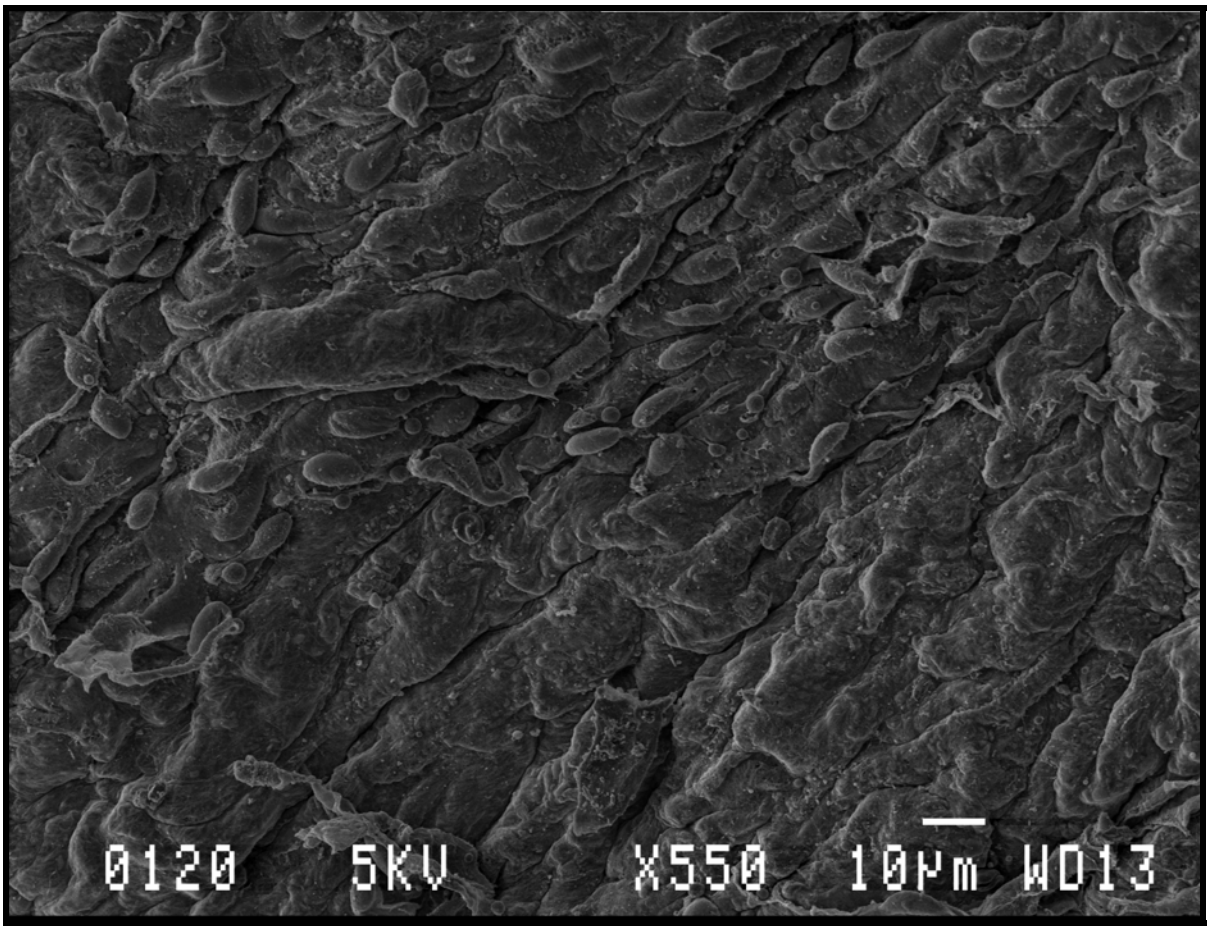


Figure 7.9a and 7.9b illustrates the endothelial lining of the same rabbit as in figure 7.8, only at x3 500 magnification.

Figure 7.9a: Endothelial lining of a rabbit after treatment with aspartame. Note the damage to the microvilli indicated by the circle (x3 500 magnification)

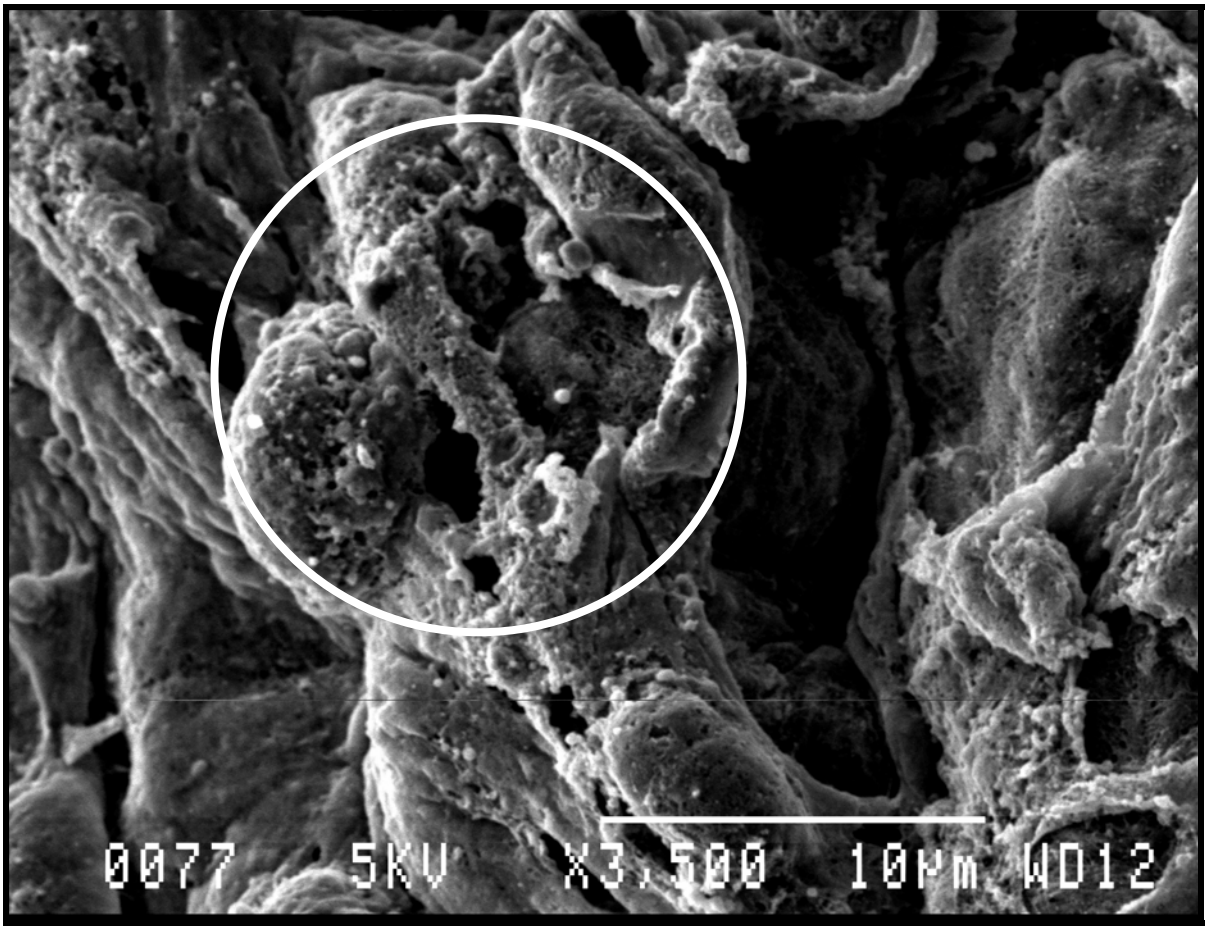


Figure 7.9b: Endothelial lining of a rabbit treated with aspartame. Arrows indicate filament fibers of the cytoskeleton. Note how contents of cell were spilling to outwards (x3 500 magnification)

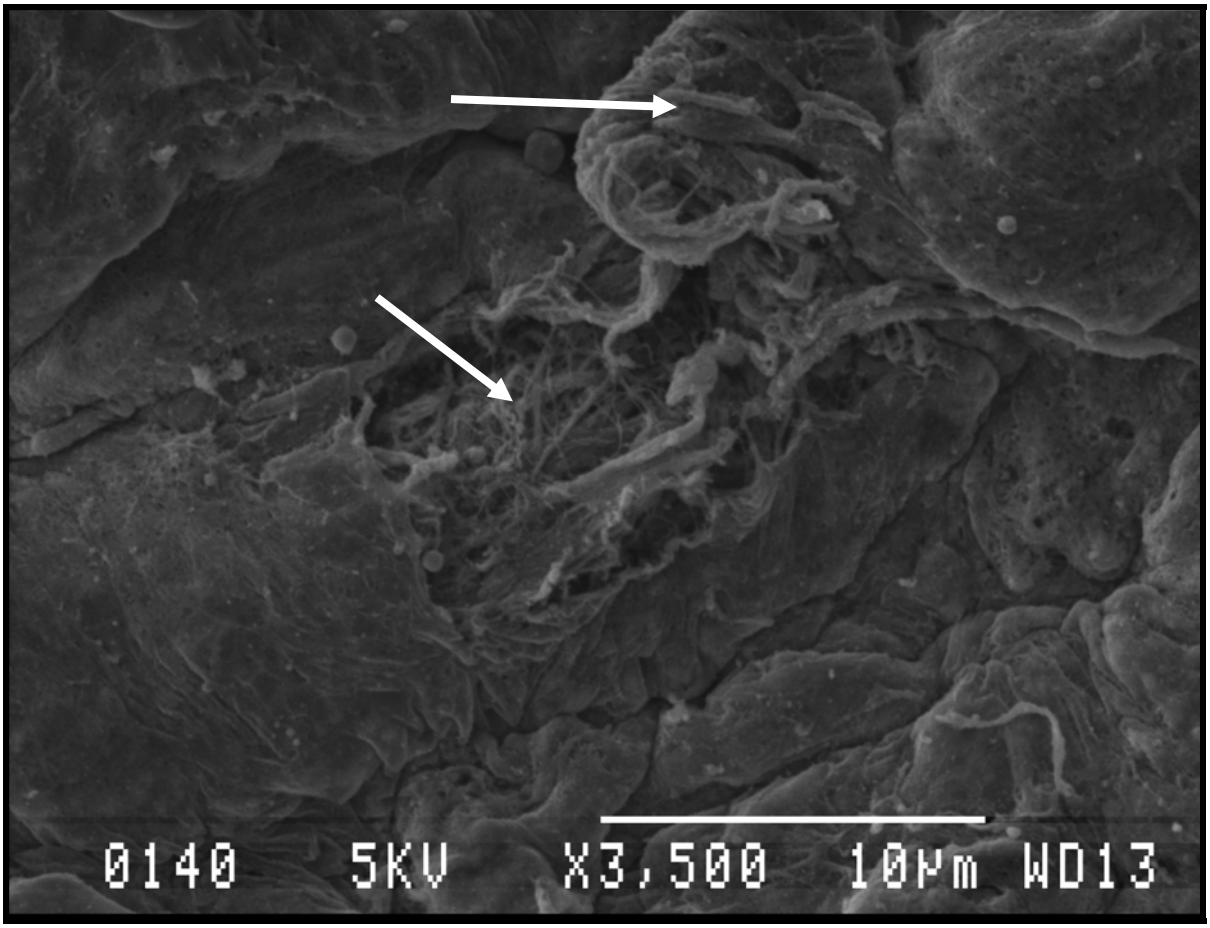


Figure 7.10 illustrates the effects of aspartame on the endothelial lining of a different rabbit after treatment.

Figure 7.20: Endothelial lining of a different rabbit after treatment with aspartame (x 550 magnification)

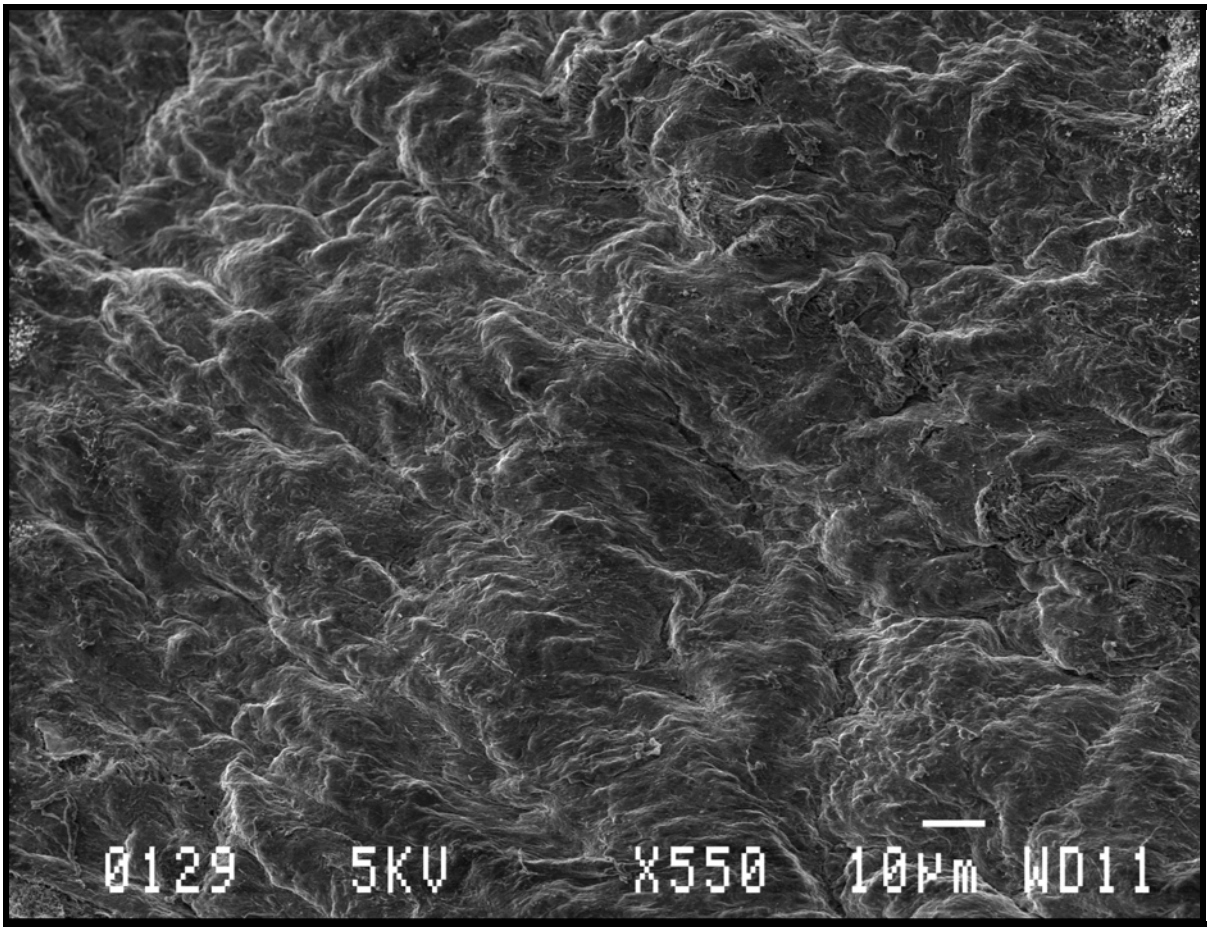


Figure 7.11 illustrates the endothelium in figure 7.10 at a higher magnification.

Figure 7.11 Endothelial lining of a rabbit after treatment with aspartame. The white arrows indicate the filament fibers of the cytoskeleton (x3 500 magnification)

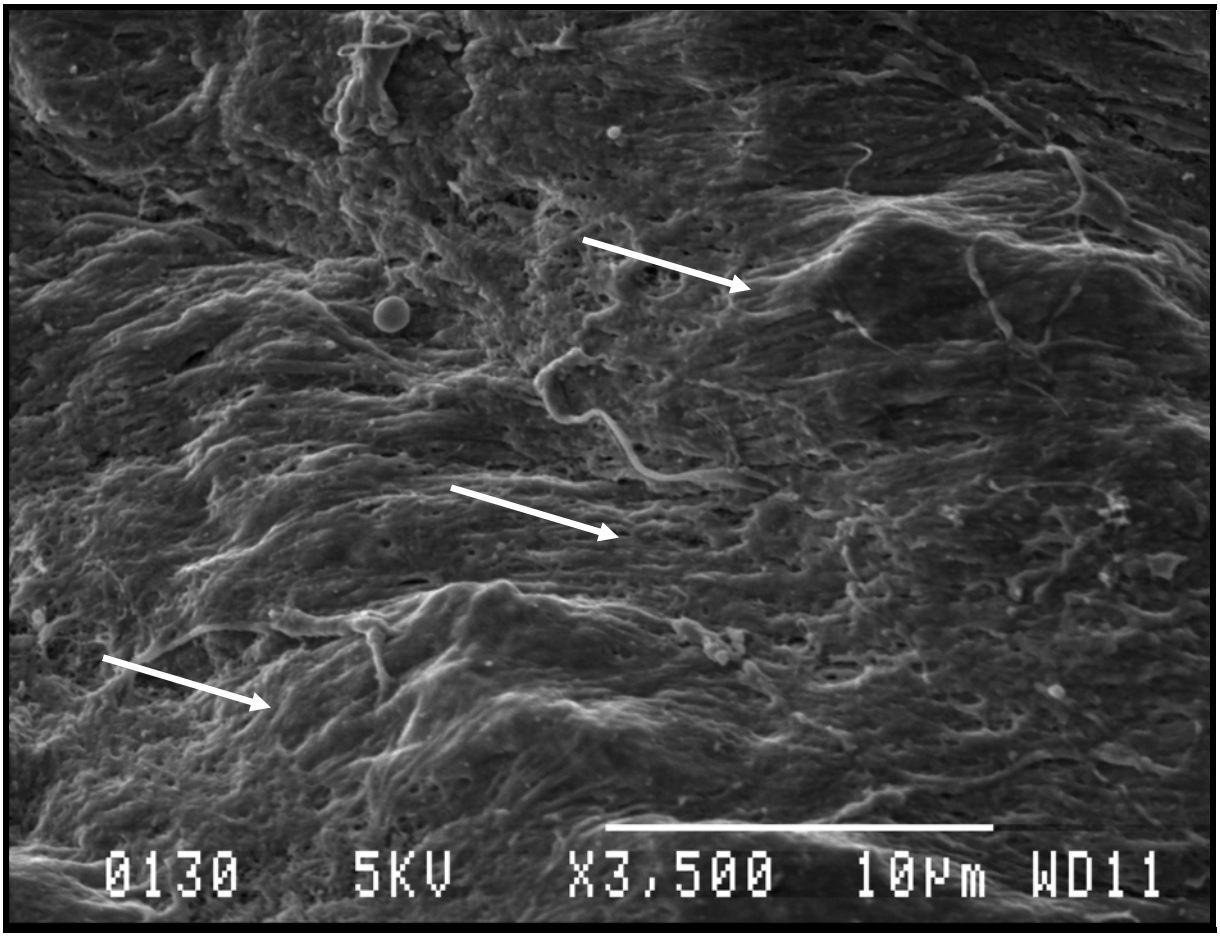


Figure 7.6a indicates the endothelial lining of the control rabbit, with the arrows indicating the microvilli found in the aorta. Fujimoto and co-workers in their study in 2005 also reported on finding endothelial microvilli in the aorta of the rabbit, particularly at the presumed site of foetal origin of the ductus arteriosus and near the origin of the subclavian artery. The samples of the aorta for this study were also taken from the area surrounding the origin of the subclavian artery. Figure 6b demonstrated normal endothelial morphology. The different endothelial cells could be distinguished with the cells exhibiting a long, elongated shape. The endothelial cells had a squamous, tile-like organization. At a higher magnification (Figure 7.7), the cell membrane had a smooth appearance. The oval indicates small hair-like projections (microvilli) from the cell membrane.

Figure 7.8 illustrates the microvilli of the endothelial lining of a rabbit treated with aspartame. At this lower magnification (x550) it seemed as though the treatment with aspartame had no adverse effect on the endothelial lining or the microvilli. When looking at this same endothelial lining at a higher magnification (x3 500) it is suggested that the aspartame did indeed adversely affect the lining, but it was only visible at a higher magnification. The microvilli of the aorta was damaged (Figure 7.9a) with holes appearing over the length of the microvilli. The membranes also appeared more porous with cytoskeleton being visible (inside circle). At a different area it also appeared as though the contents of the cell was spilling outwards (Figure 7.9b), and also here the cytoskeleton was visible, as indicated by the arrows.

Figures 7.10 and 7.11 illustrated the endothelial lining of a different rabbit after treatment with aspartame. At the lower magnification (Figure 7.10) it would appear as though the endothelial lining formed a continuous layer and no individual cells could be distinguished. The higher magnification (Figure 7.11) revealed distinct filament-like structures, as appose to the control (Figure 7.6b) where clear endothelial cells with smooth cellular membranes could be distinguished with microvilli in areas closely related to the origin of the subclavian artery (Figure 7.6a).

7.3.4 TEM study of the endothelial cells of the aorta

Figures 7.12a and 7.12b illustrate the nuclei of endothelial cells of a control rabbit.

Figure 7.12a: Illustration of the nucleus of an endothelial cell of a control rabbit. Note the clear nuclear envelope (arrow) and the even distribution of the chromatin throughout the nucleus (white arrow) (x7 500 magnification)

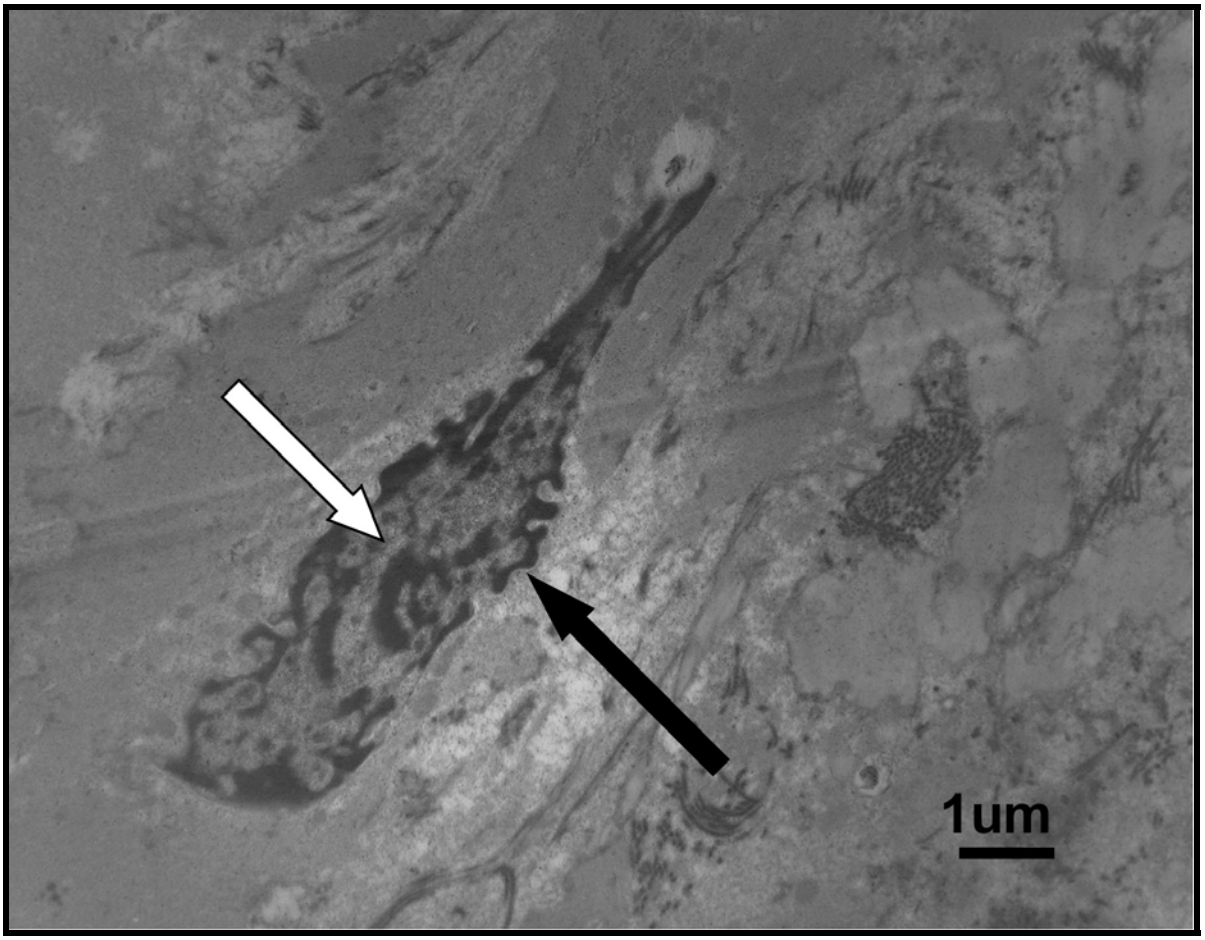


Figure 7.12b: Illustration of the nucleus of an endothelial cell of a control rabbit. Note the clear nuclear envelope (arrow), the nucleolus (white arrow) and the cytoplasm of another endothelial cell (dashed arrow) with a tight junction being visible just to the right of it (x7 500 magnification)

