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

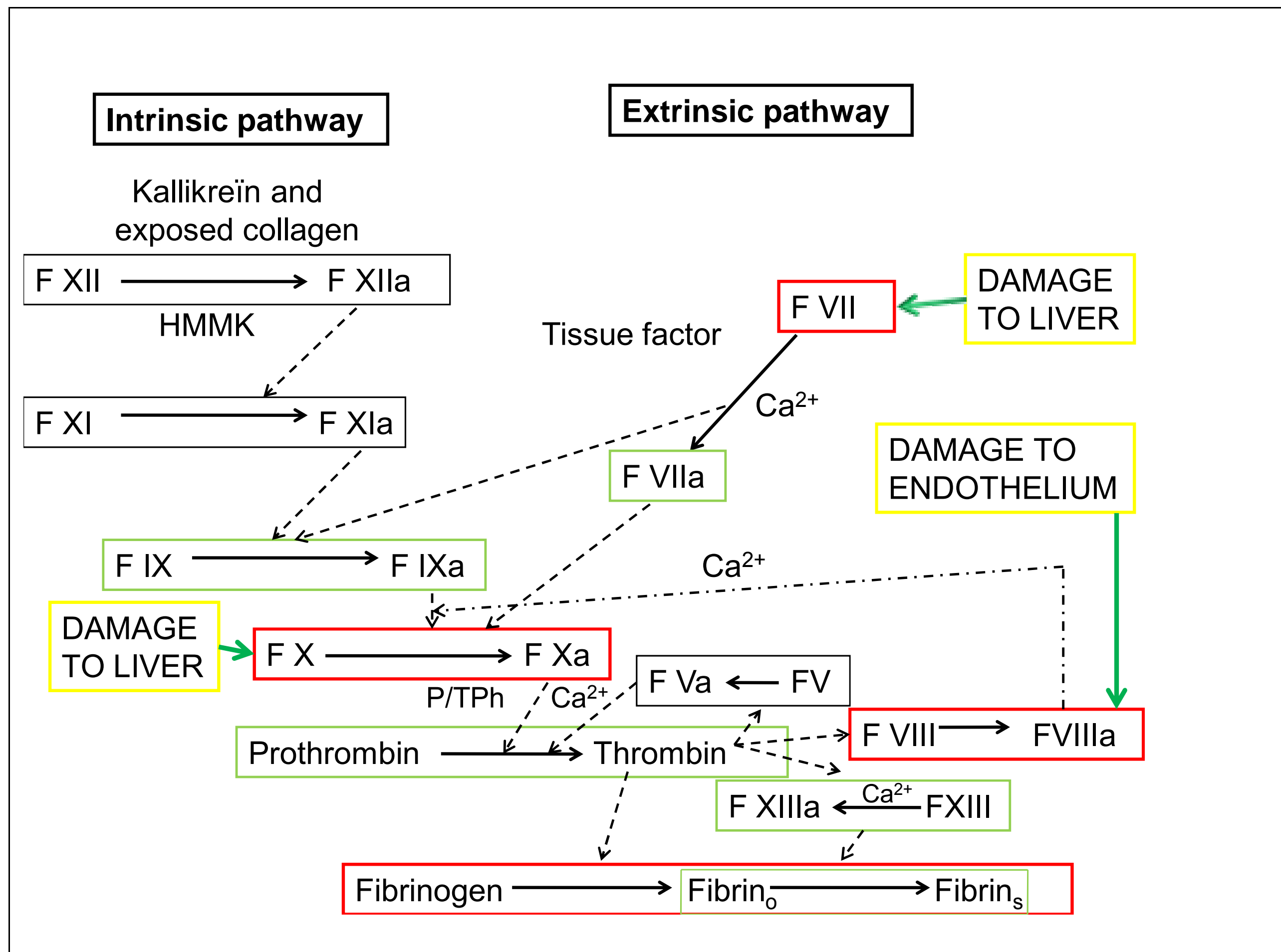
volume of blood that can be obtained from these small animals. The rabbit, however, is bigger and have a greater blood volume which makes it more suitable for obtaining blood samples. It was established in chapter 3 that blood can be drawn quite easily from the marginal ear vein of the rabbit with minimum 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.