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).