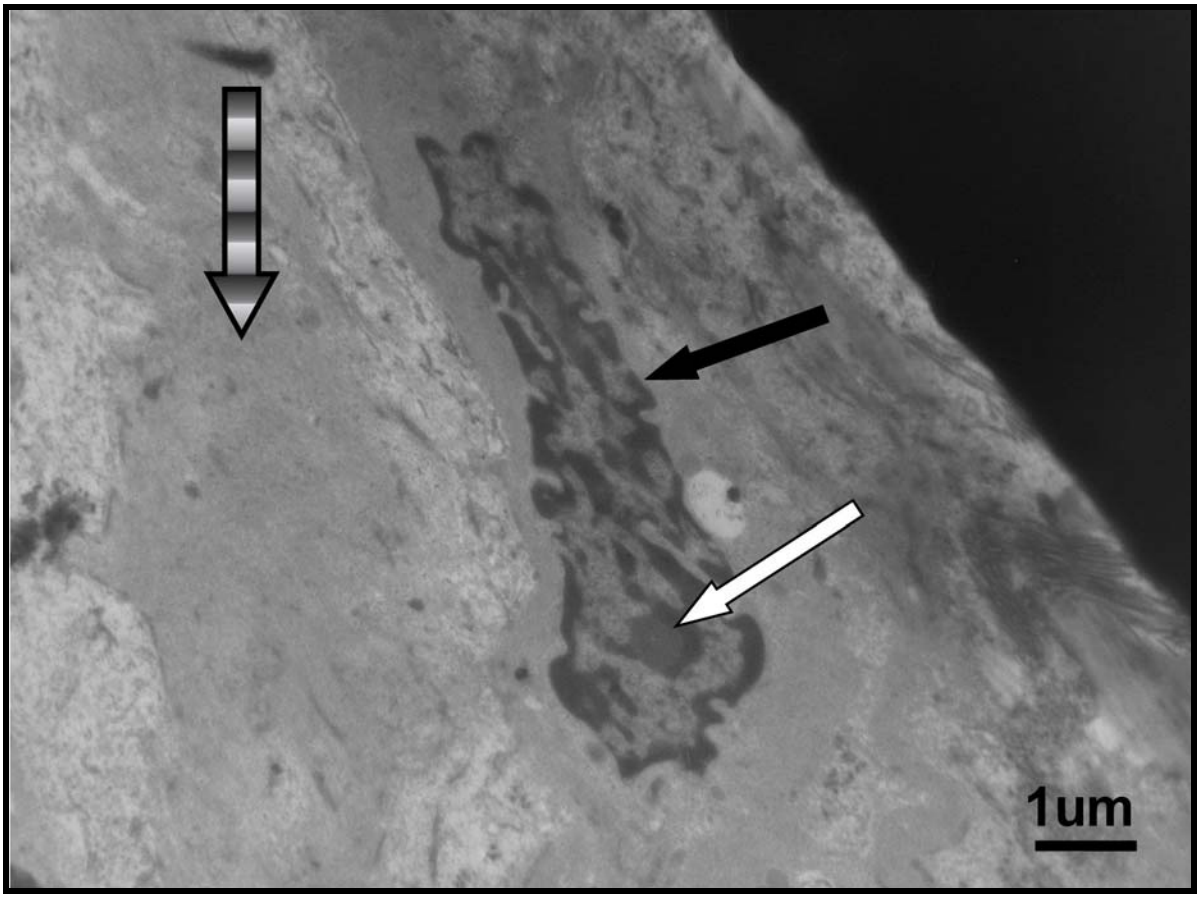
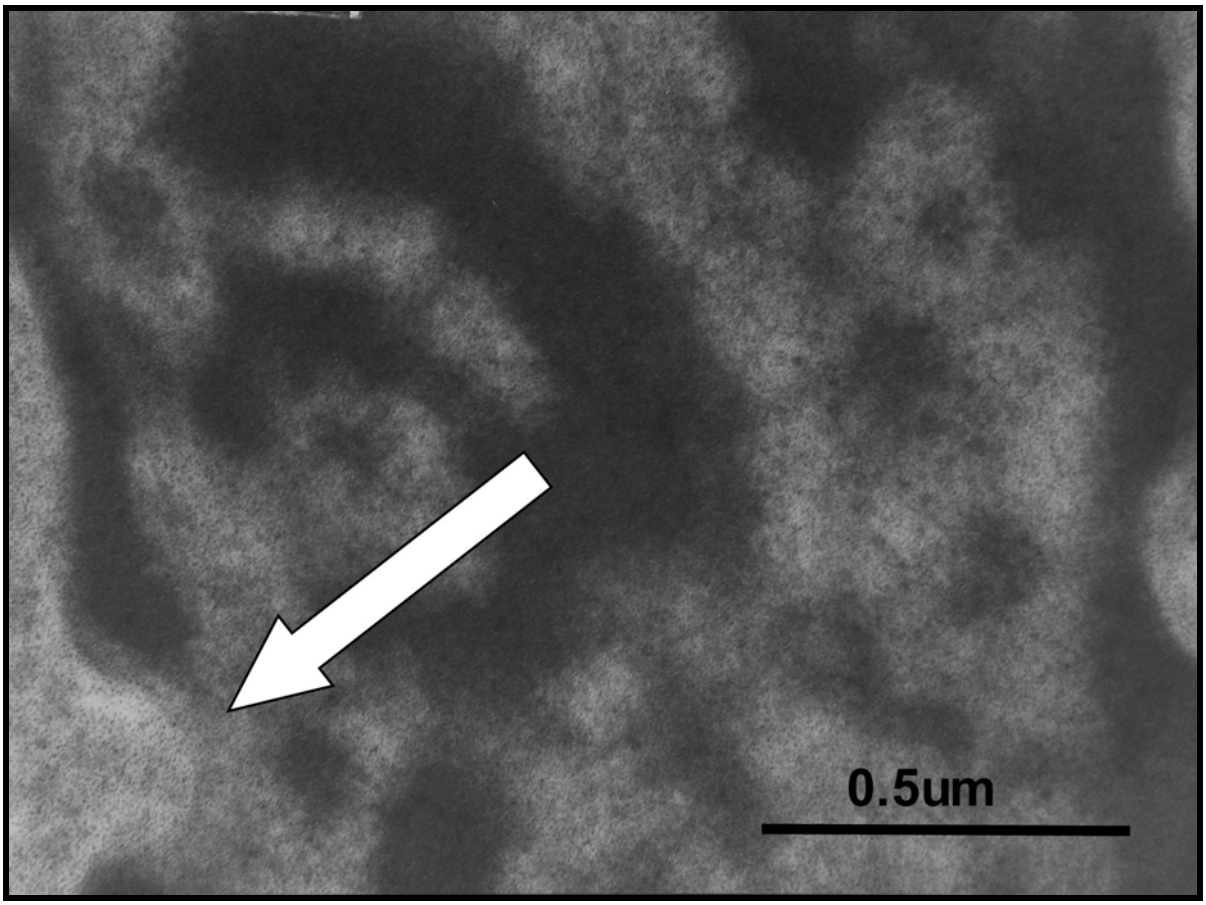


Figure 13 illustrates the nuclear envelope and chromatin of an endothelial cell of a control rabbit.

Figure 7.13: The chromatin of an endothelial cell of a control rabbit. Note the clear nuclear envelope (white arrow) and the even distribution of the chromatin (x59 000 magnification)



Figures 7.14a and 7.14b illustrates the nuclei of endothelial cells of a rabbit after treatment with aspartame.

Figure 7.14a: Illustration of the nucleus of an endothelial cell of a rabbit after treatment with aspartame. Note the fact that the nuclear envelope is visible on some areas (arrow), and absent in other (dashed arrow). The chromatin visibly underwent nuclear envelope marginalization (white arrows) (x7 500 magnification)

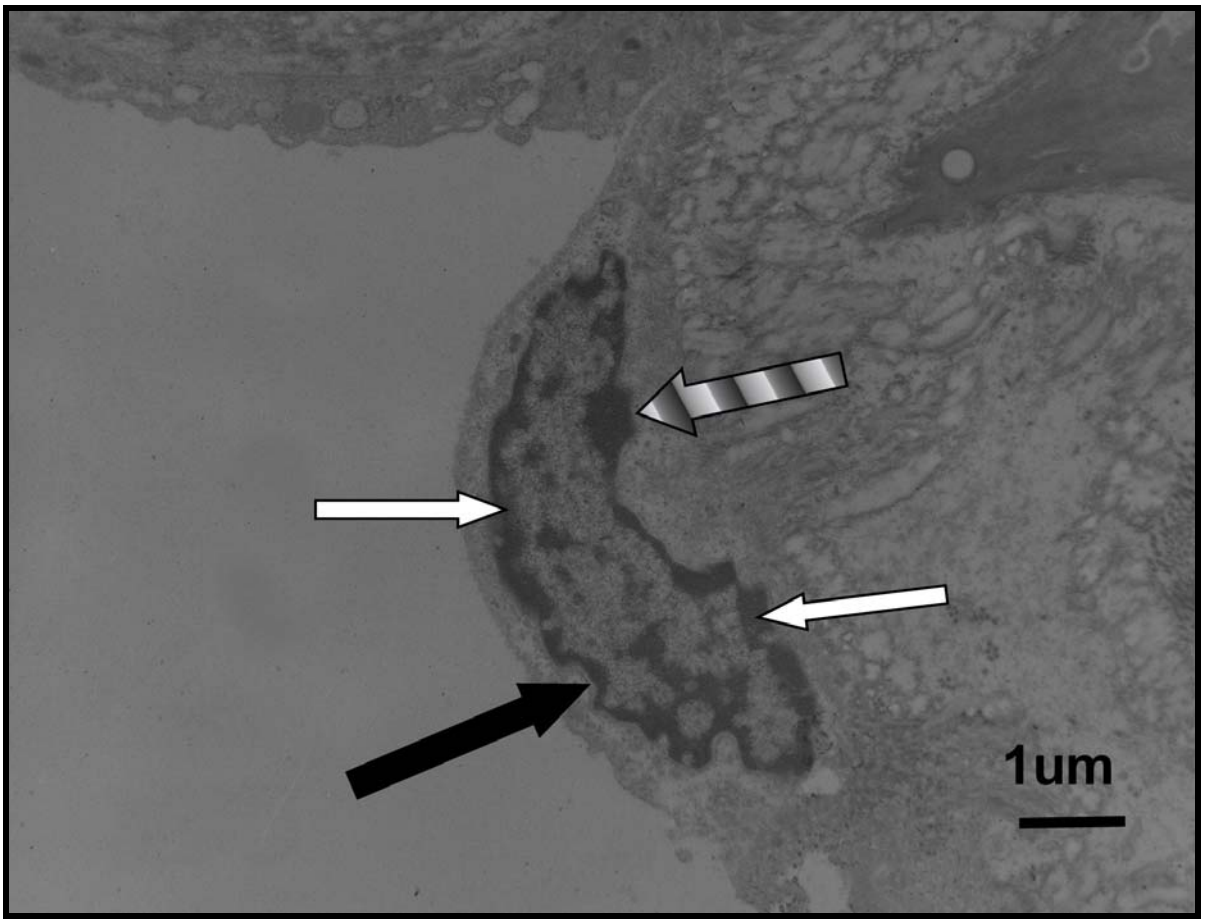


Figure 7.14b: Illustration of the nucleus of an endothelial cell of a rabbit after treatment with aspartame. Note the nuclear envelope marginalization of the chromatin (white arrows), with nuclear envelope being visible in some areas (arrow) and absent in other areas (dashed arrow). Also it appears as though some of the chromatin was spilling from the nucleus into the cytoplasm (dashed arrow). Rupture of the cellular membrane was clearly visible (circle) (x7 500 magnification)

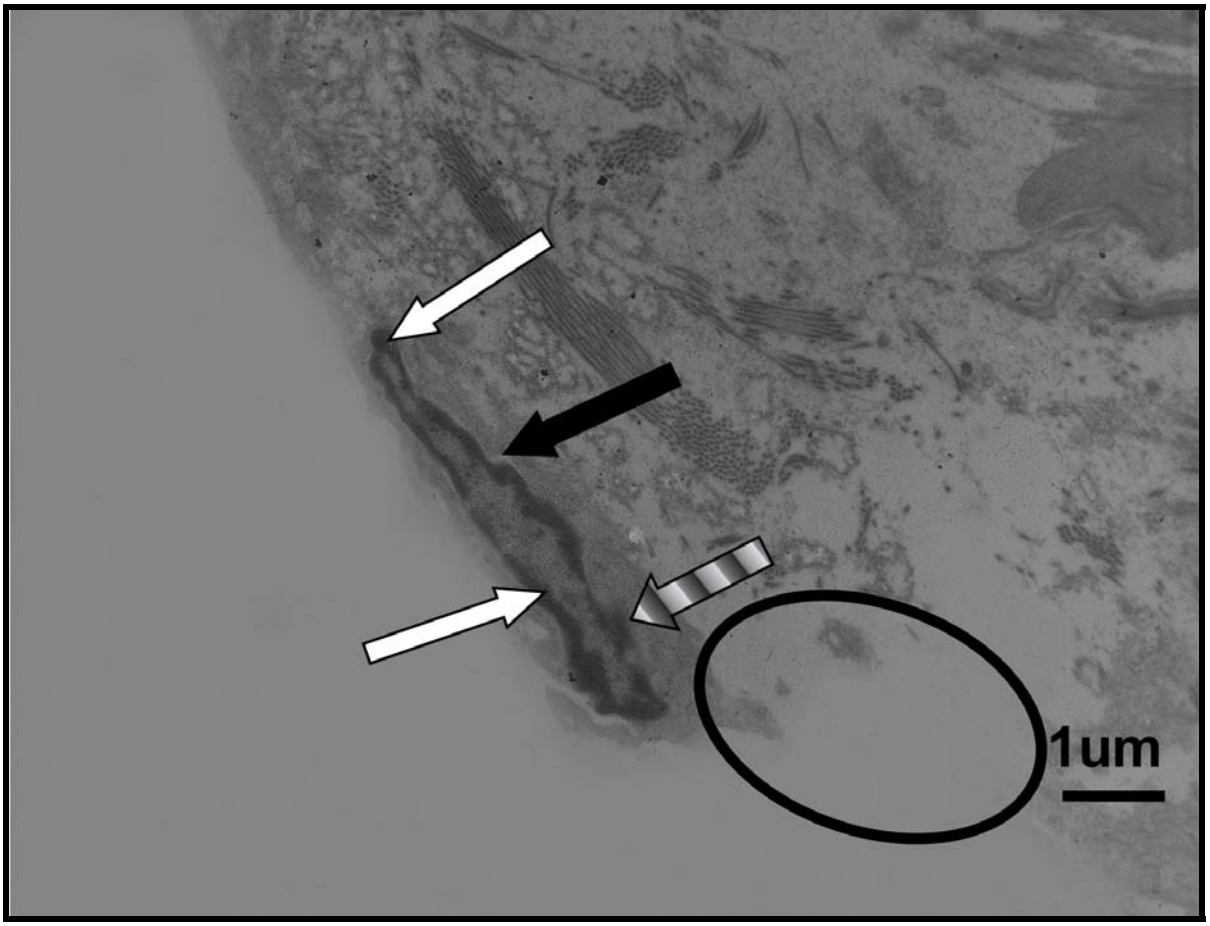
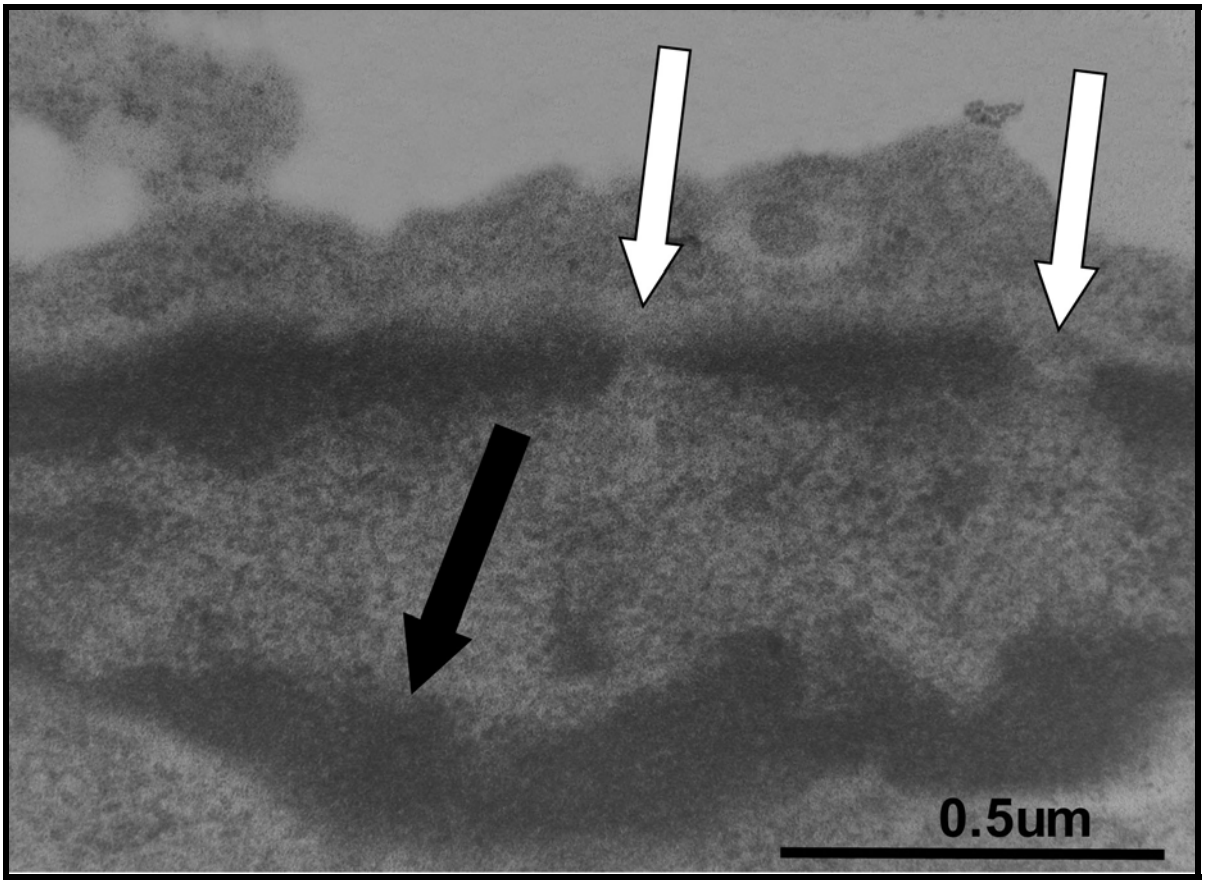


Figure 7.15 illustrates the nucleus of an endothelial cell at a higher magnification.

Figure 7.15: The chromatin inside the nucleus of an endothelial cell of a rabbit after treatment with aspartame. Chromatin marginalization towards the nuclear envelope was clearly visible (arrow), with little to no chromatin towards the centre of the nucleus. The nuclear envelope was also not clearly distinguishable. Damaged areas of the nuclear envelope was visible at two areas (white arrows) (x59 000 magnification)



Figures 7.12a and 7.12b illustrated the nucleus of an endothelial cell of a control rabbit. The nuclear envelope was clearly visible in both illustrations with the chromatin of the nuclei being evenly distributed. The nucleolus of the nucleus and the cell membrane of the endothelial cell were also easily distinguishable in figure 7.12b. At a higher magnification (Figure 7.13) the chromatin of the endothelial cell was clearly visible and evenly distributed. The nuclear envelope could also be distinguished, with no apparent damage.

Figure 7.14a illustrated an endothelial cell of a rabbit after treatment with aspartame. The nucleus was clearly visible, with the chromatin appearing condensed and marginalized towards the nuclear envelope. Little to no chromatin was visible in the centre of the nucleus. The nuclear envelope was visible in certain areas and absent in others. Damage to the cellular membrane of the endothelial cell was clearly visible in figure 7.14b with the contents of the cytoplasm appearing to spill outwards. The chromatin of the nucleus of this endothelial cell, obtain from a rabbit fed aspartame, also demonstrated the nuclear envelope marginalization of the chromatin. It appeared as though some of the chromatin was spilling into the cytoplasm in an area where the nuclear envelope was not visible. The nuclei of these endothelial cells also appeared smaller than those of the control (Figures 7.12a and 7.12b). At a higher magnification of the nucleus of an endothelial cell after treatment with aspartame (Figure 7.15), the nuclear envelope marginalization was clearly visible with possible areas of damage to the nuclear envelope. The chromatin was also not evenly distributed through the whole of the nucleus.

7.4 SUMMARY AND EXPLANATION

7.4.1 Light microscopic studies of the morphology of the leukocytes and the number of different leukocytes counted

Photos were taken of the different leucocytes to compare possible morphological differences between the controls and aspartame treated rabbits. The results obtained for the control leukocytes can be summarized as per table 7.4.

Table 7.5: Comparison between the morphology of the leukocytes of the control and aspartame treated groups

Cells	**Nuclei and chromatin**	**Cytoplasm**	**Granules**	**Cell membrane**
Lymphocytes **Controls**	Stained dark blue with the chromatin more densely packed	Very little visible	None	Visible
Aspartame	Stained lighter blue	Close to cell membrane stained darker blue		
Monocytes **Control**	Stained dark blue with chromatin densely packed	Light blue, with vacuoles present	None	Visible
Aspartame	Stained dark blue and was bean-shaped	Cytoplasm more visible, almost transparent. No vacuoles present		
Basophils	Bi-lobar	Control stained a deeper blue than aspartame rabbit	Dark blue	More clearly visible

Table 7.4 (continue): Comparison between the morphology of the leukocytes of the control and aspartame treated groups

Cells	Nuclei and chromatin	Cytoplasm	Granules	Cell membrane
Eosinophils **Controls**	Bi-lobar with distinct dark blue stain	Red	Red granules visible	More clearly visible in the control
Aspartame	Chromatin more densely packed closer to nuclear envelope		Stained more brilliantly	
Heterophils	Brilliantly blue with up to 5 lobes visible		Red granules, stained more brilliantly in control; individual granules visible in control	More clearly visible in control, staining light blue
	Chromatin of aspartame treated rabbit more densely packed		Red granules less in aspartame rabbits and not as clearly distinguishable	

It would appear as though aspartame affected some of the morphology of the different leucocytes, but the size of the cells were not adversely affected. The cytoplasm of the basophils of the aspartame treated rabbit did not stain as dark blue as that of the control, which could be an indication that the number of granules (secrete heparin and histamine) inside the cytoplasm decreased. Heparin, binds to enzyme inhibitor antithrombin III (AT III) leading to conformational change which result in its active site being exposed. The activated AT III then inactivates thrombin and other proteases involved in blood clotting, most notably factor Xa. The rate of inactivation of these proteases by AT III increases 1000-fold due to the binding of

heparin (Bjork and Lindahl, 1982). From results obtained in chapter 5 regarding the coagulation profile of the rabbits after treatment with aspartame, it was concluded that the amount of thrombin decreased leading to bleeding tendencies. Heparin assist in the process of thrombin inhibition, thus it is hypothesized that an immune response was triggered and that the number of granules inside the basophils decreased in order for less heparin to be secreted which would further increase the inability for coagulation.

The granules of the eosinophils of the aspartame treated rabbits, on the other had, stained more brilliantly red which leads to the hypothesis that the number of granules increased after treatment with aspartame. Eosinophils are known to modulate allergic reactions by secreting the substances in their granules which neutralize histamine and inhibits degranulation of mast cells (contain heparin and histamine) (Meyer and Meij, 1996). It was also shown, in table 7.3, that the number of eosinophils increased by 30.33% when compared to counts obtained for the controls. Thus, it is hypothesized that the increase in the amount of eosinophils and thus also the amount of granules (due to increase in number of eosinophils and as seen in figure 7.4b) were due to an immune response to correct the decrease in coagulation, caused directly by the decrease in the concentration of factors VII, X and VIII after treatment with aspartame as discussed in chapter 5 of this thesis.

The number of granules in the heterophils of the aspartame treated rabbits appeared less and they were not as clearly distinguishable as compared to that of the controls. The granules inside the heterophils are known to be lysozymes and phagocytin, two antibacterial agents (Archer *et al*., 1963). Results also indicated (Table 7.3) that the number of heterophils decreased by 42.85% when compared to numbers obtained for the control rabbits. Thus, when taking into account that the number of cells and granules or levels of antibacterial agents in the granules appeared to decrease (less brilliant staining of the granules in cytoplasm), the ability of the cells to protect against bacterial infection would decrease. Thus it is hypothesized that the rabbits might have suffered from a degree of suppressed immunity and that bacterial infections could occur more easily (due to lower number of heterophils and granules inside cytoplasm).

The increase in the number of zones that had to be counted to obtain the 100 leukocytes in the aspartame treated rabbits reflects that the total number of leucocytes decreased. This could be an indication that the rabbits suffered from a degree of suppressed immunity. The number of eosinophils, however, increased as an immune defence mechanism to prevent further inability for coagulation.

7.4.2 SEM study of the endothelial lining of the aorta

The tendency of blood to coagulate intravascularly is prevented by a number of mechanisms and any coagulate that does form will be dissolved in time. Intravascular coagulation is prevented by the following:

- The smooth endothelial lining of the cardiovascular system;
- The continuous circulation of blood;
- Prostacyclins (produced by endothelial cells) that prevent platelet aggregation;
- Anti-thrombin III – circulating factor that binds to thrombin, inactivating it;
- Heparin, a natural anti-coagulant which is secreted by mast cells and basophils (Guyton and Hall, 2006) – improves inactivation of thrombin by antithrombins. (Meyer and Meij, 1996).

The results obtained in this study indicate that the endothelial lining of the aorta was damaged after treatment with aspartame. The microvilli present in the aorta were also damaged (Figure 7.9a) and it appeared as though the cellular contents were spilling outwards (Figure 7.9b) with the cytoskeleton also being visible. Another, but similar picture was painted by figure 7.10 that illustrated that no individual endothelial cells were visible. Filament-like structures were visible on the higher magnification (Figure 7.11), thus the cytoskeleton of the cells was exposed. The endothelial cell integrity was disrupted during treatment with aspartame, which made the aorta samples from the aspartame treated rabbit more prone to damage during the critical point drying procedure, disrupting the cellular membranes of the endothelial cells. This disruption of cell membranes were not observed in the control samples (Figure 7.6b and 7.7). As seen from above, endothelial cells play an important role in maintaining haemostasis in the vascular system, thus damage to the endothelial cells could lead to an increased amount of coagulation. The endothelial cells are also responsible for the synthesis and release of F VIII into the blood stream (Rubin and Leopold, 1998). Thus, it is hypothesized that the decrease in the

concentration of circulating F VIII, as described in chapter 4 of this thesis could be due to the extend of the damage to the endothelial cells (Figures 7.9a, 7.9b and 7.11).

7.4.3 TEM study of the endothelial lining of the aorta

Results obtained during further ultra-structural analysis of the endothelial cells with the TEM confirmed results obtained by the SEM. Damage to the nucleus was clearly visible, with regards to the distribution of the chromatin (marginalization towards the nuclear envelope) and damage to the nuclear envelope itself (not visible in all areas). The cellular membrane of the endothelial cells was also damaged (Figure 7.14b), which confirms the results obtained on the SEM, also indicating cellular membrane damage (Figures 7.9a and 7.9b). The nuclei of the endothelial cells after treatment with aspartame also appeared smaller (Figures 7.14a and 7.14b).

Characterization of apoptosis mainly derives from morphological and ultra-structural observations (Kerr, Wyllie and Currie, 1972). Intracellular and plasma membrane structural modifications have been widely recognized as crucial factors involved in cell injury and cell death. Changes in nuclear morphology and in organelle structure as well as specific phenomena at the cell surface level, namely surface smoothing and surface blebbing, are often considered as markers associated with cell pathology (Allen, 1987). In addition, it must be recalled that these structural findings are intimately related to the cascade of biochemical and physiological events leading to changes in cellular homeostasis, to the loss of cell volume regulation, to some modifications of macromolecule synthesis and, finally the loss of cell viability (Malorni, Fais and Fiorentini, 1998). A single intracellular event can be extensively analysed by using, in parallel, biochemical, molecular or ultra-structural approaches. The complex sequence of structural modifications ultimately leading to cell death can be recognized by light and electron microscopy techniques. These analyses are mainly qualitative and can indicate: i) the different features of the apoptotic process in terms of appropriate markers, e.g. histo-type associated;, and ii) the staging of the process, e.g. early or late phases (also called secondary necrosis). However, quantitative analysis, i.e. cytometric and morphometric, can also be performed by using light microscopy, fluorescence microscopy, confocal microscopy, SEM and in some conditions TEM (Bellomo *et al*., 1994; Malorni *et al*., 1993; Allen, 1997).

A cell undergoing apoptosis shows characteristic morphology that can be observed microscopically:

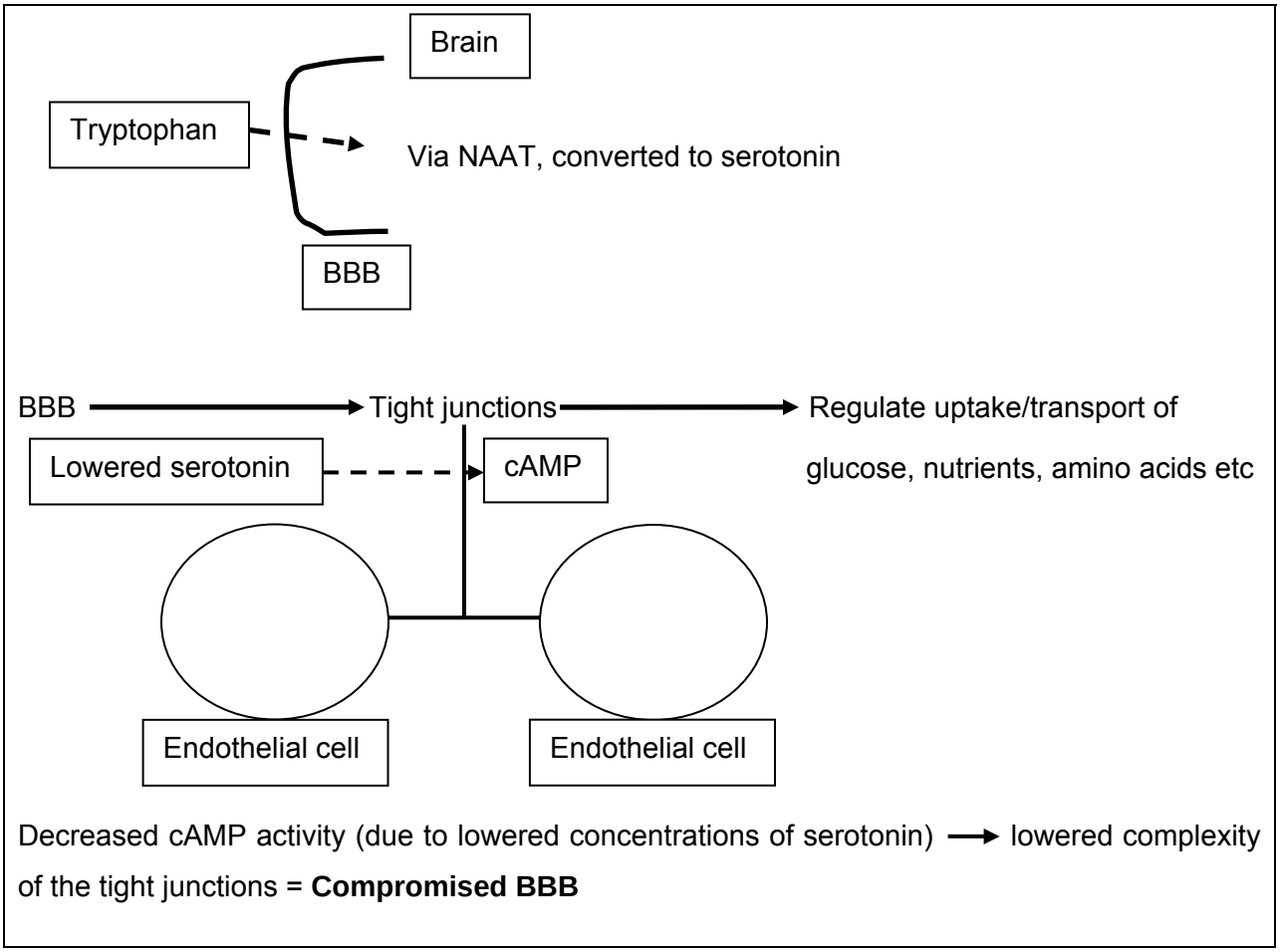
1. Cell shrinkage and rounding due to the breakdown of the protein-like skeleton by caspases
2. The cytoplasm appears dense, and the organelles appear tightly packed
3. Chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis
4. The nuclear envelope becomes discontinuous and the DNA inside it is fragmented in a process referred to as karyorrhexis. The nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the degradation of DNA
5. The cell membrane shows irregular buds known as blebs
6. The cell breaks apart into several vesicles called apoptotic bodies, which are then phagocytosed (Susin *et al*., 2000; Kihlmark, 2001; Nagata, 2000).

Thus, it could be summarized that the endothelial cells were experiencing cellular pathology as changes in their nuclear morphology (condensation and marginalization of chromatin; damage to nuclear envelope) and cell surface smoothing (Figure 7.10) were visible. Also, cell pathology leads to modifications in the synthesis of macromolecules. Thus the production and secretion of F VIII by the endothelial cells could have decreased, as proven by the results obtained in chapter 5. The two changes in the nuclear morphology is also hallmarks for apoptosis (bullets 3 and 4 mentioned above), thus it was clear that the endothelial cells were undergoing apoptosis.

Endothelial cells and their tight junctions form an integral part of the blood brain barrier (BBB) (Leeson, Leeson and Paparo, 1988). Phenylalanine, constituting 50% of the metabolites of aspartame, binds to a neutral amino acid transporter (NAAT) to be carried over the BBB. Other amino acids namely tryptophan, methionine, branched-chain amino acids and tyrosine, necessary for production of important neurotransmitters, also have to bind to the NAAT for transport over the BBB. Thus there is competition for NAAT to be carried over the BBB, so that the amino acids can be converted to their respective neurotransmitter counterparts.

Phenylalanine is also converted to tyrosine (precursor of DOPA and dopamine) by the enzyme phenylalanine hydroxylase in the liver. Thus, a certain amount of circulating phenylalanine will be converted to tyrosine, but a large amount of the phenylalanine will occupy the NAAT (due to lager concentrations in the blood after intake of aspartame with no synchronised administration of valine [Maher and Wurtman, 1987]), making it impossible for other amino acids to be carried over the BBB. Tryptophan, one of the amino acids necessary for the production of serotonin in the brain, also utilise the NAAT for transport. It has been proven that phenylalanine from aspartame decreases the concentration of serotonin produced due to the competition between phenylalanine and tryptophan for transport via NAAT over the BBB, with phenylalanine almost always occupying NAAT due to its high concentrations (Sharma and Coulumbe, 1987). Also it was shown in chapter 6 of this thesis that platelet aggregation was decreased (SEM), due to a direct decrease in the amount of circulating thrombin necessary for the degranulation of the platelets (dense granules contain serotonin, necessary for platelet-platelet aggregation), thus indirectly decreasing the amount of circulating serotonin. cAMP plays an integral part in the complexity of the tight junctions between the endothelial cells which form part of the BBB. A decreased concentration of circulating serotonin lowers the activity of cAMP, and decreased cAMP activity leads to a decrease in the complexity of the tight junctions between the endothelial cells (Humphries *et al*., *In Press)*. Thus, it is hypothesized that due to the lowered concentrations of serotonin present (decreased transport of tryptophan over BBB and decreased thrombin concentrations, as described above), the BBB was compromised as both the tight junctions could have been affected (lowered activity of cAMP due to decrease in concentration of circulating serotonin) and the apoptotic appearance of the endothelial cells (TEM).

Diagram 7.1: Effects of inability to convert tryptophan to serotonin on the cAMP activity



Adapted from Humphries *et al*., *In Press*

7.5 CONCLUSION

Three of the five intravascular anticoagulation mechanisms (endothelial lining; AT III and heparin) and the normal coagulation pathway (decrease in F VII, X and VIII as described in chapter 5 of this thesis) were adversely affected by treatment with aspartame, either directly or indirectly. The effects of aspartame on the coagulation system, indirectly leading to decreased concentrations of thrombin (confirmed by results obtained on the platelet aggregation, chapter 6 of the thesis) affect coagulation which could lead to bleeding disorders. The intravascular anticoagulation system was also affected by treatment with aspartame, both directly (endothelial lining) and indirectly (no secretion of heparin or AT III). Thus it would seem as though an immune response was triggered by the administering of aspartame (increase in eosinophils, no

increase in basophils). The rabbits treated with aspartame also appeared to have suppressed immunity, as indicated by an overall decrease in the amount of leukocytes counted (Table 7.3). The effect of the decrease in the coagulation factors (bleeding disorders) causes decreased coagulation. A further decrease in coagulation was probably prevented by the increase in eosinophils which hindered the secretion of heparin from mast cells. Thus, AT III was also not increased to prevent a further decrease in coagulation.

The decreased amount of circulating thrombin, as indicated indirectly affects the concentration of serotonin (degranulation of platelets, Chapter 6). As seen from the summary and explanation, phenylalanine (one of metabolic constituents of aspartame) also decreases the amount of serotonin be preventing the conversion of tryptophan to serotonin. It was also shown that serotonin plays an integral part in the complexity of the tight junctions between two adjacent endothelial cells by attenuating the activity of cAMP. Thus it is hypothesized that aspartame compromises the BBB as the endothelial cells were apoptotic (TEM) and the amount of circulating serotonin decreased (decreased platelet aggregation as seen with SEM) affecting the complexity of the tight junctions by lowering the activity of cAMP.

This chapter therefore concludes that a defence mechanism exists within individuals and that the effect of aspartame on the normal coagulation, leukocyte numbers and intravascular anticoagulation system are in balance. This would however, not be the case with individuals genetically predisposed towards bleeding disorders. It is hypothesized that aspartame will affect normal coagulation and circulating concentrations of coagulation factors to a much higher degree in people with bleeding tendencies, and the effects caused by aspartame will not be balanced by the normal intravascular anticoagulation mechanisms, even if no AT III or heparin is secreted. Also, that using aspartame at abuse doses (150mg/kg aspartame and above) could lead to suppressed immunity (lowered counts of leukocytes), bleeding disorders (decreased coagulation) and a possible compromised BBB (lowered activity of cAMP due to decrease in serotonin).

Chapter 8 –

Light microscopic study of the histological morphology of the liver and kidney to determine the effects of aspartame

RESEARCH QUESTION 6:

How does aspartame affect the normal histological morphology of the liver and kidney?

8.1 INTRODUCTION

The components of blood (plasma with coagulation factors, fibrin fibres, and platelet aggregates and leukocytes) were studied during the first part of this thesis. It was found that all the constituents of the coagulation system, from the coagulation factors to the intravascular anti-coagulation system were affected by the treatment with aspartame. This being the case, the aim of this chapter of the thesis was to determine whether two of the major filtration organs in the body, namely the liver and kidneys were adversely affected by the treatment with aspartame.

Ranney and co-workers in 1976 undertook a comparative study of the metabolism of aspartame in experimental animals and humans. The metabolism of aspartame was studied in mice, rats, rabbits, dogs, monkeys and humans. Ranney and co-workers found that the compound was digested in all species in the same way as are natural constituents of the diet. Hydrolysis of the methyl group by internal esterases yielded methanol, which was oxidized in the one-carbon metabolic pool of carbon dioxide (CO_2). The resultant dipeptide was split at the mucosal surface by dipeptidases and the free amino acids were absorbed. The aspartic acid moiety was transformed in large part to CO_2 through its entry into the tricarboxylic acid cycle. Phenylalanine was primarily incorporated into body protein either unchanged or as its major metabolite, tyrosine.

Adult male rats were given an oral dose of 10mg/kg aspartame 14C-labelled in the methanol carbon. At times intervals of up to 6 hours, the radioactivity in the plasma and several organs was investigated. Most of the radioactivity found (0.98% in plasma, 0.75% in liver) was bound to protein. Label present in the liver, plasma and kidney was in the range of 1-2% of the total radioactivity administered per g or ml, changing little with time. Other organs (brown and white adipose tissue, muscle, brain, cornea and retina) contained levels of label in the range of 1/12 to 1/10th of that of the liver. In all, the rat retained, 6 hours after administration about 5% label, half

of it in the liver. The specific radioactivity of the tissue protein, RNA and DNA was quite uniform. The protein label was concentrated in amino acids, different from methionine, and largely coincident with the result of protein exposure to labelled formaldehyde. DNA radioactivity was essentially in a single different adduct base, different from the normal bases present in DNA. The nature of the tissue label accumulated was, thus, a direct consequence of formaldehyde binding to tissue structures. The administration of labelled aspartame to a group of cirrhotic rats resulted in comparable label retention by tissue components, which suggests that liver function (or its defect) has little effect on formaldehyde formation from aspartame and binding to biological components. The chronic treatment of a series of rats with 200 mg/kg of non-labelled aspartame during 10 days resulted in the accumulation of even more label when given the radioactive bolus, suggesting that the amount of formaldehyde adducts coming from aspartame in tissue proteins and nucleic acids may be accumulative. It was concluded that aspartame consumption may constitute a hazard because of its contribution to the formation of formaldehyde adducts (Trocho *et al.*, 1998).

The renal cells were visualized by means of three-dimensional NaOH maceration. The three-dimensional fine structure of the cells composing the renal tissue was demonstrated by SEM after removal of extracellular matrices by NaOH maceration. Takahashi-Iwanaga (1992) focussed on glomerular mesangial cells, extraglomerular mesangial cells, and epithelial cells in the thin limb's of Henle's loop in the rat, rabbit and dog. Mesangial cells revealed a rough surface covered by short microvilli. The cells extended long branching processes in close association with the glomerular capillary, suggesting a role for them of regulating the capillary calibre. The mesangial cells interdigitated with each other by means of their microvilli, forming an intercellular labyrinth. Extraglomerular mesangial cells at the glomerular hilus were also covered with microvilli, which forms narrow labyrinthine spaces between the cells. The labyrinth among the mesangial cells and that among the extraglomerular mesangial cells connected with each other at the hilus, giving rise to a channel system leading from the periphery of the glomerulus through the hilus to the interstitial space outside the glomerulus. Renal tubule cells displayed complicated intra- and intercellular interdigitations on the basal aspect. The pattern of epithelial interdigitations was specific to each tubular segment. The descending and ascending thin limbs of the long loops demonstrated a striking contrast to each other. The former was characterized by moderate intercellular interdigitation and by numerous microvilli on the lateral

and basal surfaces; the latter was marked by elaborate, pectineal interdigitations, and by smooth basolateral surfaces (Takahashi-Iwanaga, 1992).

8.2 MATERIALS AND METHODS

8.2.1 Exposure of animals to aspartame

Rabbits in both the control and aspartame group were exposed to double distilled water and aspartame, respectively, for 25 exposures in 37 days for the three consecutive concentrations (34mg/kg; 100mg/kg and 150mg/kg aspartame). A two week resting period was allowed after completion of a concentration. Approximately one week after the last concentration was completed, the rabbits were terminated and dissected for their liver and kidney.

8.2.2 Termination of the study

After the treatment periods were completed, all the rabbits were euthanized by means of intra-peritoneal injection with sodium pentobarbitonehydrochloride (2ml) by a skilled person experienced in this technique (Mrs Annette de Freitas, animal technologist at the Medunsa campus of the University of Limpopo). All the adult rabbits not utilised for dissection were cremated. Five rabbits, 3 controls and 2 treated with aspartame (with negative/adverse clotting profiles) were dissected for the soft tissue of their livers and kidneys for light microscopic studies. After harvesting of the necessary specimens, the carcasses were cremated at the University of Limpopo, Medunsa campus.

8.2.3 Dissection and fixing of samples

Five rabbits (3 controls and 2 aspartame-treated) were given a general anaesthetic (Atravet) to make them sleepy, where-after each were euthanized by means of injection with 2ml phenobarbitone when needed for dissection. The thoracic and abdominal cavities were opened and the required organs were identified: liver and kidneys. The organs were removed and the tissue samples collected from each organ. The liver and kidney sections were fixed in 4% Formaldehyde. Tissue was obtained from the left and right lobes as well as the hilus of the liver and from the cortex and medulla of the kidney.

8.2.4 Pre-embedding procedures

After approximately a month in fixative, the tissue (liver and kidney) was removed from the fixative and serially dehydrated in 70% and 90% ethanol, followed by three changes of absolute ethanol. After dehydration, the tissue was placed in xylene (clearing agent) overnight. The tissue was then transferred into increasing concentrations of paraffin wax.

8.2.5 Embedding

Wax was melted in an embedding apparatus and the bottom of the metal embedding holders was covered with pure paraffin wax. The tissue (liver and kidney samples) was orientated and placed into the holder before complete filling with wax. The holder was covered with a plastic label, containing a record of the tissue, and allowed to set completely.

8.2.6 Sectioning

Special precautions were used to make sure that the wax blocks were of high standard before sectioning of the tissue commenced. Excess wax was trimmed away by using a scalpel. The tissue blocks, containing the liver and kidney samples was orientated and the wax block placed in the microtome for sectioning. Sections of between 5µm – 10µm (optimal 8µm) were made, and the ribbons of tissue (either liver or kidney, depending on the imbedded tissue) was transferred to a warm water bath to remove excess wax and straighten tissue sections. Sections were collected onto a glass slide and left to dry. Slides were properly labelled and numbered.

8.2.7 Approach to obtaining results from liver and kidney sections

Table 8.1: Number of views per slide for each section of tissue (liver and kidney) for each of the 5 different rabbits (3 controls and 5 aspartame treated rabbits) studied under the microscope

Tissue studied	Section of tissue	Number of sections per ribbon/slide	Number of slides	Total viewed
Liver	Left lobe	5 Serial sections	5 Slides	15 sections (Every second slide in a series with all five serial sections per slide)
	Centre of liver	5 Serial sections	5 Slides	15 sections (Every second slide in a series with all five serial sections per slide)
	Right lobe	5 Serial sections	5 Slides	15 sections (Every second slide in a series with all five serial sections per slide)
Kidney	Cortex	5 Serial sections	5 Slides	15 sections (Every second slide in a series with all five serial sections per slide)
	Medulla	5 Serial sections	5 Slides	15 sections (Every second slide in a series with all five serial sections per slide)

8.2.8 Staining with Haematoxylin and Eosin stain

Haematoxylin is a nuclear stain that stains the nuclei of the cell blue to black. The counter stain, Eosin, is responsible for the pinkish colour observed in the cytoplasm and connective tissue. Tissue were deparaffinized, rehydrated and stained with Haematoxylin for 10 minutes and counterstained in Eosin for 3 minutes. The tissue was then dehydrated and mounted for viewing under the Nikon Optiphod transmitted light microscope (Nikon Instech Co., Kanagawa, Japan).

8.3 RESULTS AND DISCUSSION

8.3.1 Microscopic study of the histological morphology of the liver

Sections from the different parts of the liver, (left lobe of liver; right lobe of liver and centre of liver) were dissected, thus it was mentioned from which part of the liver the illustration were taken, indicating possible differences and for the purpose of orientation.

Figure 8.1a and 8.1b illustrates a section through the right lobe of the liver of a control rabbit, indicating a blood vessel with hepatocytes surrounding it.

Figure 8.3a: Section through the right lobe of the liver of a control rabbit illustrating a blood vessel in the centre of the photo. Note the clear cellular membranes of the hepatocytes (black arrows) with the darker staining nuclei (circles). Dashed lines indicate polygonal shape of the hepatocytes

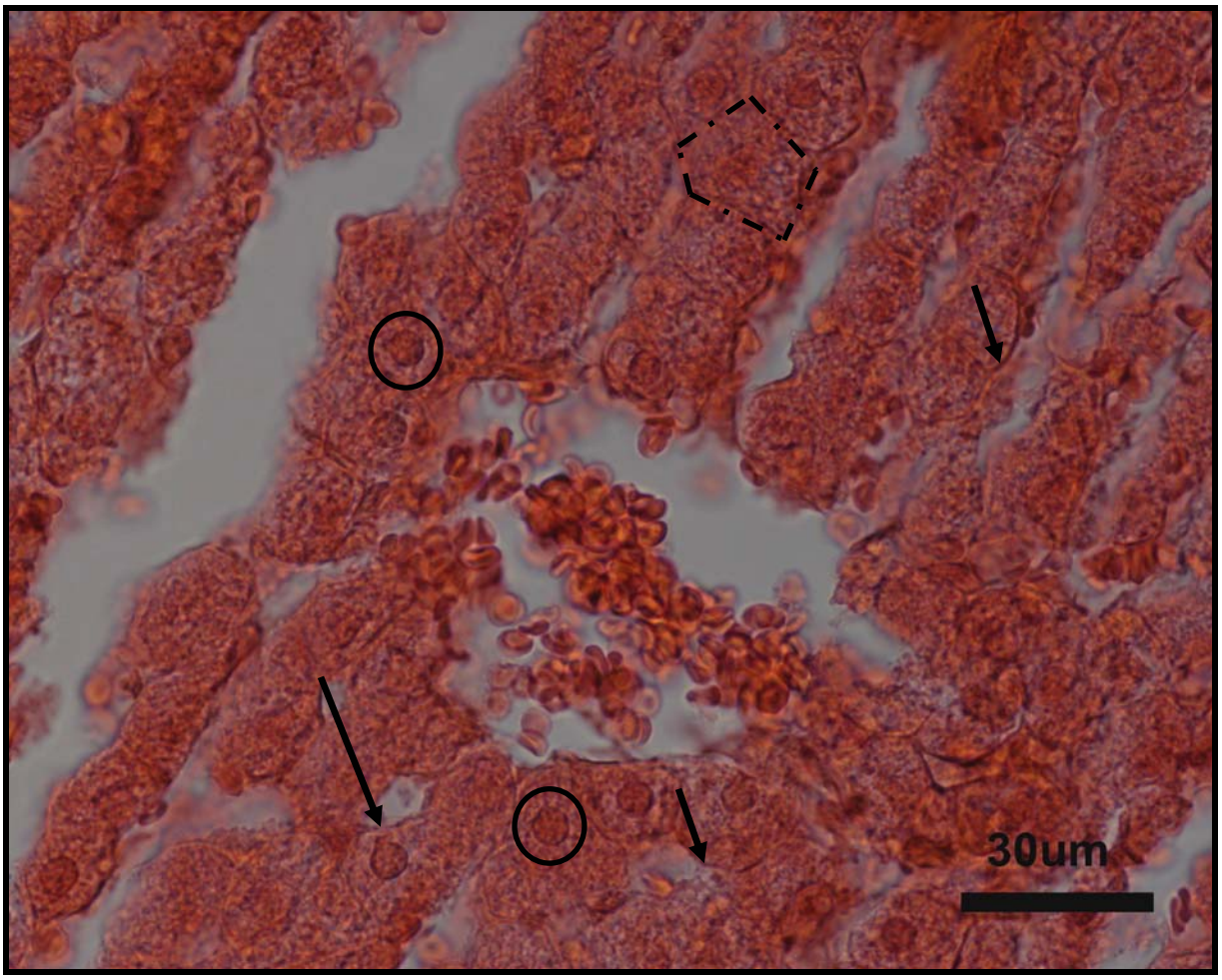


Figure 8.1b: Section through the right lobe of the liver of a control rabbit. Note the blood vessel in the centre of the photo, with two ducts. Note the clear cellular membranes of the hepatocytes (arrows) and the cuboidal epithelium lining the ducts (white arrows). Also, the endothelial lining of the blood vessel was clearly visible (dashed black arrow)

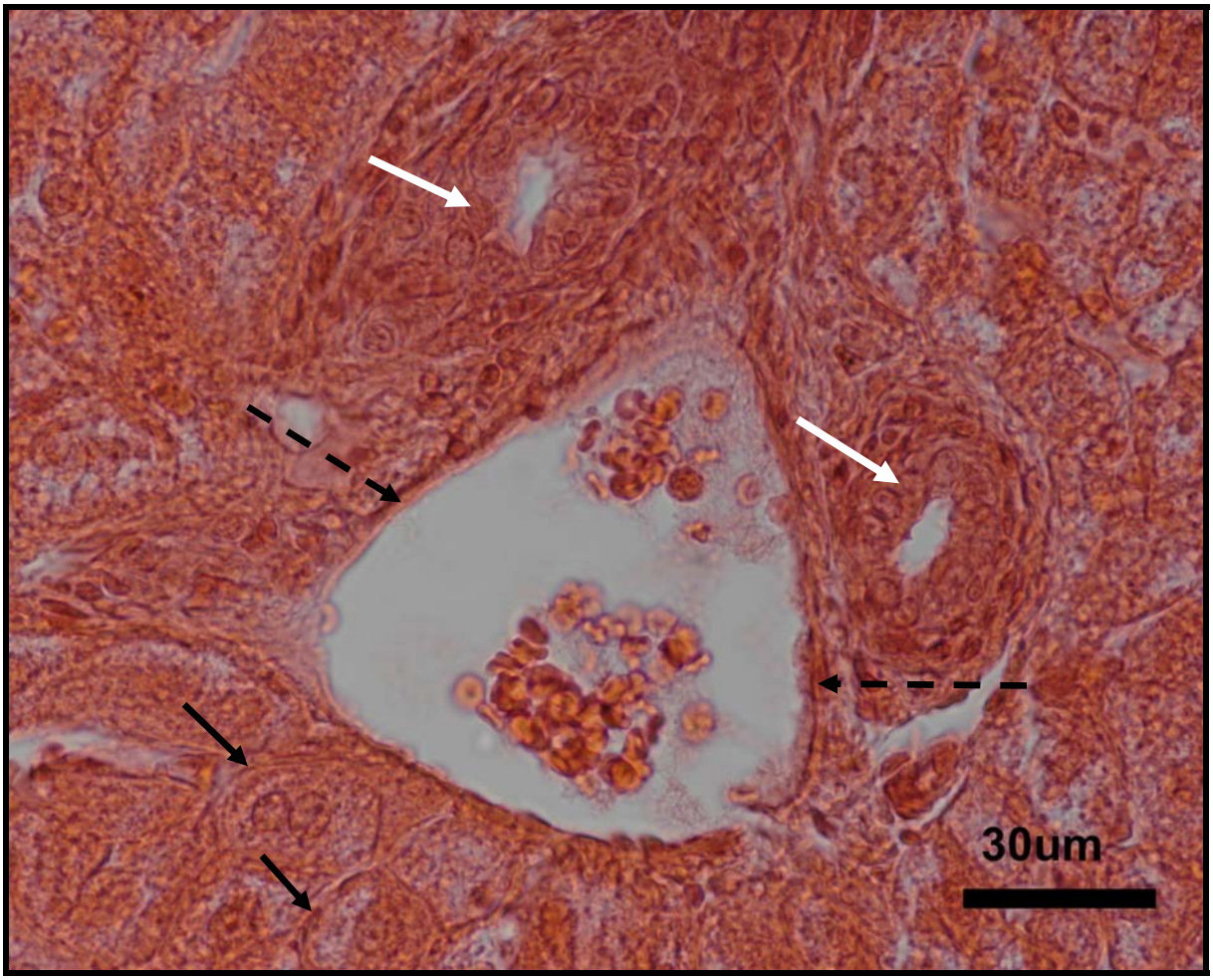


Figure 8.2 illustrates the cords of the liver, also from the right lobe, in the control rabbit.

Figure 8.4: Section through the right lobe of the liver of a control rabbit illustrating the cords that are present in the liver. Note the clear cellular membranes of the hepatocytes (arrows) and the organization of the hepatocytes in clearly distinguishable rows (dashed rectangles). Nuclei were clearly visible in the hepatocytes (circles). Endothelial lining of the blood vessel clearly visible (white arrow)

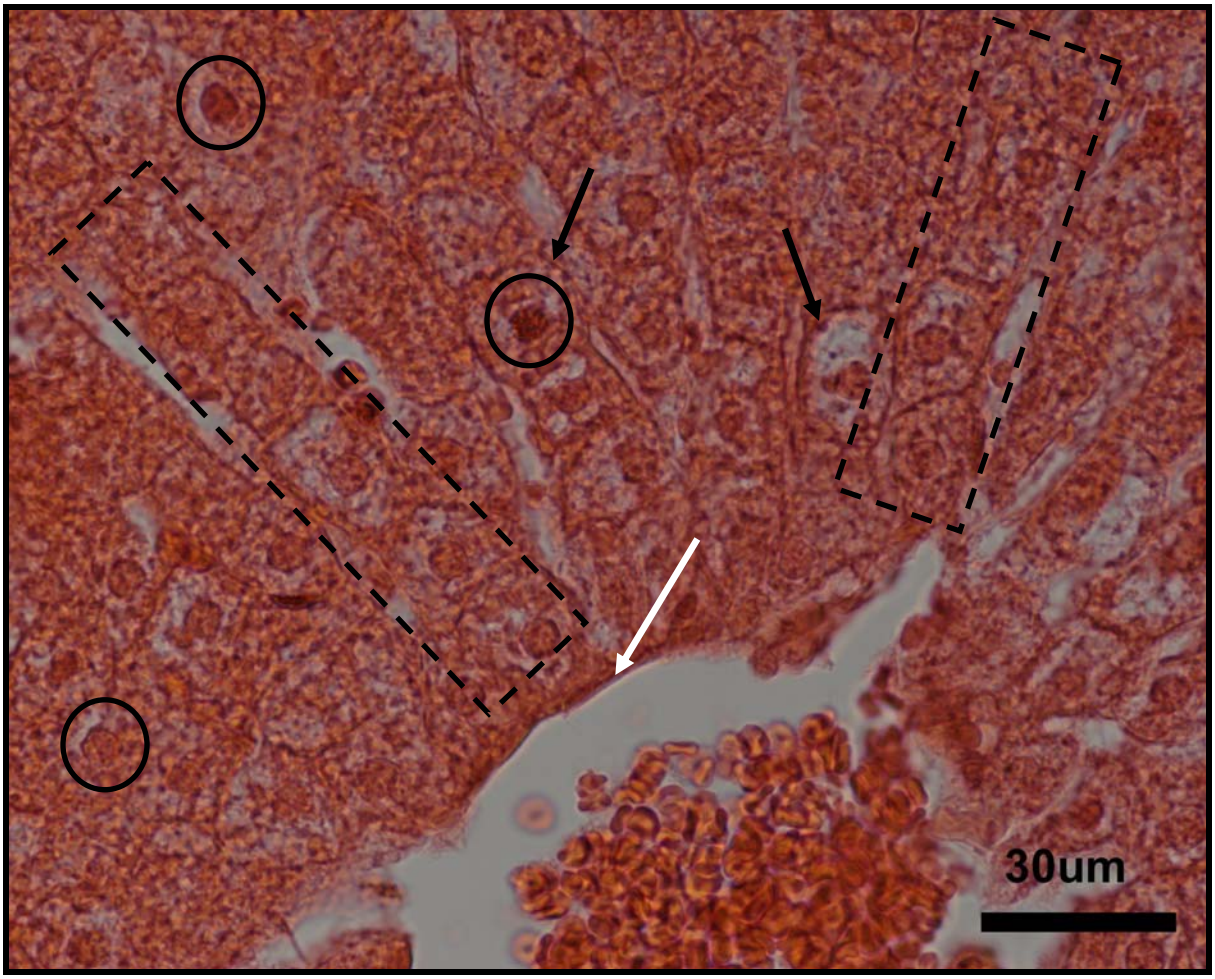


Figure 8.3a illustrates a section through the centre of the liver of a rabbit after treatment with aspartame.

Figure 8.5a: Section through the centre of the liver of a rabbit after treatment of aspartame. Note the absence of clearly distinguishable hepatocytes with no cell membranes being visible. Cords of hepatocytes were present, but appeared more like the cytoplasma of the hepatocytes formed a continuous layer. Endothelial lining of the blood vessel also not clearly distinguishable

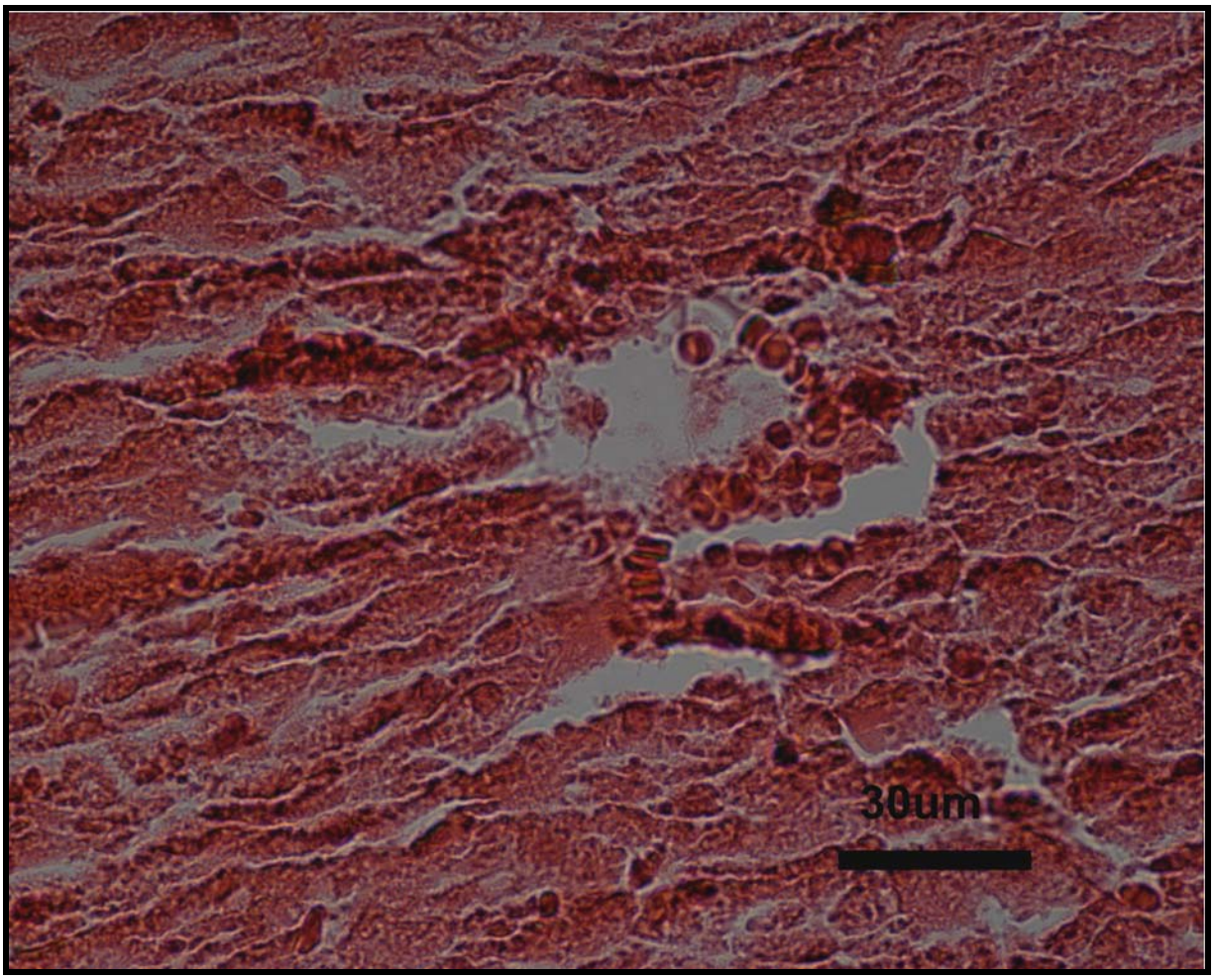


Figure 8.3b illustrates a section through the right lobe of the liver of a rabbit after treatment with aspartame.

Figure 8.3b: Section through the right lobe of the liver of a rabbit after treatment with aspartame. Cords were visible with the cellular membranes also being clear (black arrows). The cuboidal epithelium lining the duct (white arrow) was clearly distinguishable. Endothelial ling of the blood vessel appeared thinner (dashed black arrow). It appeared as though the cytoplasm of the hepatocytes were starting to become more granular, with lace-like appearance becoming more spaced and broken (circles)

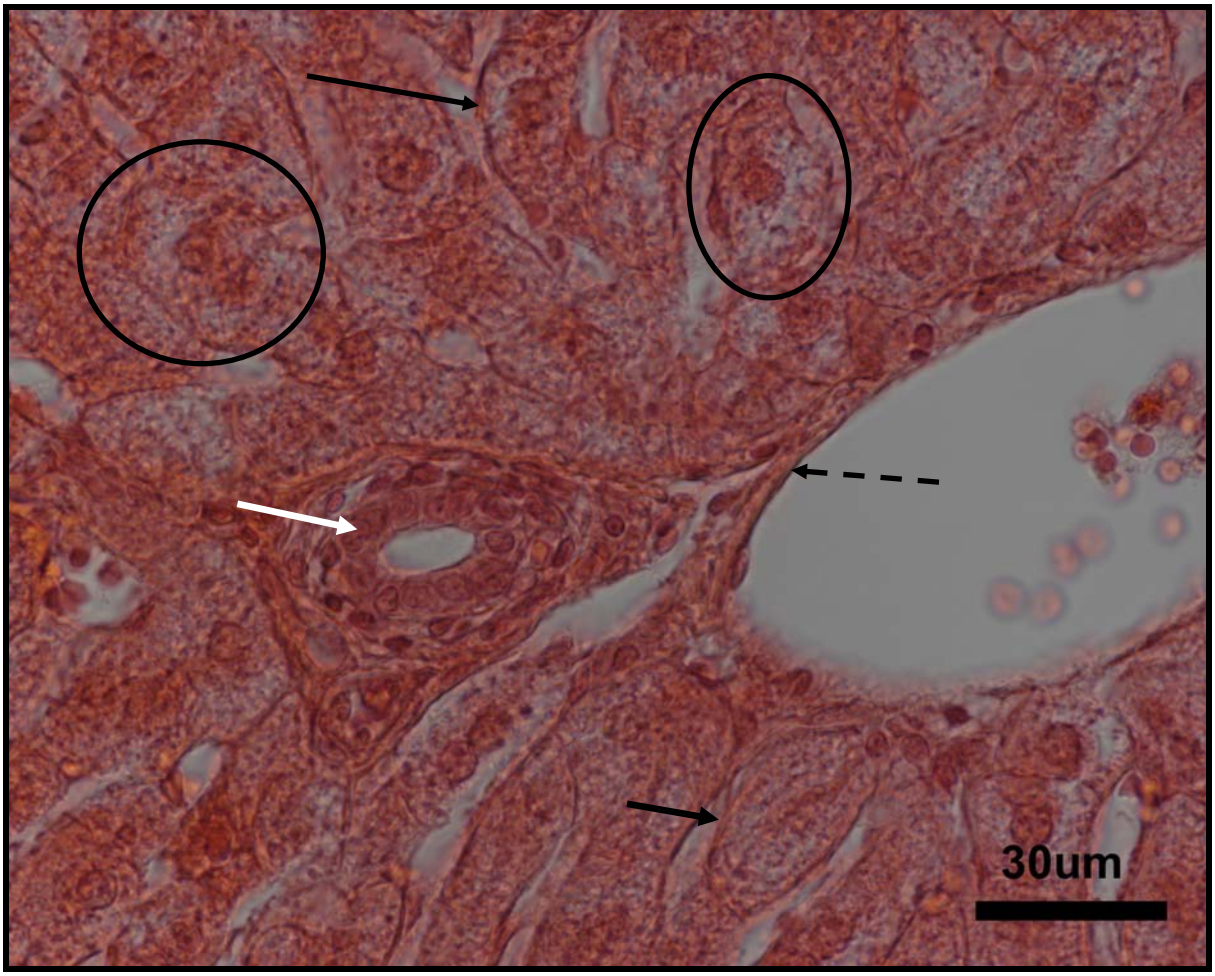


Figure 8.4a and 8.4b illustrates a section through the left lobe of the liver of a rabbit treated with aspartame.

Figure 8.6a: Section through the left lobe of the liver of a rabbit after treatment with aspartame. The circles indicate hepatocytes which appear to have been damaged with the cytoplasm appearing granular, chromatin of the nuclei condensed and the cytoplasm appear to be retracting from the nuclei

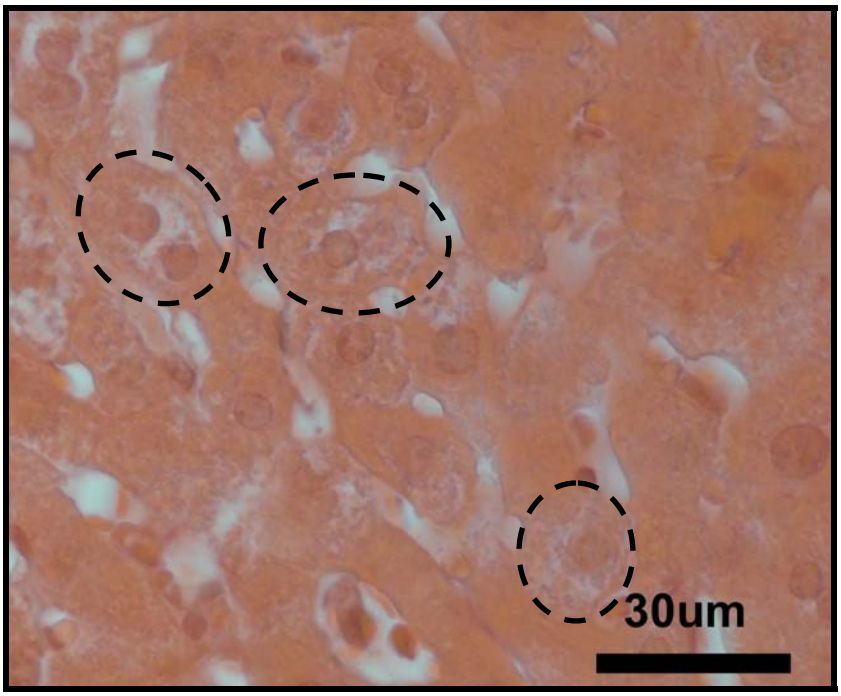


Figure 8.4b: Section through the left lobe of the liver of a rabbit after treatment with aspartame. Note the circled hepatocyte, with a clearly damaged nucleus. Chromatin appeared fragmented

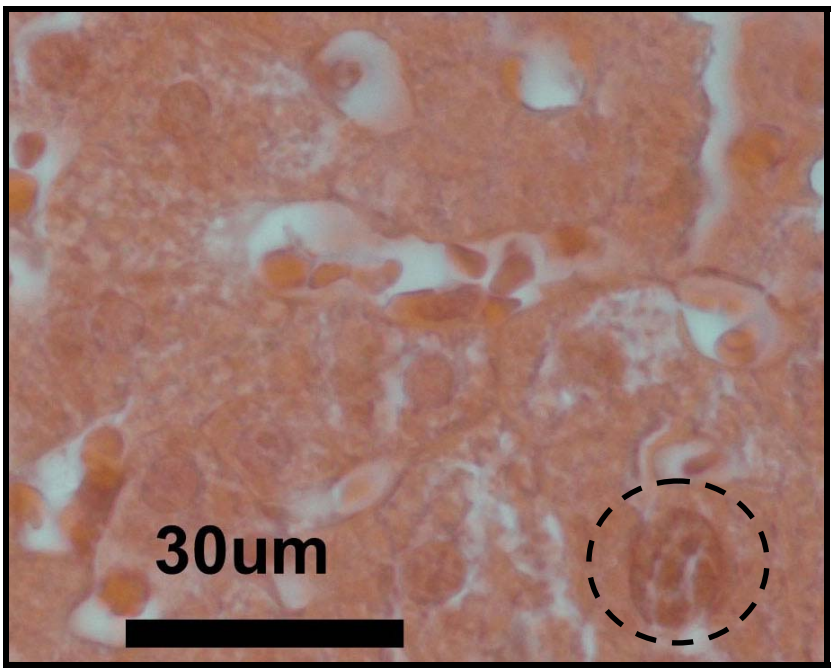


Figure 8.5 illustrates a section through the right lobe of the liver of a control rabbit.

Figure 8.5: Section through the right lobe of the liver of a control rabbit indicating a number of hepatocytes, of which three were bi-nucleate hepatocytes (circles)

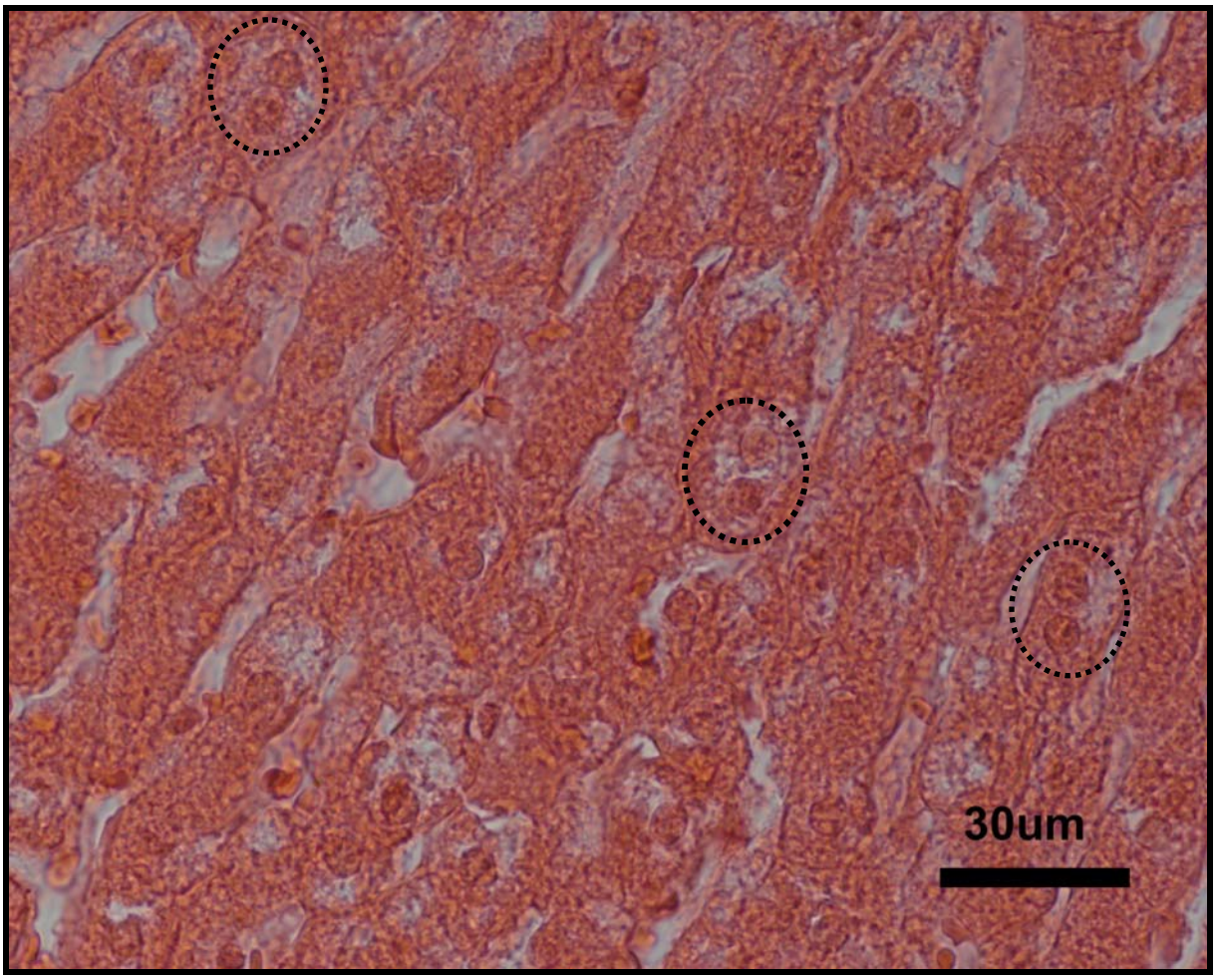


Figure 8.6 illustrates a section through the centre of the liver of a rabbit after treatment with aspartame.

Figure 8.6: Section through the centre of the liver of a rabbit after treatment with aspartame. Note the number of hepatocytes with two nuclei (circles). Cytoplasm of hepatocytes appeared more granular and chromatin inside nuclei more condensed

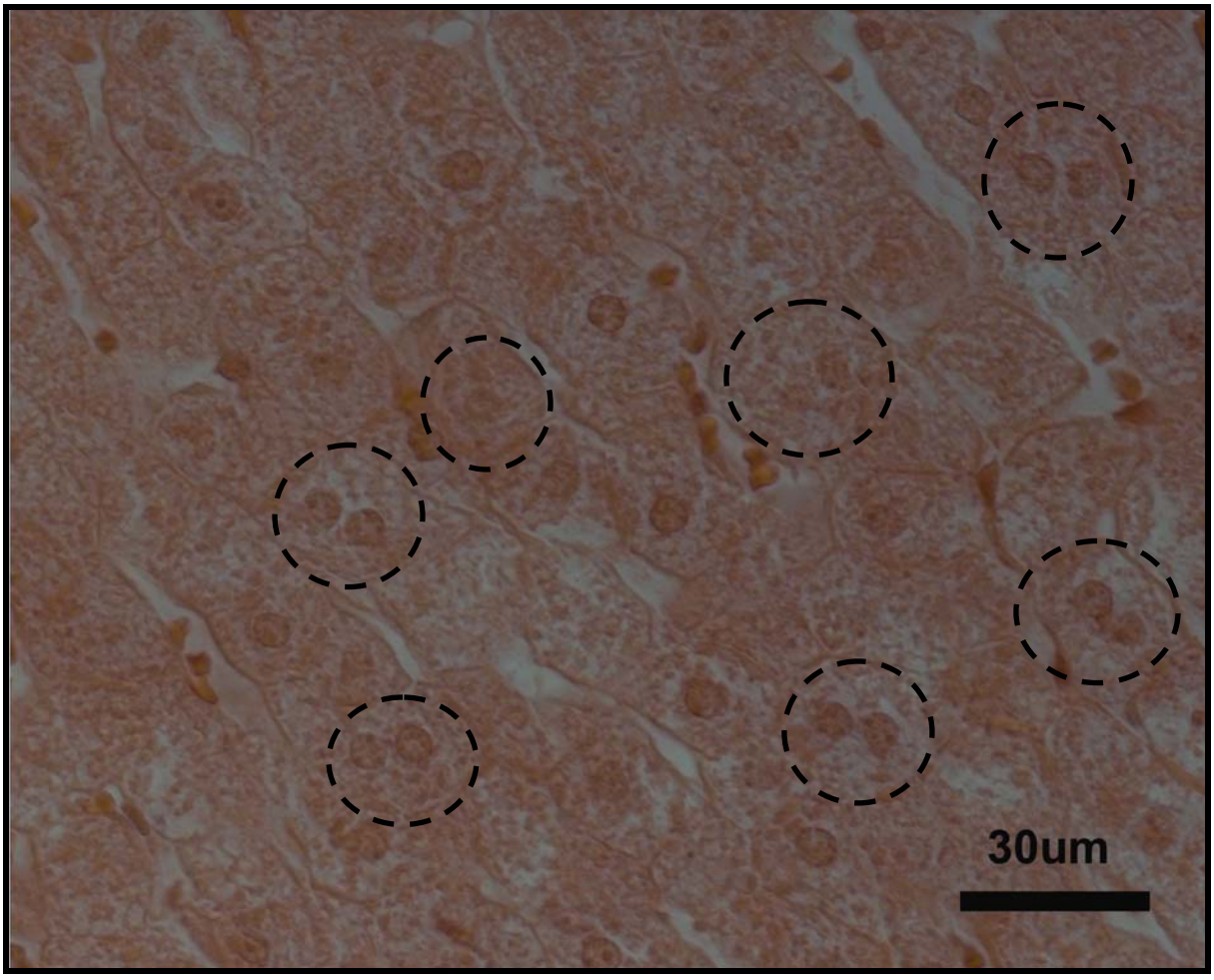


Figure 8.1a and 8.1b illustrated a section though the right lobe of the liver of a control rabbit. A central vein was visible in the centre of the illustrations containing a number of red blood cells. The endothelial lining was clearly visible (Figure 8.1b). The hepatocytes had a polygonal shape (dashed lines; Figure 8.1a) with the cellular membranes clearly visible for the different hepatocytes (Figure 8.1a and 8.1b; arrows). The nuclei of the hepatocytes had a spherical to ovoid shape with prominent, scattered chromatin granules (circle; Figure 8.1a). The cytoplasm of the hepatocytes had a lace-like appearance, possibly due to glycogen storage. Two ducts were visible in figure 8.1b, with the simple cuboidal epithelial cells having clear cellular membranes and rounded nuclei. Figure 8.2 was a section through the right lobe of the liver indicating the cords of hepatocytes that were present in the liver (dashed rectangles). Cellular membranes of the hepatocytes were clearly distinguishable, with the nuclei being prominent.

Figure 8.3a illustrated a section through the centre of the liver of a rabbit after treatment with aspartame. The cell membranes of the hepatocytes could not be identified, with the polygonal shape of the hepatocytes being absent. The nuclei of the hepatocytes were not clearly visible, with the shape ranging between round, ovoid and elongated and chromatin appeared condensed. No cords could be distinguished and it appeared as though the cytoplasm of the different hepatocytes had flown together to form one continuous layer. The endothelium of the central vein, present in the illustration could not be distinguished. Figure 8.3b was from a section through the right lobe of the liver of a rabbit after treatment with aspartame. Cords were visible with hepatocytes arranged in clear rows and cell membranes were clearly visible (arrows). The hepatocytes retained their polygonal shape, with the nucleoli being visible in the nuclei. The chromatin appeared condensed with the shape of the nuclei ranging from round to ovoid in shape (Figure 8.3b). The lace-like appearance of the cytoplasm of the hepatocytes, which had a constant pattern with a dark pink colour in the controls (Figure 8.1a), were more spaced and broken with more transparent areas being present. The cell membranes of the cuboidal epithelium lining the duct were clearly visible, with the nuclei being round (white arrow). The cytoplasm of the hepatocytes store glycogen, causing the lace-like appearance. Thus it is hypothesized that the spaces were bigger due to an increased storage of glycogen and that the pattern of the lace-like appearance could be disturbed due to damage to the hepatocytes by treatment with the aspartame. The endothelium of the central vein was clearly visible and appeared slightly thickened.

Figures 8.4a and 8.4b illustrate a section through the left lobe of the liver of a rabbit after treatment with aspartame. The hepatocytes had their characteristic polygonal shape with their nuclei appearing round to ovoid in shape. The spaced and broken appearance of the lace-like cytoplasm of the hepatocytes appeared to be more intense than the right lobe or centre of the liver, with the cytoplasm retracting/moving towards the cellular membranes. Figure 8.4b illustrates damage to the nucleus (inside circle), with the normal appearance of the scattered chromatin granules being more prominent and transparent areas starting to become visible.

The hepatocytes of the liver have a marked variation in size (polyploidy) with up to 25 percent of the cells being bi-nucleate. These bi-nuclear cells arise due to mono-nuclear cells undergoing endomitosis when nuclear volume and DNA content have approximately doubled. Mitosis is rare in adult liver cells (1 mitosis per 15 000 cells), but numerous mitotic figures can be found during repair following injury (Leeson *et al*., 1988a). More bi-nucleate cells were visible in the two aspartame treated rabbits than in the three control rabbits, after viewing under the microscope. Figure 8.5 and 8.6 represent a single eye field of a control rabbit and aspartame treated rabbit respectively. A comparison was done between the total number of cells present in both figures (Figure 8.5 and 8.6) according to nuclei that was clearly distinguishable. The following results were obtained:

Table 8.2: Comparison between the number of bi-nucleate cells in the control and aspartame treated rabbits

	Controls	Aspartame treated rabbits
Average number of cells/single eye field	35	45
Total number of single eye fields studied	15	15
Average number of bi-nucleate cells	3	7
Percentage bi-nucleate cells	8.57%	15.55%

8.57% of the hepatocytes were bi-nucleate in the control rabbit. If taken as an experimental count, this could be an indication of the normal number of bi-nucleate hepatocytes as counted in for a control in a single eye-field. It is therefore hypothesized that the increase from 8.57% to 15.55% (increase of 6.8%) could be due to mitosis to repair the damage and injury to the hepatocytes as indicated by figure 8.4a and 8.4b after treatment with aspartame.

Three sections of the liver (left lobe, right lobe and centre) were utilised for determining the effects of aspartame on the liver. For the control rabbits, there was no visible difference between the different sections of the liver. This appeared not to be the case for the rabbits treated with the aspartame. The left and right lobe showed the same level of damage, exhibiting a granular cytoplasm (lace-like appearance spaced and broken, with the cytoplasm appearing to retract from the nuclei and the chromatin becoming denser (chromatin granules more scattered) (Figures 8.3b, 8.4a and 8.4b). However, cell membranes and cords of hepatocytes were still visible. The effect of the aspartame on the centre of the liver was far more pronounced as the effect on the left and the right lobes. The cell membranes of the hepatocytes could not be distinguished with the cytoplasm of the different hepatocytes appearing as one continuous layer. The cords of the hepatocytes could not be distinguished (Figure 8.3a). Figure 8.5 illustrated the centre of the liver with seven different hepatocytes

having bi-nucleate nuclei. If the hypothesis that there was an increase in the number of bi-nucleate hepatocytes between the control and aspartame treated rabbits was accepted, three of the seven bi-nucleate hepatocytes (8.57% of 45 hepatocytes subtracted from seven hepatocytes with bi-nucleate nuclei) was busy undergoing mitosis to repair the damage and injury brought about by the treatment with aspartame. These result lead to the hypothesis, that the larger the distance from the centre of the liver, the less outspoken the effect of aspartame on the hepatocytes, but an effect will be present and due to the damage to the hepatocytes that the liver attempts to replace the damaged hepatocytes. Results obtained in chapter 5 of this thesis, support the microscopic findings that the liver must have been damaged as three of the five factors (F VII, F X and fibrinogen) produced and secreted by the liver were affected after treatment with aspartame. In humans, if both F VII and F X concentrations are decreased, prolonged hepatitis is diagnosed (Package insert – STA Deficient X; Ref 00738), thus it is hypothesized that this could also have been a possibility in this study. Phenylalanine is converted to tyrosine in the liver by phenylalanine hydroxylase. Thus it is hypothesized that due to the damage to the hepatocytes, the liver could be unable to convert aspartame to tyrosine, thus the level of phenylalanine in the blood was high, thus increasingly suppressing tryptohan conversion to serotonin as tryptophan cannot be carried over the BBB.

8.3.2 Microscopic study of the histological morphology of the kidney

8.3.2.1 Cortex of the kidney

Figure 8.7a and 8.7b illustrates renal corpuscles and the medullary rays of the kidney of a control rabbit.

Figure 8.7a: Cortex of the kidney of a control rabbit illustrating a number of renal corpuscles (circles) and tubules

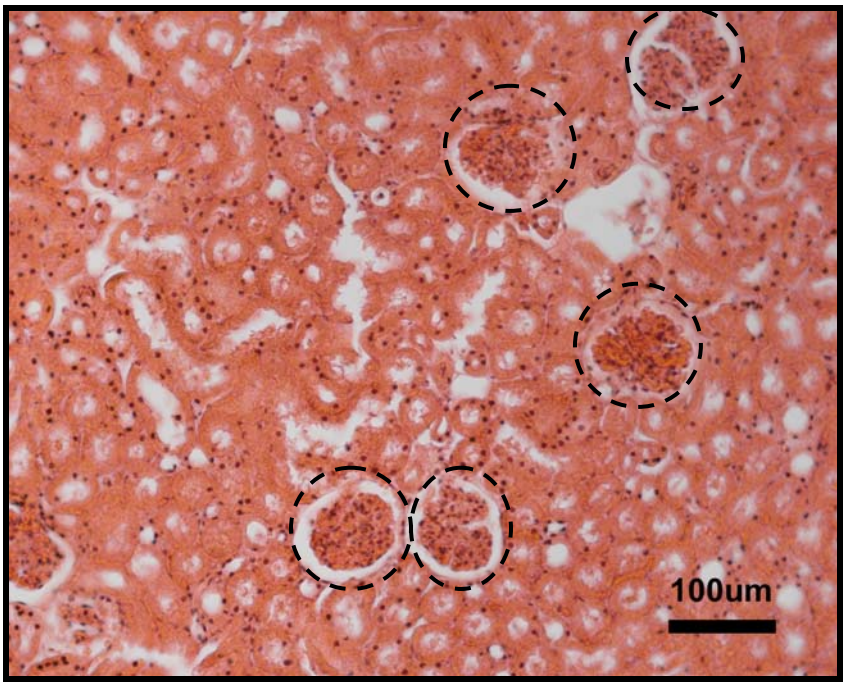


Figure 8.7b: Cortex of the kidney of a control rabbit illustrating renal corpuscles (circles) and the medullary rays (arrows) present in the cortex

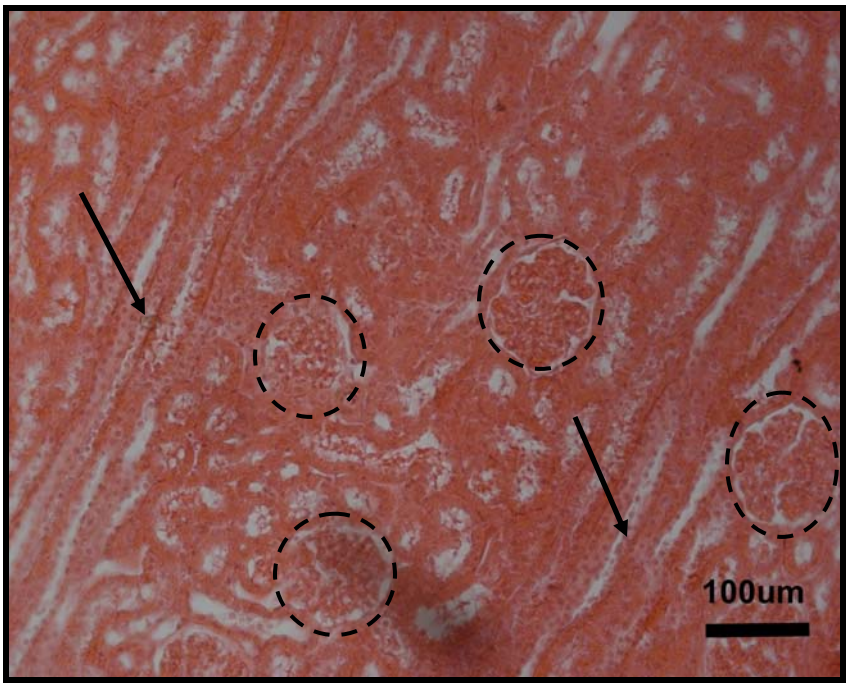


Figure 8.8 illustrates the renal corpuscle of a control rabbit at a higher magnification.

Figure 8.8: Renal corpuscle of a control rabbit. Label A – extraglomerular mesangial cells; Label B – vascular pole; Label C – glomerulus; Label D – mesangial cells; Label E – urinary space; Label F – parietal layer of Bowman's capsule; Label G - Nucleus of podocyte

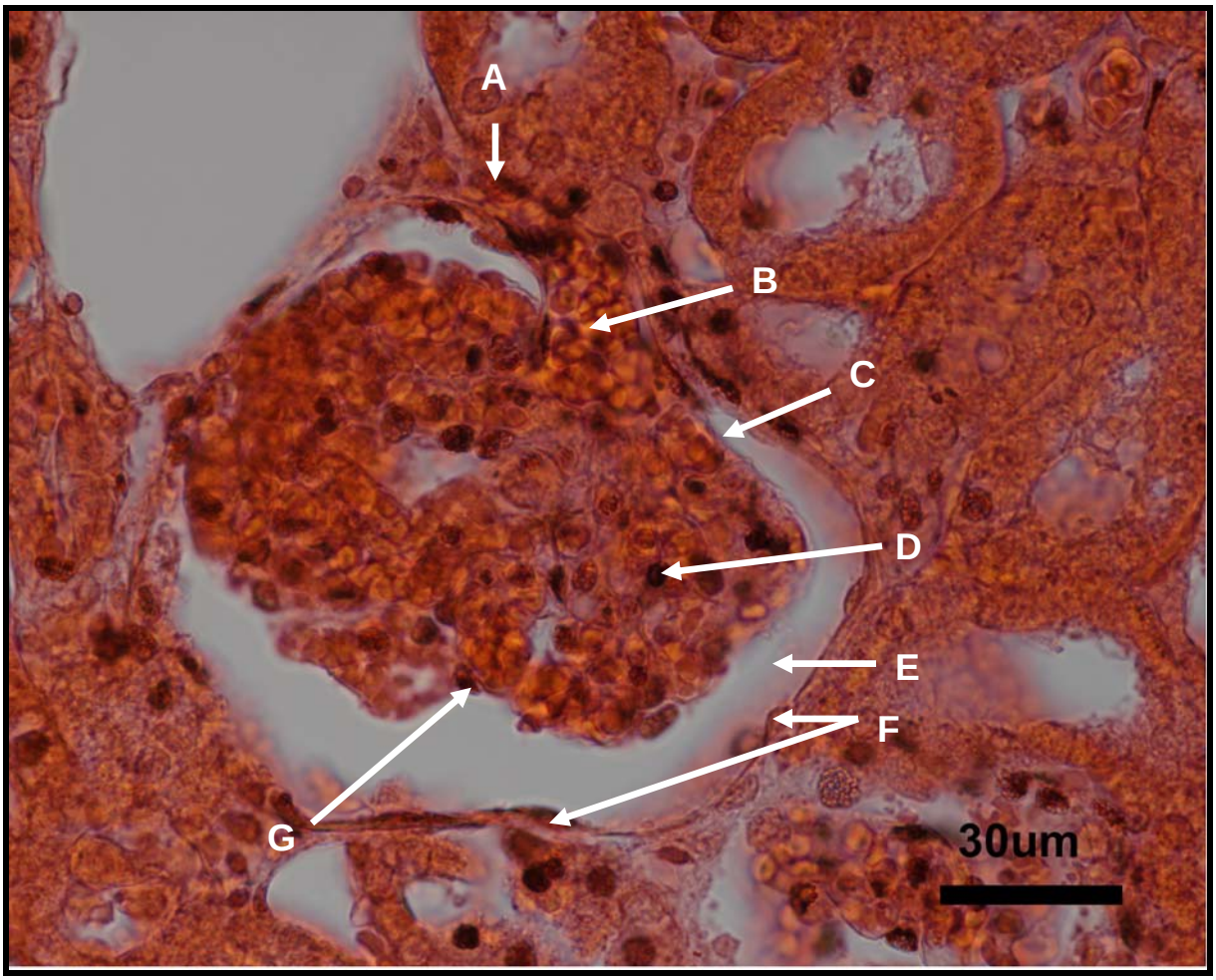


Figure 8.9a and 8.9b illustrates the layers of Bowman's capsule of a control rabbit at a higher magnification.

Figure 8.9a: Parietal layer of the capsule of Bowman in a control rabbit (arrows)

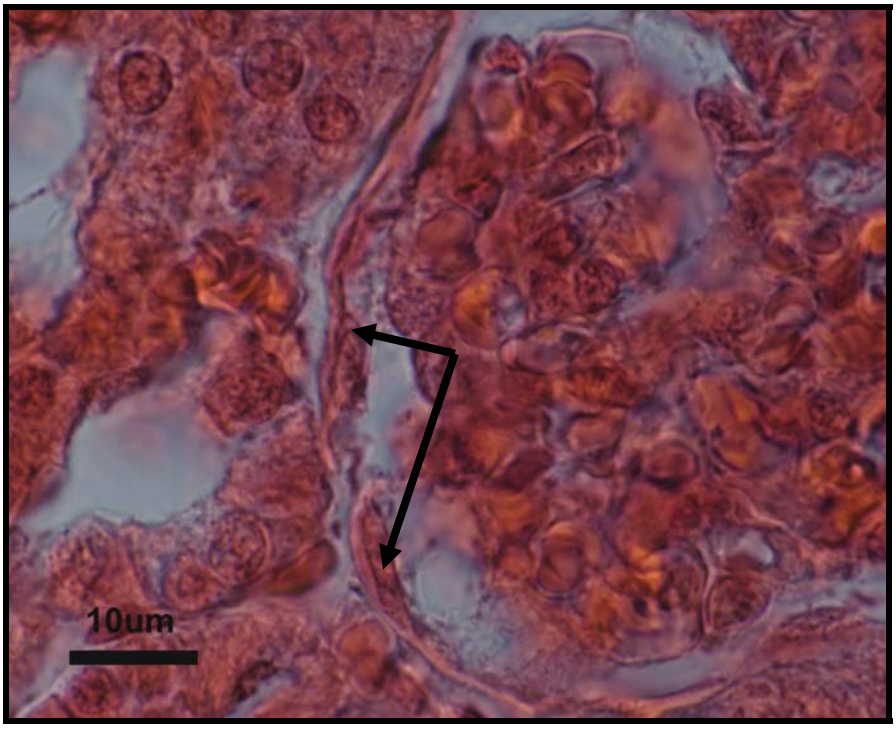


Figure 8.9b: Visceral layer of the capsule of Bowman in the control rabbit (arrows). The dashed arrow indicates the nucleus of a podocyte

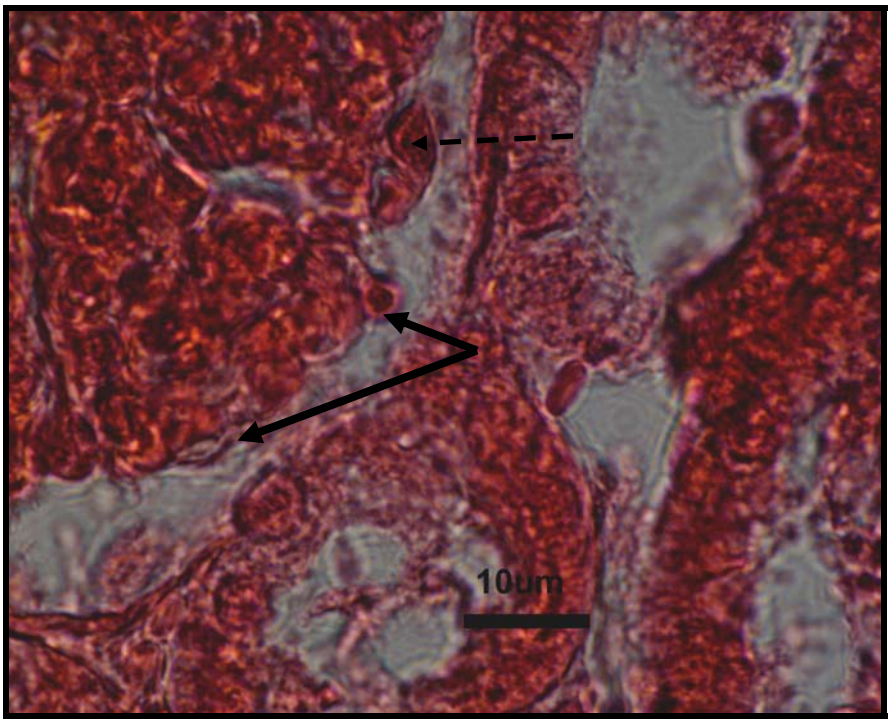


Figure 8.10 illustrates the renal corpuscles and medullary rays of a rabbit after treatment with aspartame.

Figure 8.10: Cortex of the rabbit treated with aspartame, illustrating the renal corpuscles (circles) and the medullary rays (arrows). The dashed arrow indicates damage to cellular membranes of cuboidal epithelium lining the tubules

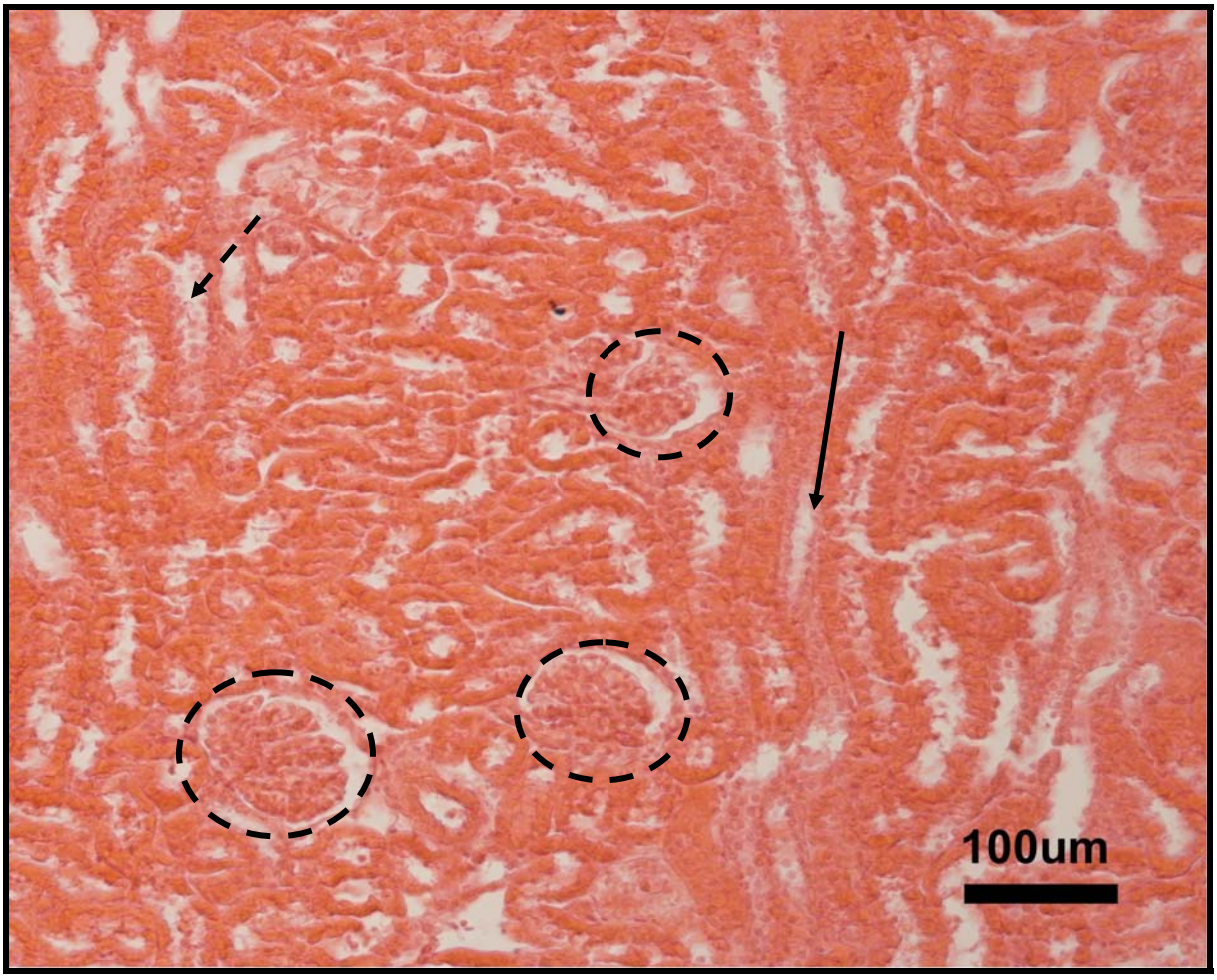


Figure 8.11 illustrates the renal corpuscle of an aspartame treated rabbit.

Figure 8.11: Glomerulus of an aspartame treated rabbit. Label A – extraglomerular mesangial cells; Label B – vascular pole; Label C – glomerulus with mesangial cells; Label D – urinary space; Label E – parietal layer of Bowman's capsule; Label F – nucleus of podocyte

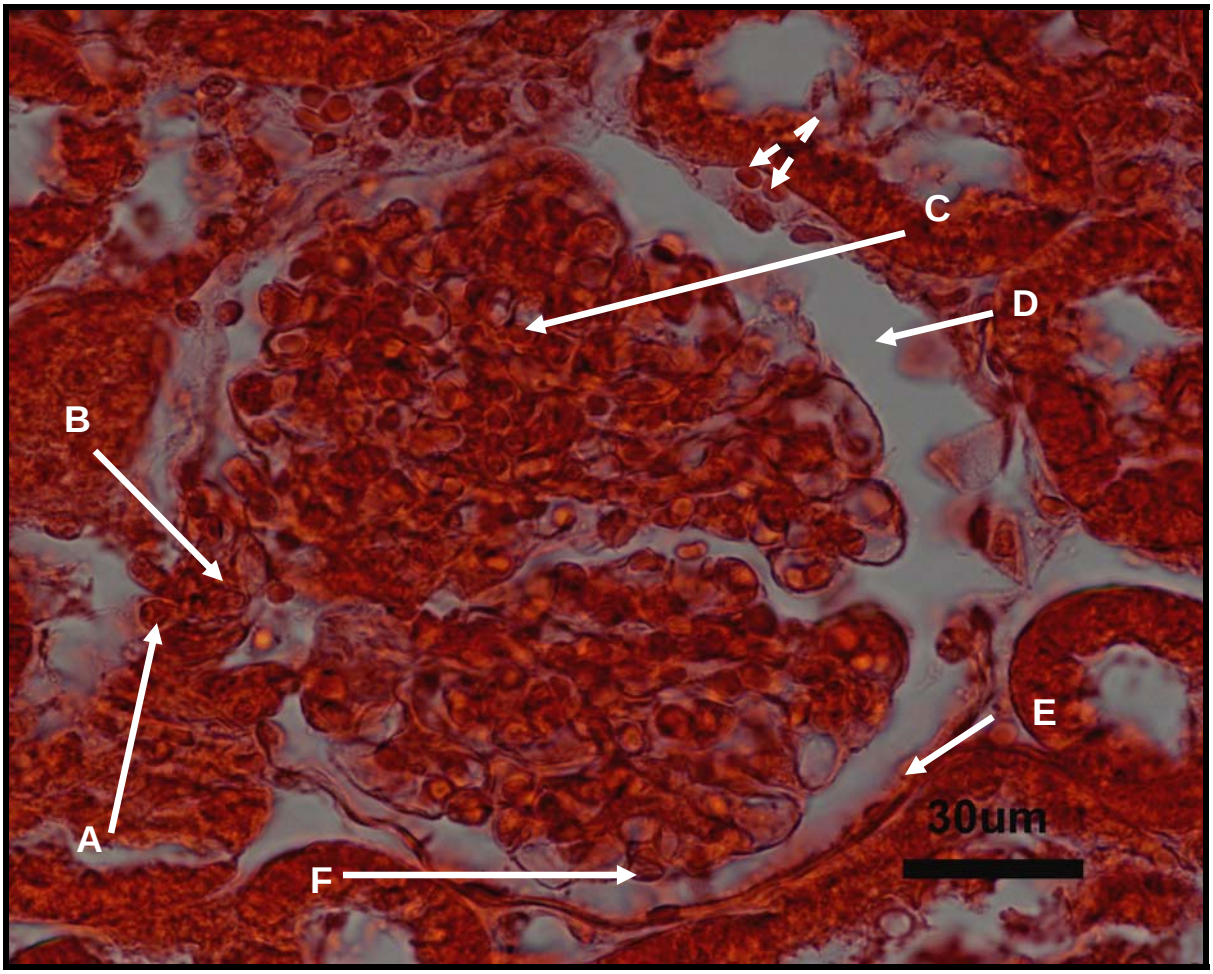


Figure 8.12a and 8.12b illustrates the layer of Bowman's capsule at a higher magnification.

Figure 8.12a: Parietal layer of the capsule of Bowman of a rabbit after treatment with aspartame (arrows)

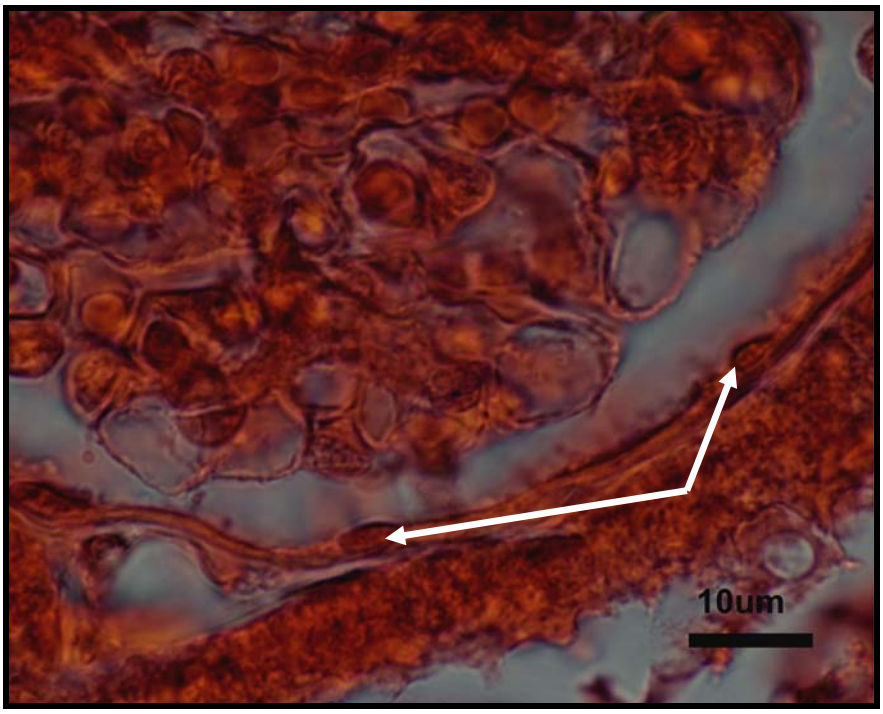


Figure 8.12b: Visceral layer of the capsule of Bowman of a rabbit after treatment with aspartame (arrows)

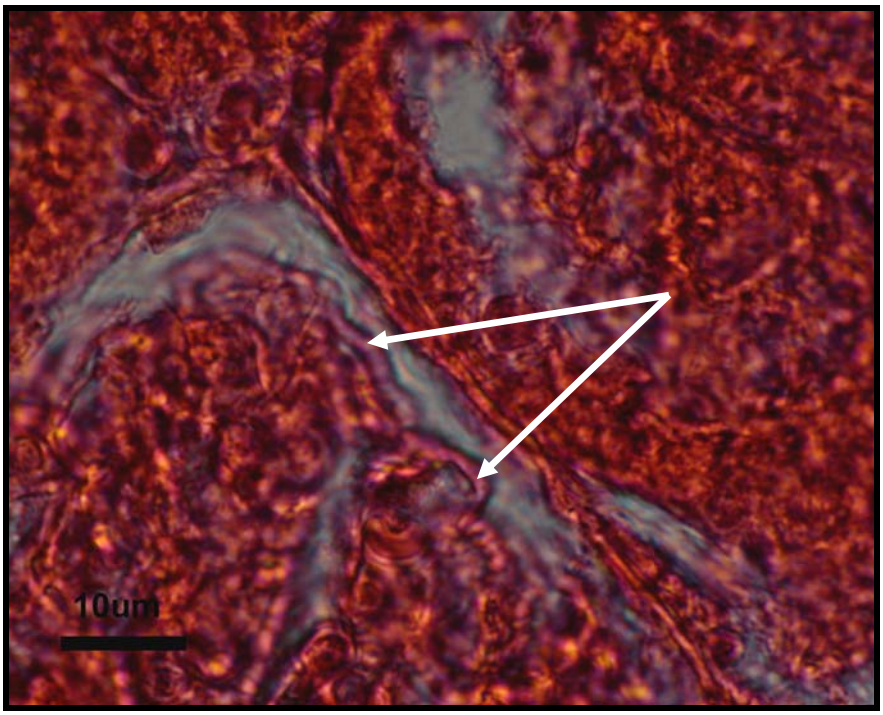


Figure 8.7a and 8.7b illustrated the renal corpuscles and medullary rays that were present in the cortex of a control rabbit. The urinary space surrounding the glomeruli of the renal corpuscles was clearly visible, even at low magnification. Figure 8.8 illustrated a renal corpuscle of a control rabbit at a higher magnification. The extra-glomerular mesangial cells (Label A) were visible at the vascular pole (Label B) of the renal corpuscle and the urinary space (Label D) could also be distinguished. The parietal layer of the capsule of Bowman (Label E) was noted with the simple squamous epithelium having clear flat nuclei which very slightly protruded into the urinary space. A nucleus of a podocyte (Label F) of the visceral layer of the capsule of Bowman was visible. Figure 8.9a and 8.9b illustrated the different layers of the capsule of Bowman, only at a higher magnification. The simple squamous epithelium with the flattened nuclei could be distinguished (arrow; Figure 8.9a). The visceral layer, consisting of podocytes (dashed arrow; Figure 8.9b) was visible as a very thin line on the outer surface of the glomerulus.

Figure 8.10 illustrated the cortex of a rabbit after treatment with aspartame. The renal corpuscles were clearly visible, with the urinary space (circles) appearing enlarged. The tubules found in the medullary rays (arrow) appeared damaged (dashed arrow) as the cellular membranes could not be distinguished. It appeared as though the nuclei of the simple cuboidal epithelium lining the tubules were spilling into the lumen of the tubules.

Figure 8.11 illustrates a renal corpuscle at a higher magnification. Extra-glomerular mesangial cells were visible (Label A) at the vascular pole (Label B) of the renal corpuscle. A number of mesangial cells were visible between the capillaries of the glomerulus (Label C), which appeared to have increased (more nuclei visible than in control) and both layers of Bowman's capsule were very clear. Erythrocytes were visible underneath the parietal layer of Bowman's capsule (dashed arrows; Label E) and the layer itself appeared thickened. The visceral layer of the capsule of Bowman (Label F) investing the capillaries of the glomerulus were clearly visible (Label G). When these layers were studied at a higher magnification (Figures 8.12a and 8.12b), both the parietal and visceral layers appeared to have thickened. The visceral layer that was not visible at first glance for the control rabbit (Figure 8.9b), was very clearly distinguishable after treatment with aspartame (Figure 8.12b).

8.3.2.2 Medulla of the kidney

Figure 8.13 illustrates the medulla with the collecting tubules in a control rabbit.

Figure 8.13: Collecting tubules in the medulla of a control rabbit. Arrows indicate the cuboidal epithelium of the proximal convoluted tubules. Note the clearly distinguishable cellular membranes

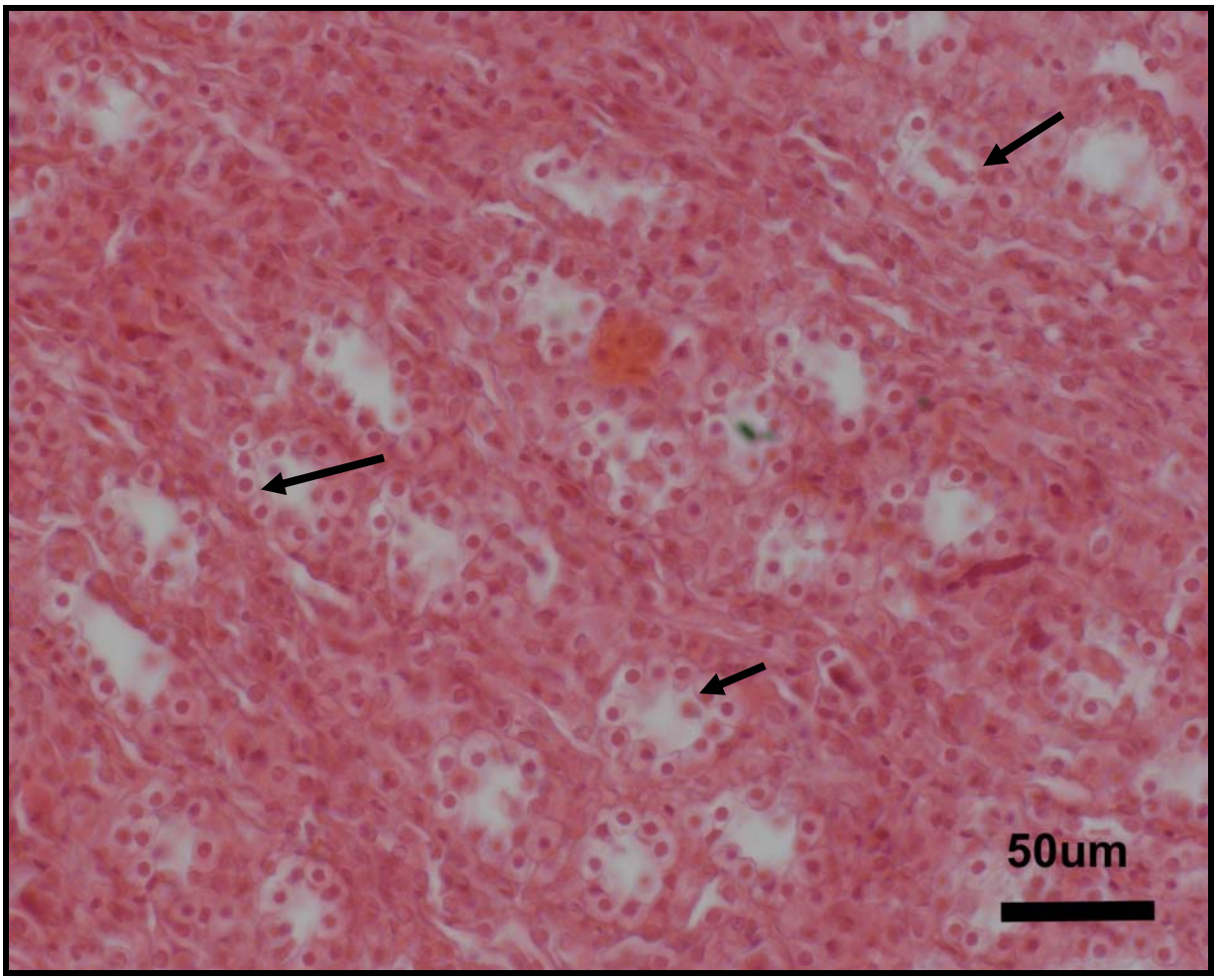


Figure 8.14 indicates the squamous epithelium of thin segment of the Loop of Henle's in a control rabbit.

Figure 8.14: Squamous epithelium of the thin segment of the loop of Henle's in the control rabbit (arrows)

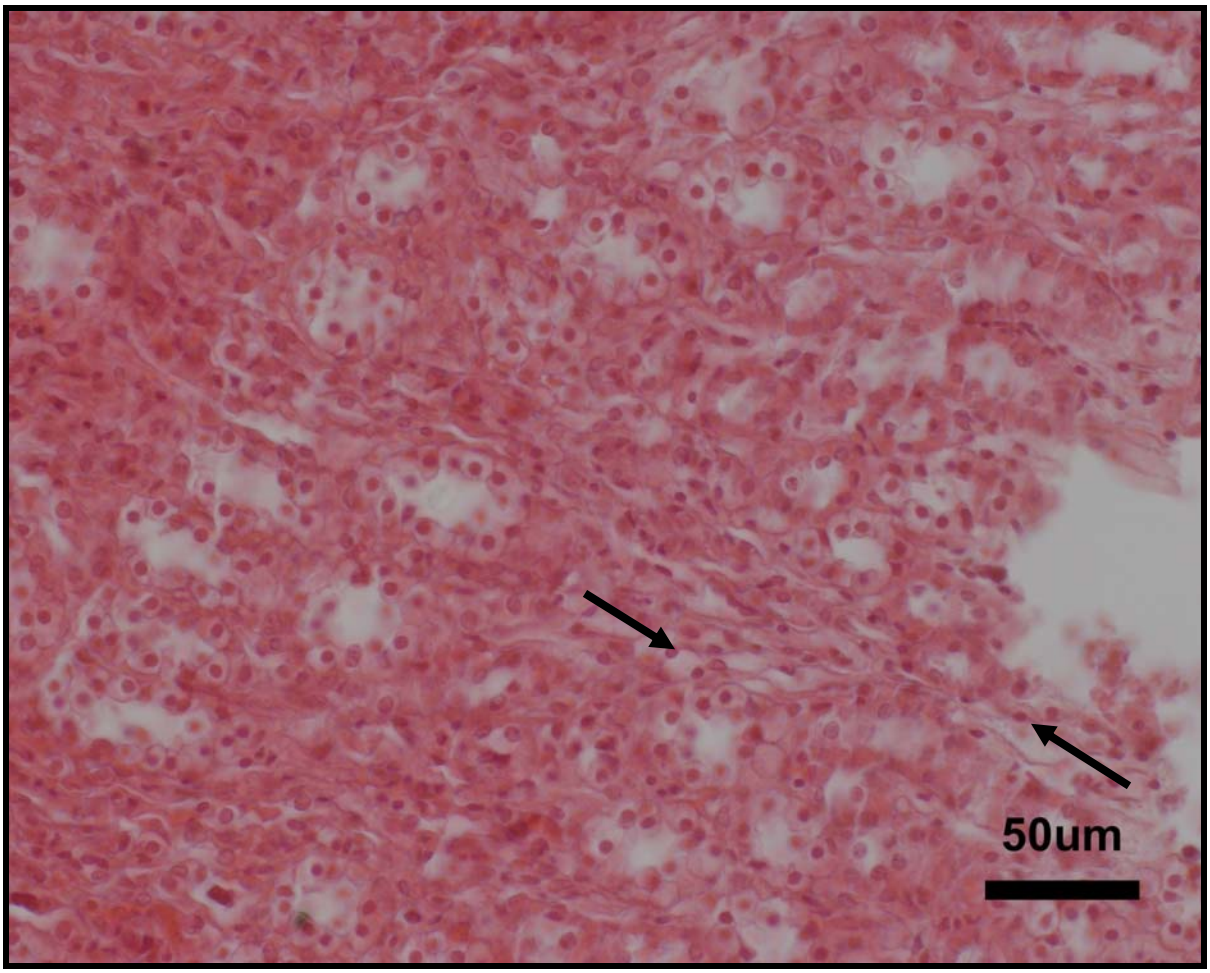


Figure 8.15a and 8.15b illustrates the cuboidal and squamous epithelium of the proximal convoluted tubule and thin segment of the loop of Henle's respectively in a control rabbit at higher magnifications (x40 and x 100 respectively). Figure 8.15c illustrates an endothelial cell of a control rabbit.

Figure 8.17a: Arrow indicates the cuboidal epithelium of the proximal convoluted tubule and the dashed arrow indicate the squamous epithelium of the thin segment of the loop of Henle's in a control rabbit

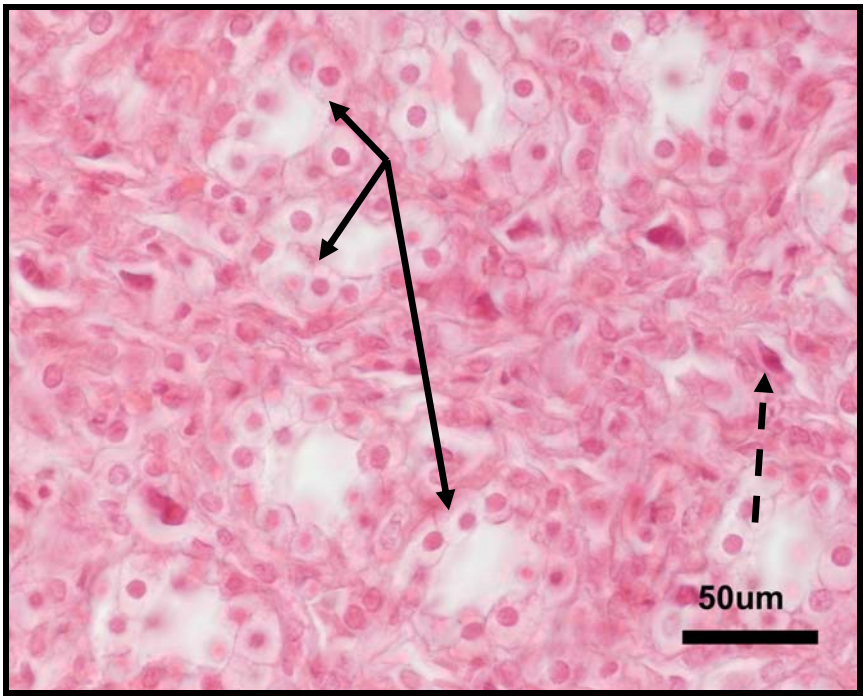


Figure 8.15b: Arrow indicates the cuboidal epithelium of the proximal convoluted tubule in a control rabbit. Note the clearly distinguishable cellular membranes. The dashed arrow indicates the squamous epithelium of the thin segment of the loop of Henle's

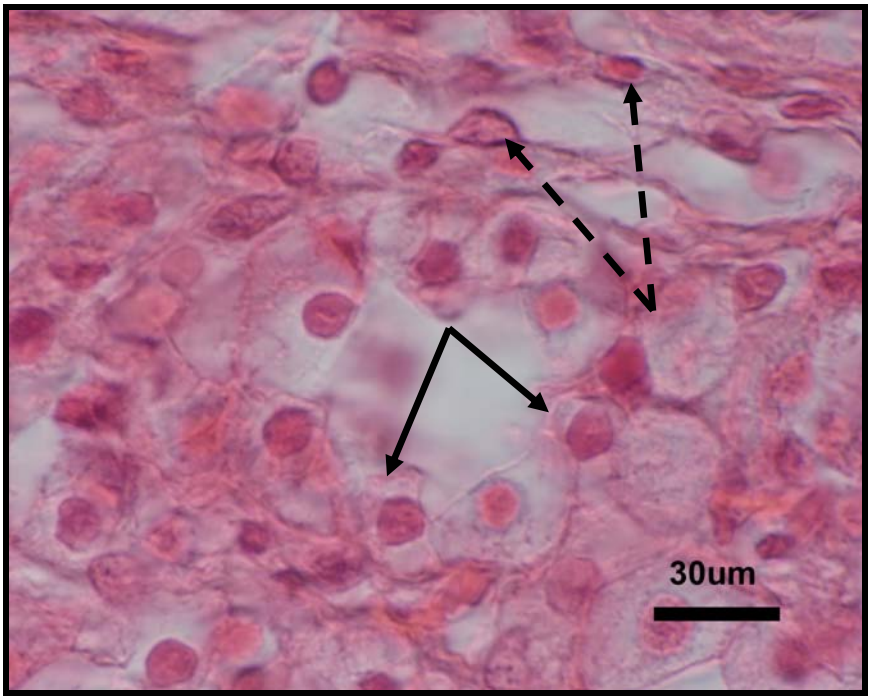


Figure 8.15c: Endothelial cell (arrow) inside the vasa recta of a control rabbit. Note the flattened, ovoid shape of the nucleus

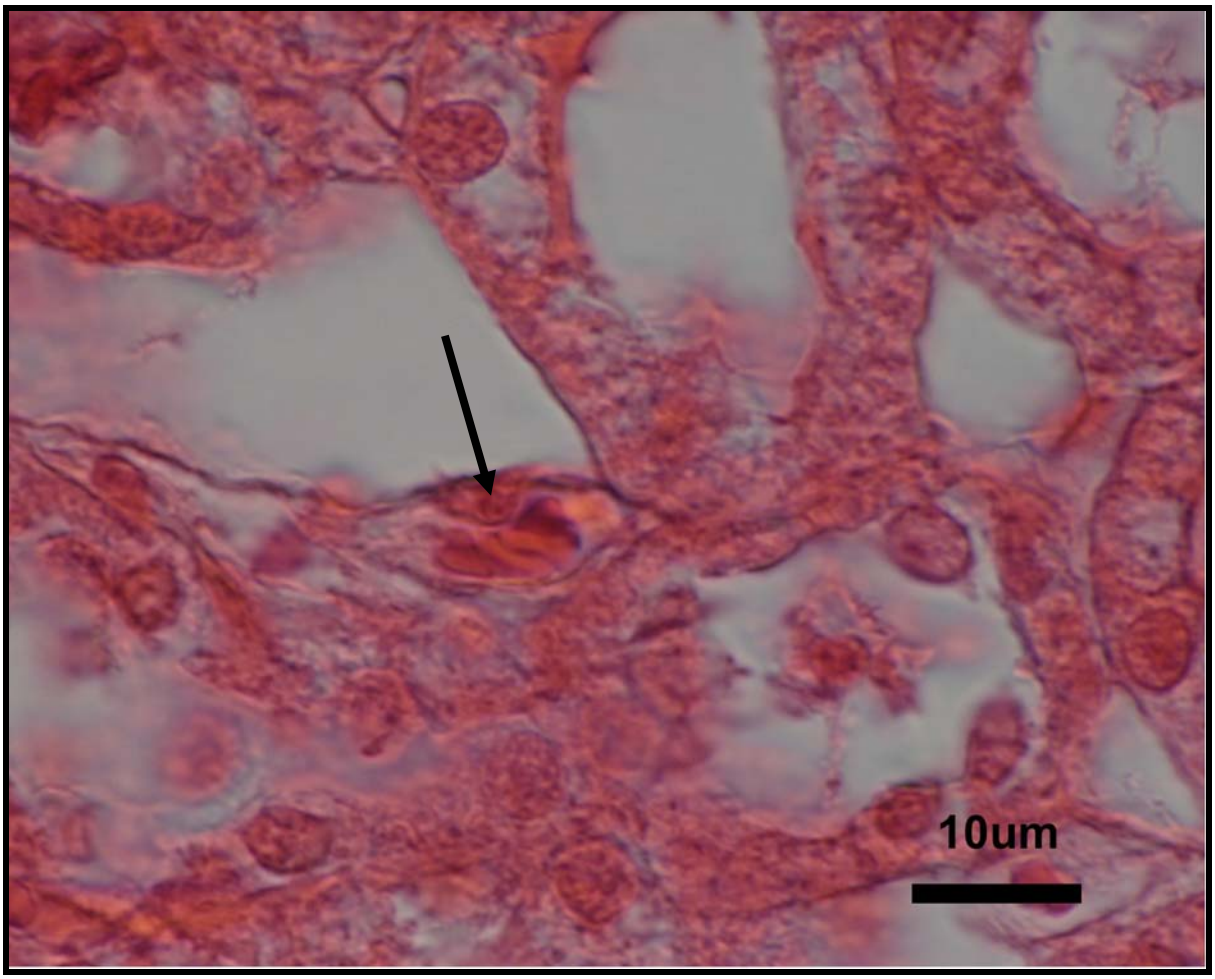


Figure 8.16 illustrates the medulla with the collecting tubules in an aspartame treated rabbit.

Figure 8.16: Collecting tubules in the medulla of an aspartame treated rabbit. Arrows indicate the cuboidal epithelium of the proximal convoluted tubules. Note that the cellular membranes have suffered damage

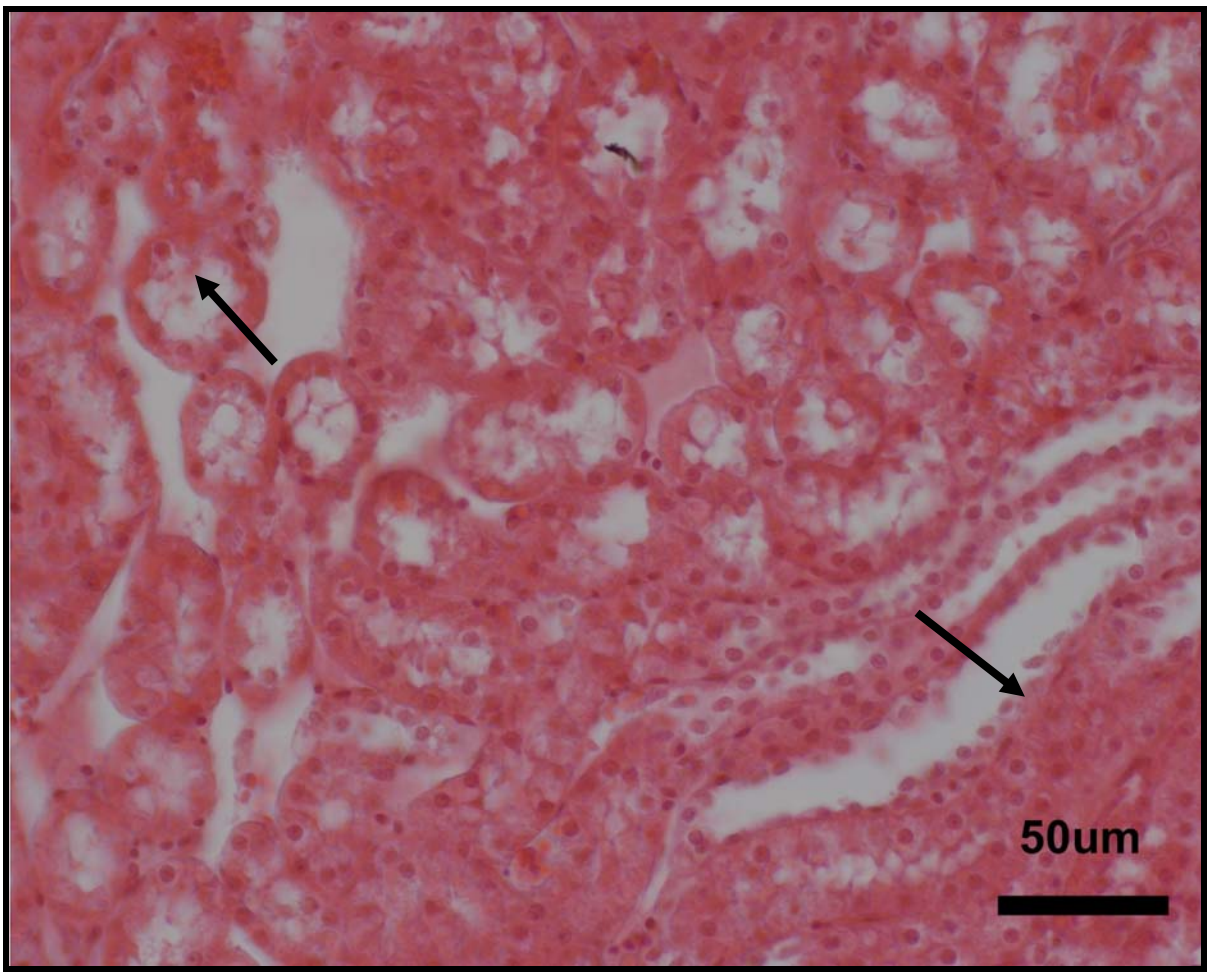


Figure 8.17 indicates the squamous epithelium of thin segment of the Loop of Henle's in a aspartame treated rabbit.

Figure 8.17: Squamous epithelium of the thin segment of the loop of Henle's in the aspartame treated rabbit (arrows). Note the rounded nucleus of an endothelial cell (dashed arrow) inside the vasa recta

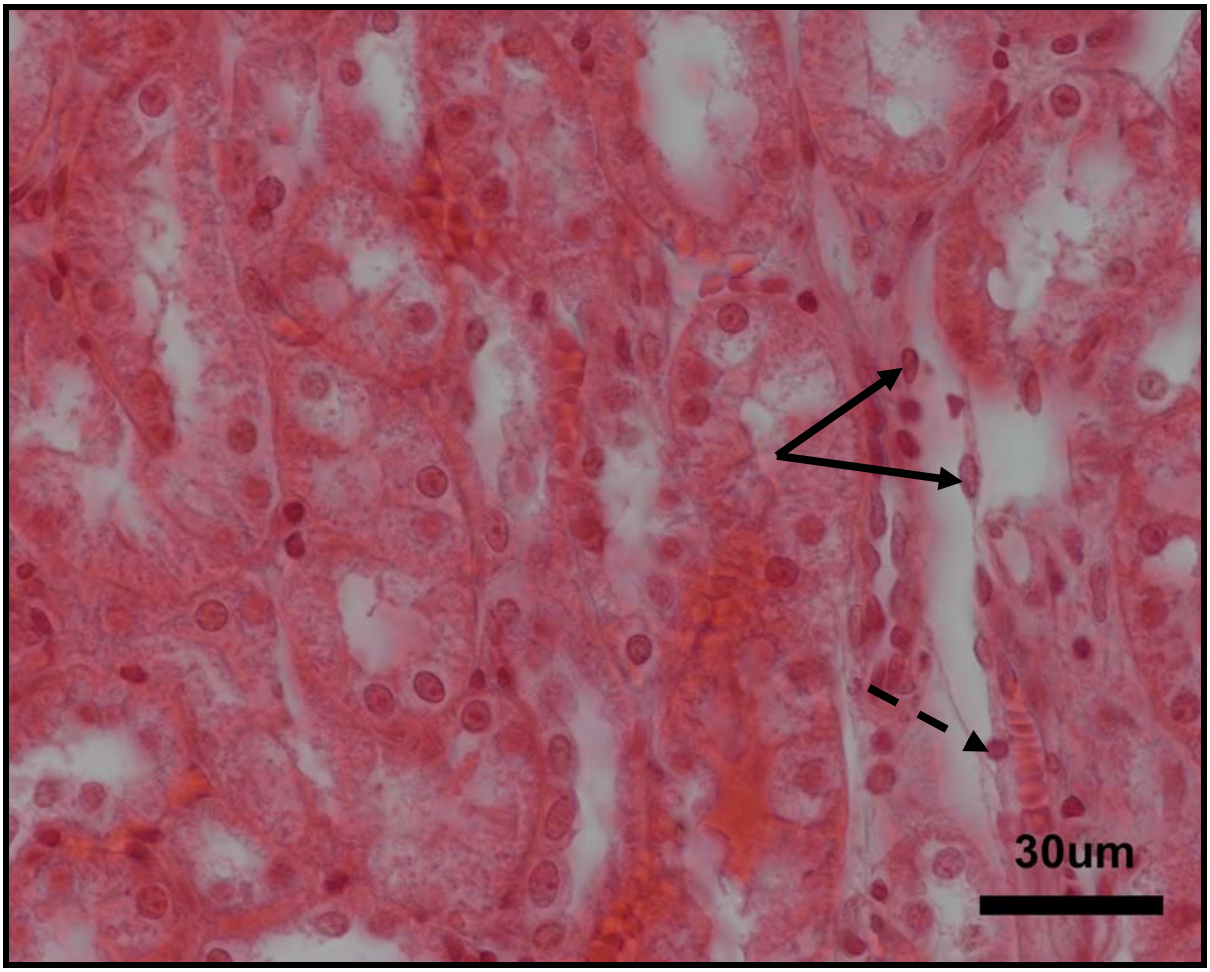


Figure 8.18a illustrates the cuboidal and squamous epithelium of the proximal convoluted tubule and thin segment of the loop of Henle's respectively and figure 8.18b illustrates the endothelium lining in an aspartame treated rabbit at higher magnifications (x40 and x 100 respectively).

Figure 8.18a: Cuboidal epithelium of the proximal convoluted tubule (arrows) and squamous epithelium of the thin segment of the loop of Henle's (dashed arrows) in the aspartame treated rabbits. The cytoplasm of the cuboidal cells appeared washed-out. Damaged cellular membranes of the cuboidal epithelium were also present (white arrows)

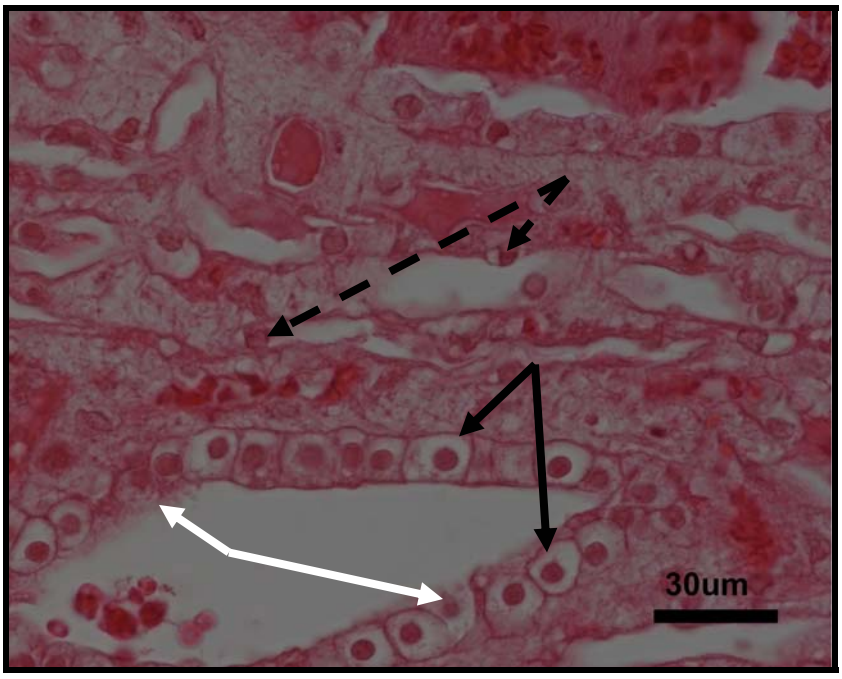


Figure 8.18b: Endothelial lining of the vasa recta in an aspartame treated rabbit. Note the rounded, not flat, nucleus of the endothelial cell (arrow)

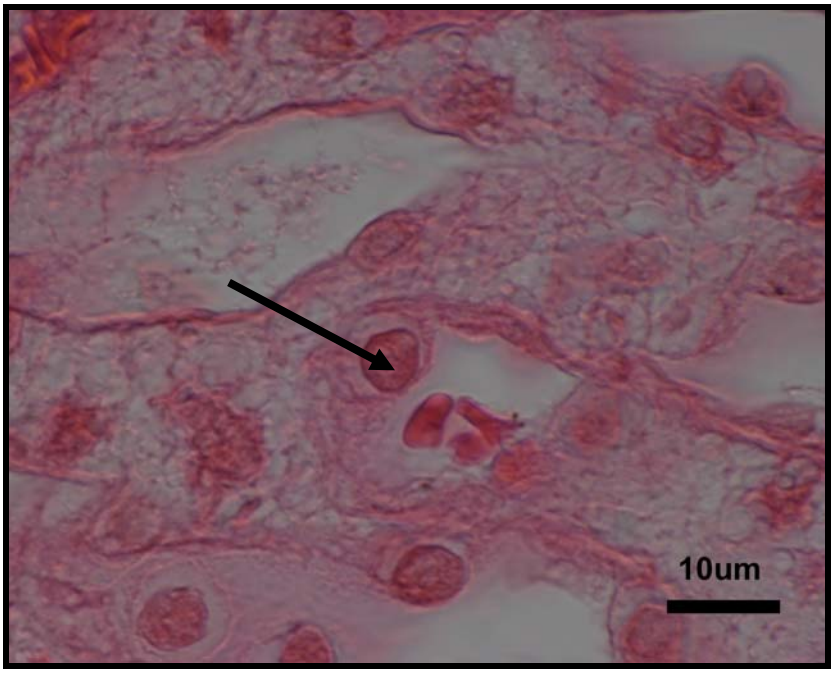


Figure 8.13 illustrated the collecting tubules in the medulla of a rabbit after treatment with aspartame. The cuboidal epithelium lining the proximal convoluted tubule (arrows) could be distinguished, with clearly visible cellular membranes. Figure 8.14 illustrated the simple squamous epithelium lining (arrows) the thin segment of the loop of Henle's in the medulla of the kidney of a control rabbit. The cellular membranes were clearly visible and their flattened nuclei could be easily distinguished. Figure 8.15a and 8.15b illustrated the cuboidal (arrows) and squamous epitheliums (dashed arrows) of the proximal convoluted tubule and thin segment of the loop of Henle's respectively at a higher magnification. Cellular membranes were clearly visible for both epitheliums. The cytoplasm of the cuboidal epithelium stained light pink with filament-like structures being visible. The nuclei of the squamous epithelium had their characteristic flattened, elongated shape. Figure 8.15c illustrated the nucleus of an endothelial cell of the vasa recta, with its characteristic flattened ovoid shape.

Figure 8.16 illustrated the collecting tubules in the medulla of the kidney of a rabbit after treatment with aspartame. The cuboidal epithelium lining the proximal convoluted tubule appeared damaged as no clear cellular membranes could be distinguished (arrows). Figure 8.17 illustrated the squamous epithelium that lines the thin segment of the loop of Henle's, with the nuclei appearing more rounded in shape (arrows). The nucleus of an endothelial cell of the vasa recta was also visible (dashed arrow), but it did not have its characteristic flattened shape, it was round. At a higher magnification, the cytoplasm of the cuboidal epithelium appeared transparent (Figure 8.18a) and the filament-like structures that were present in the controls (Figure 8.15b) were very few to absent in the rabbits after treatment with aspartame. The nuclei of the squamous epithelium appeared rounder (Figure 8.18b) as when compared to those in the controls (Figure 8.15b). Figure 8.18b illustrated the nucleus of an endothelial cell of the vasa recta in a rabbit after treatment with aspartame. The nucleus had lost its characteristic flatted shape and became round.

8.4 SUMMARY AND EXPLANATION

Table 8.3 summarizes the differences found in the livers of the control and aspartame treated rabbits.

Table 8.3: Comparative summary between the control and aspartame treated rabbits after treatment with aspartame with special attention to the sections of the liver from which it was obtained

	Control	Aspartame – Centre of liver	Aspartame – Right lobe liver	Aspartame – Left lobe liver
Endothelium of central vein	Clearly visible (Right lobe liver; Fig. 8.1b)	Could not be distinguished (Fig. 8.3a)	Clearly visible; appeared thickened (Fig. 8.3b)	No vein present
Shape of hepatocyte	Polygonal shape (Right lobe liver; Fig. 8.1a)	Could not be distinguished (Fig. 8.3a)	Polygonal shape (Fig. 8.3b)	Polygonal shape (Fig. 8.4a)
Shape of nuclei of hepatocytes	Spherical to ovoid in shape	Not clearly visible; nuclei had round, ovoid elongated shape (Fig. 8.3a)	Round to ovoid in shape with nucleoli visible (Fig. 8.3b)	Round to ovoid (Fig. 8.4a)
Chromatin of nuclei of hepatocytes	Prominent scattered chromatin granules (Right lobe liver; Fig. 8.1a)	Condensed (Fig.8.3a)	Condensed (Fig. 8.3b)	Damage to nuclei – scattered pattern of chromatin granules more prominent with transparent areas becoming visible (Fig. 8.4b)
Cellular membranes of hepatocytes	Clearly visible (Right lobe liver; Fig.8.1a and 8.1b)	Could not be distinguished (Fig. 8.3a)	Clearly visible (Fig. 8.3b)	Clearly visible (Fig. 8.4a)
Cytoplasm of hepatocytes	Lace-like appearance (Right lobe liver; Fig. 8.1a)	Flown together (Fig. 8.3a)	Lace-like appearance more spaced and broken; transparent areas present (Fig. 8.3b)	Spaced and broken appearance more intense; retracting towards cellular membranes (Fig. 8.4a)

Table 8.3 (continue): Comparative summary between the control and aspartame treated rabbits after treatment with aspartame with special attention to the sections of the liver from which it was obtained

	Control	Aspartame – Centre of liver	Aspartame – Right lobe liver	Aspartame – Left lobe liver
Simple cuboidal epithelium of ducts	Clear cellular membranes with rounded nuclei (Right lobe liver; Fig. 8.2)	No ducts present	Clearly visible with round nuclei (Fig. 8.3b)	Not present
Cords of the hepatocytes	Cellular membranes clearly distinguishable; nuclei prominent (Right lobe liver; Fig. 8.2)	No cords could be distinguished (Fig. 8.3a)	Visible; Hepatocytes in clear rows	Not present

Table 8.4 illustrates a comparative summary of the cortex of the kidney of the control and aspartame treated rabbits to illustrate the possible effects of aspartame.

Table 8.4: Comparative summary of the cortex of the kidney of both the control and aspartame treated rabbits to illustrate the possible effects of aspartame

	Control rabbits	**Aspartame treated rabbits**
Urinary space	Clearly visible (Fig. 8.7a and 8.7b)	Appears enlarged
Extra-glomerular mesangial cells	Clearly visible at vascular pole (Fig. 8.8; Label A and B)	Visible at vascular pole (Fig. 8.11; Label A and B)
Mesangial cells	Visible (Fig. 8.8; Label D)	Visible; appeared to have an increase in the number of cells (more nuclei visible) (Fig. 8.11; Label C)
Parietal layer of the capsule of Bowman	Simple squamous epithelium with flattened nuclei (Fig. 8.8; Label E) (Fig. 8.9a)	Erythrocytes captured below parietal layer (dashed arrows; Fig. 11; Label E); appeared thickened (Fig. 8.12a)
Visceral layer of the capsule of Bowman	Nucleus of podocyte visible Fig. 8.8; Label F). Layer very thin (Fig. 8.9b)	Clearly visible (Fig. 8.11; Label G); appeared thickened (Figure 8.12b)

Table 8.4 illustrates a comparative summary of the medulla of the kidney of the control and aspartame treated rabbits to illustrate the possible effects of aspartame.

Table 8.5: Comparative summary of the medulla of the kidney of both the control and aspartame treated rabbits to illustrate the possible effects of aspartame

	Control rabbits	Aspartame treated rabbits
Simple cuboidal epithelium of proximal convoluted tubule		
Cellular membranes	Clearly visible (Fig. 8.13)	Damaged (Fig. 8.16)
Cytoplasm	Light pink with filament-like structures (Fig. 8.15a and 8.15b)	Transparent; Filament-like structures few to absent (Fig. 8.18a)
Nuclei	Round to oval (Fig. 8.15a and 8.15b)	Round to oval (Fig. 8.18a)
Simple squamous epithelium of the thin segment of the loop of Henle's		
Nuclei	Flattened and elongated (Fig. 8.15a and 8.15b)	More rounded (Fig. 8.17 and 8.18b)
Endothelial lining of the vasa recta		
Nuclei	Flattened, ovoid shape (Fig. 8.15c)	Round nucleus (Fig. 8.18b)

8.5 CONCLUSION

Blood circulated through both the liver and kidneys as to detoxify and to form urine respectively. It was seen in the previous chapters that the different components of blood was adversely affected by aspartame (leukocytes, coagulation factors, fibrin networks and platelet aggregation), thus it is hypothesized that the different filtering organs (liver and kidneys) might also be adversely affected.

8.5.1 Morphology of the liver

Damage to the cellular membranes were observed (Figure 8.3a) after treatment with aspartame and distinct changes were observed in the cytoplasm of the hepatocytes. The lace-like appearance of the cytoplasm changed to a spaced and broken appearance with the filament-like structures becoming less or was even absent (Figures 8.3a, 8.3b and 8.4a). The nuclei of the aspartame treated hepatocytes retained the scattered pattern of their chromatin granules, but their granules became more prominent with transparent areas becoming visible between the granules (Figure 8.4b). Three sections of the liver were studied: centre, left lobe and the right lobe. No differences was observed between the three sections in the control rabbits, but distinct differences were observed between the centre of the liver and left and right lobes of the liver respectively. It appeared as though the left and right lobes were not as severely affected by the treatment with aspartame as the centre of the liver. It is therefore hypothesized that the further the distance from the centre of the liver (main blood supply) the less the effects of aspartame on the liver.

A number of bi-nucleate hepatocytes were present in both the control (Figure 8.5) and aspartame treated liver (Figure 8.6). Literature indicates that up to 25 percent of hepatocytes in the liver could be bi-nucleate (Leeson *et al*., 1988a). It was determined that the control had 8.57% bi-nucleate hepatocytes and this value was set as the normal value. In total, 45 hepatocytes were counted (clearly visible nuclei) in the aspartame treated rabbit and 7 of the 45 hepatocytes were bi-nucleate. Thus, 8.57% of the 7 bi-nucleate hepatocytes in the aspartame treated rabbits were equal to four. Thus four of the seven hepatocytes were bi-nucleate, the other three hepatocytes were undergoing mitosis to repair the damaged caused by the aspartame as discussed above. In humans, if both factor VII and X are decreased, the individual suffers from prolonged hepatitis (Package insert – STA Deficient X; Ref 00738). Thus it is hypothesized that this was the case here after treatment with aspartame, as damage to the liver was histologically established, confirming why the concentration of both factors VII and X could be decreased (chapter 5). A possible explanation for why factors II, V and IX were not affected by the aspartame could not be determined, but further studies into this phenomenon should be undertaken.

The endothelial lining of the central vein (Figure 8.3b) was damaged, as the cells could not be distinguished. Factor VIII is secreted by vascular endothelium, thus this could be an indication as to why the levels of factor VIII were decreased. The damage to the endothelium in the liver together with the damage to the endothelium observed in the aorta (chapter 7) explains why the total concentration of circulating factor VIII was decreased (chapter 4). The subsequent damage to the liver could be the direct cause of the high level of circulating phenylalanine in the blood, resulting in a decreased amount of phenylalanine being converted to tyrosine via phenylalanine hydroxylase. Thus, phenylalanine is more likely to occupy the NAAT, inhibiting the transport of tryptophan (precursor for serotonin) over the BBB. Thus it is hypothesized that the damage can cause lowered level of serotonin, needed for optimal functioning of cAMP. Thus the damage to the liver could indirectly lead to the compromised BBB effect.

8.5.2 Morphology of the kidneys

Normal morphology of the renal corpuscle and urinary space were observed in the control rabbits in the cortex of the kidneys (Figure 8.7a and 8.7b; Figure 8.8). This changed however after treatment with aspartame. The urinary space of the renal corpuscle appeared enlarged (Figure 8.10) after treatment with aspartame. Erythrocytes were captures underneath the parietal layer of the capsule of Bowman (Figure 8.11, label E) which was not observed for the controls. The parietal layer of the capsule of Bowman also appeared thickened (Figure 8.12a). An increased number of mesangial cells were visible between the capillaries of the glomerulus (Figure 8.11; label C).

The most visible change caused by the aspartame was the appearance of a thickened visceral layer of the capsule of Bowman. The visceral layer of the capsule of Bowman closely invests the glomerular capillaries, with nuclei on the capsular side of the basal laminae of the capillaries, but the cells are greatly modified and do not form a complete sheet. The cells are called podocytes and are basically stellate in form, with their cell bodies rarely in contact with the basal lamina. The podocytes have a number of primary and secondary processes or pedicles. Pedicles of adjacent podocytes and podocytic processes interdigitate in a complex manner, often with the primary processes of one prodocyte overlying the primary processes and pedicles of a neighbouring podocyte. Between pedicles is an extensive system of clefts, or

intercellular spaces, known as filtration slits. This arrangement whereby much of the capsular surface of glomerular capillaries is covered by interdigitating pedicles between which are filtration slits, proves a large area for filtration, as all slits eventually drain to capsular space and thus into the lumen of the proximal convoluted tubule (Leeson *et al*., 1988b). Thus it is hypothesized that a thickened visceral layer, caused either by changes in the endothelium, basal lamina or the podocytes, will negatively influence the rate of filtration of blood to form the glomerular filtrate.

Normal morphology was observed for the cuboidal epithelium of the proximal convoluted tubule and the squamous epithelium of the thin segment of the loop of Henle in the control rabbit. The endothelial cells had their characteristic flattened, elongated nuclei. The cuboidal epithelial cells that line the proximal convoluted tubule have structural characteristics suitable for reabsorption (Kierszenbaum, 2002). Thus, if these cells are damaged, as were the case in this study (Figure 8.16 and 8.18a), reabsorption of fluid could be affected. The endothelial cell nuclei of the vasa recta were damaged, with the nuclei being more rounded (Figure 8.18b) than those observed in the controls, also indicating that aspartame affected the endothelial lining inside the medulla of the kidney.

It can therefore be concluded that aspartame negatively influenced the morphology of both the liver and the kidney. Damage to the liver was confirmed histologically, supplying evidence of the lowered concentrations of circulating factors VII and X (chapter 4).

The histology of the kidney was also affected by the aspartame, with major effects to the visceral layer of the capsule of Bowman and the cuboidal epithelium of the proximal convoluted tubule. It is suggested however, that further ultra-structural studies must be performed on the effects of aspartame on the renal corpuscle and cuboidal epithelium of the proximal convoluted tubule as they form key components of the urinary system.

Chapter 9 –

General conclusion

In the light of the controversies relating to the effects of the dipeptide aspartame as described in the literature study, the aim of this study was to determine the effects of aspartame on the blood coagulation system of the New Zealand white rabbit by answering the following research questions:

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?
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?
3. Is there a difference between the blood clotting time, coagulation profile and different coagulation factors of the control and aspartame treated groups and how does these values compare to that of humans?
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?
5. Has the morphology and number of the leukocytes (light microscopy) changed after treatment with aspartame and how is the endothelial lining (SEM and TEM) of the blood vessels affected when treated with aspartame?
6. How does aspartame affect the normal histological morphology of the liver and kidney?

The following hypothesis was also suggested for this study:

The hypothesis of this study is that the morphology of both the platelets and the fibrin fibres will be altered by the presence of aspartame and that the concentration of the different coagulation factors will be changed. It is also thought that the morphology of the endothelial cells lining the blood vessels will be modified and if all of the above mentioned was true, the liver and kidneys which filter and detoxify the blood, will most certainly also be affected.

Experimental animals of choice for pharmaceutical studies are usually rats, mice and rabbits. However, rats and mice are small animals, which become problematic when considering the

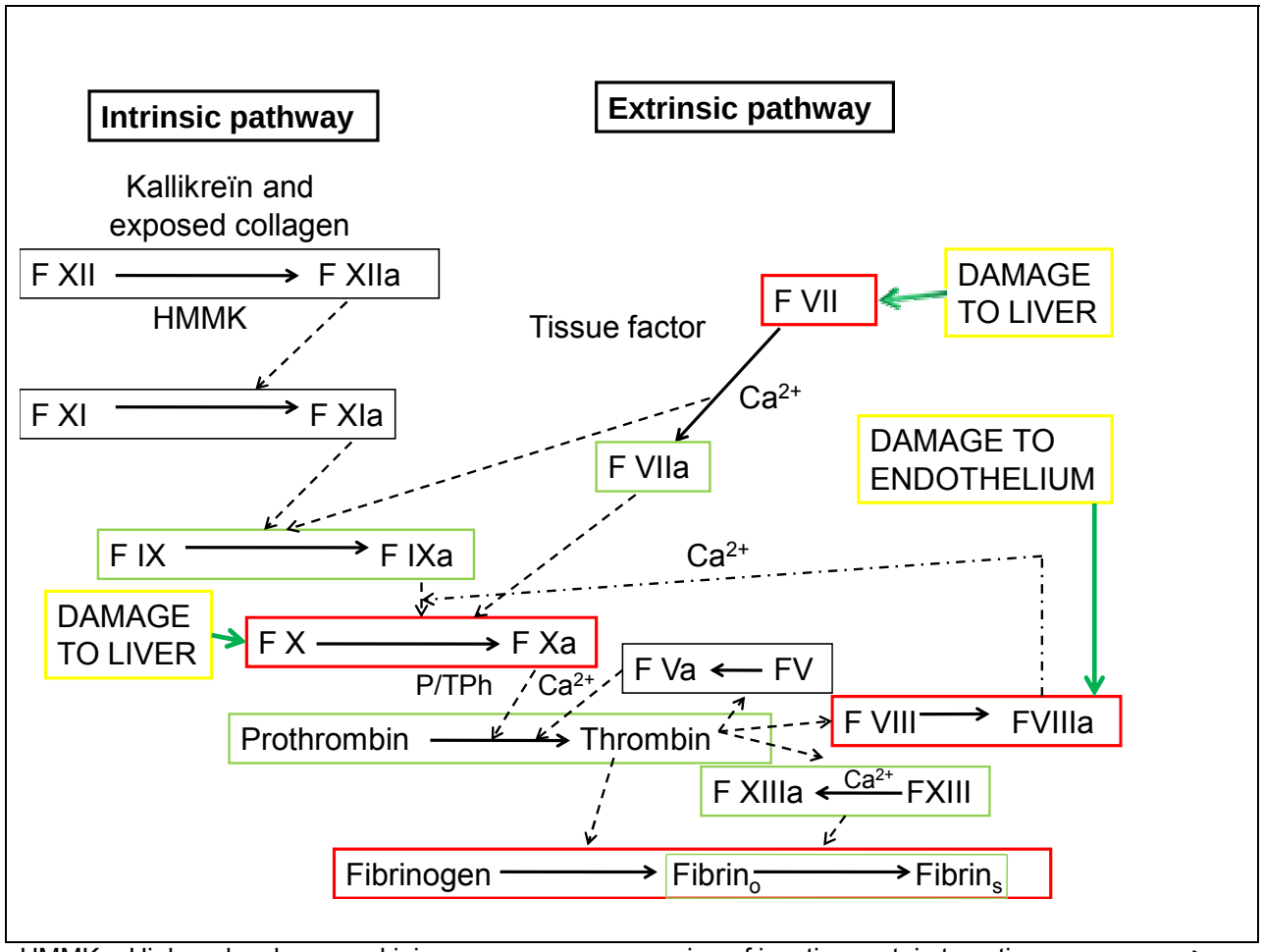
volume of blood that can be obtained from these small animals. The rabbit, however, is bigger and have a greater blood volume which makes it more suitable for obtaining blood samples. It was established in chapter 3 that blood can be drawn quite easily from the marginal ear vein of the rabbit with minimum 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 are too high. This leads to difficulty in long term studies, particularly in coagulation studies as blood can only be drawn once from rats and mice. After the animal of choice was determined, aspartame had to be administered without loss of any of the aspartame solution. A few methods were tested but inserting of a syringe into the back of the mouth was proven to be most successful.

The next question that had to be answered was whether or not the rabbit could be successfully used and implemented as a model for studying the blood coagulation system. Chapter 4 of this thesis compared the ultra-structure of the fibrin networks and platelet morphology of the human, mouse and rabbit. After performing ultra-structural analysis of the fibrin networks and platelet morphology it was concluded that the rabbit model provided to be a better option to study, particularly for coagulation and haemostasis processes, as the fibrin network and the platelet aggregate ultra-structure seemed comparable to that of humans. Also, both major and minor fibre thickness compared well to that of human tissue. It was suggested that the rabbit model also complied 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 was 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 (chapter 5).

The next step towards determining whether or not aspartame adversely affects the coagulation system was by studying the coagulation metabolomics and the change of the different factors due to the intake of aspartame.

Diagram 9.6: Coagulation pathway with red squares indicating factors affected by intake of aspartame, yellow squares indicating damaged tissue/organ resulting in decreased factors. Solid green arrow indicate effects of damage to the tissue



HMMK – High molecular mass kininogen; ⟶ - conversion of inactive protein to active enzyme; - - -> Active enzyme as cofactor for activation of inactive protein; ·····> - active factor VIII working as cofactor on active factor IX to activate factor X; P/TPh – platelet or tissue phospholipids; Fibrino – unstable fibrin; Fibrins – stable fibrin. Diagram adapted from Meyer and Meij, 1996.

Results obtained in chapter 5 indicated that the concentrations of factors VII, X and VIII were directly decreased (indicated in red in diagram 9.6) by the intake of aspartame. The factors highlighted in green (Diagram 9.6) indicates the factors that would indirectly be influenced by the intake of aspartame. If a decrease in factors VII, X and VIII are present, as was determined

in chapter 5, it is hypothesized that there would be a resultant indirect decrease in the amount of circulating thrombin, but not prothrombin (Factor II; results of chapter 5) as these factors are all needed for the conversion of prothrombin to thrombin. This hypothesis was proven using 2 different research techniques. Firstly by the high amount of circulating fibrinogen (g/L) obtained in chapter 5 which was confirmed by the extent to which fibrin clots formed after the addition of external thrombin (ultra-structural study on the SEM, chapter 6). Secondly, the degree to which platelet aggregation occurred in chapter 6. Thrombin is necessary for the degranulation of platelets so that platelet aggregation can occur, thus a decrease in thrombin will directly lead to a decrease in the occurrence of platelet aggregation as proven by the SEM studies performed in chapter 6 of this thesis. Thrombin is also necessary for the conversion of fibrinogen to fibrin and F XIII to its active counterpart which is then necessary for the stabilization of the fibrin clot. Thus, as proven above that the amount of thrombin was lowered, F XIII cannot be activated to stabilize any fibrinogen converted to fibrin.

The intravascular anticoagulation system consists out of five parts, of which three were altered by the intake of aspartame, either directly or indirectly:

1. The endothelial lining – was damaged by the intake of aspartame (SEM, chapter 7).

2. Constant blood flow.

3. Prostacyclins – secreted by endothelial cells which prevents platelet aggregation. Hypothesized that damage to endothelial cells could have caused an increase in secretion of prostacyclins as platelet aggregation were inhibited. Its concentration however was not determined, thus the decrease in platelet aggregation could also have been due to a decrease in circulating thrombin levels.

4. Antithrombin III (AT III) – inhibits thrombin and the active form of F X (mostly the active factors of the intrinsic pathway). This protein is secreted to maintain normal haemostasis. Thus, it would increase if there is an increase in coagulation and the other way around. Thus, it is hypothesized that the concentration of circulating AT III decreased because of the decrease in the amount of thrombin after aspartame ingestion. If any coagulation is present, it

cannot also be prevented, after a decrease in thrombin were noted, by an increase in the amount of circulating AT III.

5. Heparin – potentiate the action of AT III (inhibition of thrombin). Heparin is secreted by basophils and mast cells if there is an increase in coagulation. The amount of basophils decreased, 3 of the 5 aspartame treated rabbits had lower counts for basophils than the controls (Table 1; Chapter 7). Thus, a decreased amount of heparin could be released into the bloodstream. Granules inside the eosinophils inhibit the degranulation of mast cells, which contain heparin and histamine. It was seen that the eosinophil counts increased by 30.33% (Table 3; Chapter 7) and that the appeared as though the amount of granules inside the cytoplasm of the eosinophils of the aspartame treated rabbits increase (more granules visible; Table 4 – Chapter 7). It is therefore hypothesized that if there is a change in normal haemostasis, an immune response is triggered by altering the amount of relevant leukocytes. If there is an increase in the amount of thrombus formation, there will be an increase in the amount of basophils (increased heparin secretion) and a decrease in the amount of eosinophils, as the inhibit the degranulation of mast cells which also secrete heparin. Thus, there will be an increased secretion of heparin as a protection mechanism, which in turn will potentiate the action of AT III. AT III will inhibit the action of thrombin (fibrin formation and stabilization; platelet aggregation) and F Xa (conversion of prothrombin to thrombin). AT III normally present to maintain equilibrium between thrombus formation and anti-coagulation, but in the case of increased coagulation, more AT III will be secreted to prevent formation of thrombin (Inhibits F Xa) and inhibit the action of thrombin itself.

In this study, after treatment with aspartame, there was a decrease in the amount of coagulation due to the following:

1. Decrease in the amount of circulating factors VII, X and VIII (Chapter 5), indirectly leading to a decrease in circulating thrombin.

2. Fibrinogen increased (Chapter 5) which was also seen with the fibrin networks obtained when treated with external thrombin (Chapter 6). Platelet aggregation was

decreased (Chapter 6) due to decreased amounts of thrombin (activates platelet aggregation by platelet degranulation). Platelets contain two types of granules, namely α granules (also contain fibrinogen) and dense granules (contain serotonin). Serotonin also improves binding of two adjacent platelets. After serotonin is released from one platelet, it adheres to a receptor on 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)*, which is proven here by the lowered amounts of thrombin which inhibits the degranulation of the granules of the platelets (decreased amounts of serotonin). Thus, it could be stated that platelet aggregation is also hindered in this way by aspartame treatment. Also, the high concentration of circulating phenylalanine inhibits the conversion of tryptophan to serotonin (as discussed previously), thus it will further decrease the amount of circulating serotonin.

3. Decrease in the amount of basophils and an increase in the amount of eosinophils. The amounts eosinophils increased as part of an immune response that was triggered by the decrease in coagulation. Thus, mast cells degranulation were inhibited, and no heparin was secreted that could prevent thrombus formation. AT III was also not secreted because of the decrease in coagulation, thus the thrombin that is present was not inhibited in this way.

4. Endothelial lining was damaged due to the consumption of the aspartame (Chapter 7). The vascular endothelium is responsible for the secretion of F VIII. The concentration of circulating F VIII was decreased (Chapter 5) after treatment with aspartame. Thus the damage to the endothelial lining directly lead to the decrease in the amounts of circulating F VIII (decreased coagulation due to decreased conversion of prothrombin to thrombin). Studies on the TEM (chapter 8) also indicated that the endothelial cells were apoptotic (chromatin marginalization towards nuclear envelope; damage to nuclear envelope itself, cell surface smoothing, and decrease in synthesis of macromolecules). A decrease in the amount of circulating serotonin, due to lowered concentrations of thrombin (degranulation of dense granules of platelets containing serotonin) or high concentrations of circulating phenylalanine (metabolic constituent of aspartame) inhibiting conversion of tryptophan to serotonin, could lead to lowered activity of

cAMP. cAMP activity determines the complexity of the tight junctions between the endothelial cells, resulting in a functional BBB. Thus decreased concentrations of serotonin, will indirectly cause lowered activity of cAMP and it is therefore hypothesized that the BBB was compromised as both the endothelial cells themselves were affected by the aspartame (apoptotic) and the complexity of the tight junctions between the endothelial cells were also compromised (lowered cAMP activity due to decreased concentrations of serotonin).

After confirmation that aspartame adversely affected the coagulation system (Chapter 5 – 7), thus blood as a whole, the effects of aspartame on the liver and kidney were examined.

Morphology of the liver

Damage to the cellular membranes were observed and the lace-like appearance of the cytoplasm changed to a spaced and broken appearance with the filament-like structures becoming less or was even absent. The nuclei of the aspartame treated hepatocytes retained the scattered pattern of their chromatin granules, but their granules became more prominent with transparent areas becoming visible between the granules, thus exhibiting an apoptotic characteristic. Three sections of the liver were studied: centre, left lobe and the right lobe. No differences was observed between the three sections in the control rabbits, but distinct differences were observed between the centre of the liver and left and right lobes of the liver respectively. It appeared as though the left and right lobes were not as severely affected by the treatment with aspartame as the centre of the liver. It is therefore hypothesized that the further the distance from the centre of the liver (main blood supply) the less the effects of aspartame on the liver. A number of bi-nucleate hepatocytes were present in both the control and aspartame treated liver. Literature indicates that up to 25 percent of hepatocytes in the liver could be bi-nucleate (Leeson *et al*., 1988a). It was determined that 8.57% of the seven bi-nucleate hepatocytes in the aspartame treated rabbits were equal to four. This four of the seven hepatocytes were bi-nucleate, the other three hepatocytes were undergoing mitosis to repair the damaged caused by the aspartame. In humans, if both factor VII and X are decreased, the individual suffers from prolonged hepatitis (Package insert – STA Deficient X; Ref 00738). Thus it is hypothesized that this was the case here after treatment with aspartame, as damage to the

liver was histologically established, confirming why the concentration of both factors VII and X could be decreased (chapter 5). A possible explanation for why factors II, V and IX were not affected by the aspartame could not be determined, but further studies into this phenomenon should be undertaken.

The endothelial lining of the central vein was damaged, as the cells could not be distinguished. Factor VIII is secreted by vascular endothelium, thus this could be an indication as to why the levels of factor VIII were decreased. The damage to the endothelium in the liver together with the damage to the endothelium observed in the aorta (chapter 7, SEM and TEM) explains why the total concentration of circulating factor VIII were decreased (chapter 4).

Morphology of the kidneys

Normal morphology of the renal corpuscle and urinary space were observed in the control rabbits in the cortex of the kidneys. Treatment with aspartame changes this picture completely, as the urinary space of the renal corpuscle appeared enlarged erythrocytes were captured underneath the parietal layer of the capsule of Bowman. The parietal layer and visceral layers of the capsule of Bowman also appeared thickened. An increased number of mesangial cells were also visible between the capillaries of the glomerulus.

The most visible changes caused by the aspartame were the appearance of a thickened visceral layer of the capsule of Bowman and the damage to the cuboidal epithelium of the proximal convoluted tubule. The visceral layer of the capsule of Bowman plays an important role in filtration of blood to form the glomerular filtrate (Leeson *et al*., 1988b). Thus it is hypothesized that a thickened visceral layer, caused either by changes in the endothelium, basal lamina or the podocytes, would negatively influence the rate of filtration of blood to form the glomerular filtrate. The cuboidal epithelial cells that line the proximal convoluted tubule have structural characteristics suitable for reabsorption (Kierszenbaum, 2002). Thus, if these cells are damaged, as were the case after treatment with aspartame (chapter 8), reabsorption of water could be affected. The endothelial cell nuclei of the vasa recta were damaged, with the nuclei being more rounded, also indicating that aspartame affected the endothelial lining inside the

medulla of the kidney. It was suggested however, that further ultra-structural studies must be performed on the effects of aspartame on the renal corpuscle and cuboidal epithelium of the proximal convoluted tubule as they form key components of the urinary system

This study therefore concludes that aspartame adversely affected the coagulation system and the filtering organs (liver and kidney) of the rabbit. The protocol was successfully adapted for successfully obtaining blood samples from the rabbit as well as administering of aspartame without loss of any of the fluid. The rabbit proofed to be the best suited animal model for studying the coagulation system and haemostasis as the fibrin fibre morphology and thickness compared extremely well with that of humans. Also, aspartame affected the PT (prolonged) and decreased the concentration of circulating factors VII, X and VIII while the concentration of fibrinogen increased. It was determined that the amount of thrombin had to decrease as a direct result of the decrease in the above mentioned factors, thus fibrinogen could not be converted to fibrin. The amount of circulation fibrinogen in the rabbit also compared well with that found in humans. The morphology of the fibrin fibres and networks were affected by the aspartame, and the higher the concentration of aspartame tested, the more severe the coagulation pattern, which was ascribed to the fact of the high amount of circulating fibrinogen. The degree of platelet aggregation was also lowered, directly due to the decrease in the amount of thrombin, which is needed for the degranulation of the platelets which promote platelet aggregation. As a direct result, the amount of serotonin also decreased (via decrease in concentration of thrombin and high levels of circulating phenylalanine from the aspartame), which could lead to lowered activity of cAMP, influencing the complexity of the tight junctions between the endothelial cells. The endothelial lining of the aorta of the rabbit was also adversely affected (SEM and TEM) and cells appeared apoptotic. Thus, a combination of apoptotic endothelial cells and lowered complexity of the tight junctions between the endothelial cells indicate a compromised BBB. The morphology and number of the leukocytes were also altered by the intake of aspartame, and it is hypothesized that an immune response was triggered by the intake of aspartame as the amount of eosinophils and the granules inside individual eosinophils increased, inhibiting the possible actions of heparin and AT III. Lastly the normal morphology of both the liver and kidney was adversely affected by the treatment with aspartame. The effects of aspartame were more pronounced the closer to the main blood supply of the liver (centre of liver). The visceral layer of the capsule of Bowman was thickened, which could indicate a difficulty in producing

glomerular filtrate, and the damage to the cuboidal epithelium lining the proximal convoluted tubule, difficulty in reabsorption of fluid.

The rabbits received a total number of 75 doses of aspartame, from the lowest concentration to the highest over a period of 111days. The accumulative effect was studied after completion of all three concentrations on the leukocyte count and morphology, endothelium morphology and the morphology of the liver and kidney. The effects of each concentration of aspartame on the fibrin networks and platelets and concentration of certain coagulation factors were monitored throughout the study at specific intervals. Even at the concentration that is said to be safe for human consumption (34mg/kg aspartame) severe adverse effects was observed in the fibrin fibre morphology and amount of circulating fibrinogen. Thus, it could be said that even if a person utilized this product daily at a safe concentration (50mg/kg body weight per day; FDA), but the product is used for long periods of time (25 consecutive days) that aspartame will negatively influence the health of that individual.

The final judgment of the results obtained in this thesis regarding the consumption of abuse doses of aspartame, was that aspartame could lead to bleeding disorders (especially in genetically predisposed individuals), suppressed immunity and a compromised BBB. Trouble may also occur with formation of the glomerular filtrate and absorption of fluid from the proximal convoluted tubule, which could result in high blood pressure and excessive loss of fluid respectively.

Further suggested studies should be to determine the effects of the aspartame on the ultra-structure of the renal corpuscle and the proximal convoluted tubule. Further experiments to determine the reason why only certain of the coagulation factors were influenced by the intake of aspartame should also be undertaken.

Chapter 10 - References

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SUMMARY

The artificial dipeptide sweetener aspartame (APM; L-aspartyl-L-phenylalanine methyl ester) is present in many products, especially unsweetened or sugar free products. These products are frequently utilised by people trying to lose weight or patients with diabetes. Concern relating to the possible adverse effects have been raised due to aspartame's metabolic components, which is produced during its breakdown, namely phenylalanine, aspartic acid (aspartate), diketopiperazine (DKP) and methanol. Great controversy surrounds the usage of this product, even up to today.

Animal models are being used more frequently these days for determining the effects of pharmaceutical products, still in their preclinical trial periods. Animal models are more readily available and their environment can be controlled, unlike that of humans, especially in the case of their diets. A new approach has been established, which makes use of system biology. Systems biology used the idea of testing pharmaceutical product *in vivo*, and then determining its effects on the whole system of the experimental animal. Thus, a systems biology approach was also followed in this thesis, where proteins where quantified (coagulation factors; metabolomics).

Thus, the purpose of this study was to put a little light on the controversies relating to the effects of the dipeptide aspartame, and to focus on the effects of aspartame on the blood coagulation system of the New Zealand white rabbit.

Thus, the attention of this study was centred on determining whether the rabbit could be used and implemented successfully as an experimental animal model in obtaining blood samples to determine the effects of aspartame, and if so, how aspartame would influence the ultra-structural morphology of the fibrin networks, platelet aggregates and the endothelial lining. Would the coagulation profile of the rabbit be altered due to treatment with aspartame and could an immune response be activated by change in the morphology and counts of the different leukocytes present in blood? And if all of the above were affected by the intake of aspartame, would it cause changes in the normal histological morphology of the liver and kidney?

The hypothesis of this study was therefore that the morphology of both the platelets and the fibrin fibres would be altered by the presence of aspartame and that the concentration of the different coagulation factors would be changed. It was also thought that the morphology of the endothelial cells lining the blood vessels would be modified and if all of the above mentioned was true, the liver and kidneys which filter and detoxify the blood, would most certainly also be affected.

The protocol for obtaining blood from a rabbit as well as successful administration of aspartame was perfected. The rabbit was proven as best experimental model, when compared to a mouse, for studying the effects of aspartame on coagulation and haemostasis, as the rabbit exhibited similar fibrin fibre morphology and fibre thickness as that of the human. The effects of aspartame were determined by measuring the factors from the different coagulation pathways, namely the common pathway (factors II, V, X and fibrinogen); factors in the intrinsic pathway (factors VIII, IX), as well as factor VII, found in the extrinsic pathway. The *prothrombin time* (PT; measures how long blood takes to form a clot) and activated *partial thromboplastin time* (aPTT; measures recalcification time of plasma) was also measured. The ultrastructure of the fibrin fibre networks and platelet morphology was determined by utilizing the scanning electron microscope. The endothelial lining of the aorta was also studied by utilizing both scanning and transmission electron microscopy. Lastly the histological morphology of the leukocytes, liver and kidney were examined by means of light microscopy. The number of leukocytes was also counted after long-term treatment with aspartame to determine the accumulative effects of aspartame.

Results obtained indicated certain of the factors tested, were more than that found in humans, but the amount of circulating fibrinogen compare well with that found in humans, thus the rabbit makes a good candidate for studying coagulation. After treatment with aspartame, the results indicated that factors II, V, IX and the recalcification time was not adversely affected by ingestion of aspartame. But, F VII, X and VIII were decreased with a prolonged prothrombin time. All three these factors plays an integral part in the conversion of prothrombin to thrombin, which in turn s needed for a.) conversion of fibrinogen to fibrin; b.) conversion of F XIII to its active form, needed for stabilization of the fibrin fibres; c.) thrombin is needed for degranulation of the platelets, so that platelet aggregation can occur. The concentration of circulating fibrinogen increased significantly, which corroborated with results obtained for the ultrastructure

of the fibrin networks. The degree of fibrin fibre formation increased the higher the concentration of aspartame, with the minor fibres becoming more pronounced and starting to form a mat-like structure over the major fibres. The degree of platelet aggregation occurring, decreased with the increase of aspartame concentration, and it was hypothesized that it was due to the decreased amounts of thrombin present. It was hypothesized that the amount of circulating serotonin decreased due to the inability of the platelets to de-granulise (desne granules secrete serotonin) and the high amounts of phenylalanine from the aspartame, which inhibits the conversion of tryptophan to serotonin. The endothelial lining of the rabbits were damaged with the nuclei appearing apoptotic (chromatin marginalization against nuclear envelope; damage to nuclear envelope). The endothelial lining and their tight junctions play an integral part in the functioning of the blood brain barrier, in synchronization with cAMP which controls the complexity of tight junctions. The activity of cAMP is enhanced by the presence of serotonin, thus a decrease in serotonin causes lowered activity of cAMP, causing decreased complexity of the tight junctions between the endothelial cells. Thus apoptotic cells and less complex tight junctions could indicate a compromised blood brain barrier. The morphology of the leukocytes were altered, specifically that of the eosinophils and heterophils. The granules inside the eosinophils of the aspartame treated rabbit appeared to have increased and were more clearly visible, while the granules in the heterophils appeared to have become less. The granules contained within the eosinophils inhibit the degranulation of mast cells, which secrete heparin and histamine. Thus it appeared as though an immune response was triggered to prevent further decreases in coagulation. The total number of leukocytes also decreased, which indicated a level of suppressed immunity. The normal histological morphology of both the liver and kidney were affected by aspartame. Damage to the hepatocytes, as seen in the cytoplasm and the nuclei, and the subsequent arrangement of the hepatocytes into cords, which was also damaged, were noted. Thus, supplying corroborating evidence as to why a number of the coagulation factors were lowered. The visceral layer of the capsule of Bowman appeared thickened and the cuboidal epithelium lining the proximal convoluted tubule was also damaged. The visceral layer of the capsule of Bowman plays a key role in formation of the glomerular filtrate, thus a thickening of this layer could results in decreased formation of the filtrate. The proximal convoluted tubule forms part of the reabsorption apparatus of the kidney, thus damage to this part could lead to inability of reabsorption (dehydration).

The final judgment and conclusion of the results obtained in this thesis regarding the consumption of abuse doses of aspartame, was that aspartame could lead to bleeding disorders (especially in genetically predisposed individuals), suppressed immunity and a compromised BBB. Trouble may also occur with formation of the glomerular filtrate and absorption of fluid from the proximal convoluted tubule, which could result in high blood pressure and dehydration respectively.

Further suggested studies were to determine the effects of the aspartame on the ultra-structure of the renal corpuscle and the proximal convoluted tubule. Further experiments to determine the reason why only certain of the coagulation factors were influenced by the intake of aspartame should also be undertaken.

APPENDICES

Appendix A: Humphries P., Pretorius E., Naude H. Direct and indirect effects of aspartame on the brain. *European Journal of Clinical Nutrition*; (*In Press).*

Appenix B: Pretorius E., Humphries P. Ultrastructural changes to rabbit fibrin and platelets due to aspartame. *Ultrastructural pathology*; 31:77-83.

Appendix C: Pretorius E, Humphries P, Ekpo OE, Smit E, van der Merwe CF. (2007a) Comparative ultrastructural analyses of mouse, rabbit and human platelets and fibrin networks research. *Microscopy and Technique* (*In Press*).

Appendix A:

Humphries P., Pretorius E., Naude H. Direct and indirect cellular effects of aspartame on the brain. *European Journal of Clinical nutrition*; (*In Press)*.

Appendix B:

Pretorius, E., Humphries, P. 2007. Ultrastructural changes to rabbit fibrin and platelets due to aspartame. *Ultrastructural Pathology*; 31: 77-83.

Appendix C:

Pretorius E., Humphries P., Ekpo O.E., Smit E., van der Merwe C.F. 2007a. Comparative ultrastructural analyses of mouse, rabbit and human platelets and fibrin networks research. *Microscopy and Technique*; (*In Press*).