EFFECTS OF OESTROGEN ON THE NEURAL TISSUE, THROMBOTIC AND INFLAMMATORY PROFILES OF RATS IN TRANSIENT EXPERIMENTAL CEREBRAL ISCHAEMIA

By

WENDY JEANNETTE VAN DER SPUY

Thesis submitted in partial fulfilment of the requirement for the degree of

PHILOSOPHIAE DOCTOR

in the

FACULTY OF HEALTH SCIENCES

Department of Anatomy
Division Histology and Cell Biology
University of Pretoria

2013

Effects of Oestrogen on the Neural Tissue, Thrombotic and Inflammatory Profiles of Rats in Transient Experimental Cerebral Ischaemia

By

Wendy Jeannette van der Spuy

Supervisor: Prof. E. Pretorius

Co-Supervisor: Prof. M.C. Bosman

Degree: PhD Anatomy (Cell Biology)

Abstract

Cerebral ischaemia by mechanism of thrombosis is one of the leading causes of disability and/or death worldwide, the outcome thereof increasing in severity with advancing age. Cerebral ischaemia triggers a cascade of events including inflammation, blood-brain barrier disruption and apoptosis. It is well known that oestrogen is neuroprotective through various mechanisms including the interruption of inflammation, regulation of thrombosis and delay of apoptosis. This creates a strong factorial interconnection in predicting the consequences of cerebral ischaemia. Since platelets have a central role in thrombosis and inflammation, their ultrastructure being altered in conditions of inflammatory and thrombotic derivation, the question arises whether chemical analysis of coagulation factors and ultrastructural analyses of platelet morphology may provide further insight into the role of oestrogen during ischaemic insult associated with stroke.

Accordingly, an exclusively hyperglycaemic modification of the two-vessel occlusion model for inducing experimental cerebral ischaemia was established, since pre-ischaemic hyperglycaemia is known to intensify the outcome of cerebral ischaemic injury. Consequent neural tissue injury levels were correlated for three experimental groups (males, cyclic and acyclic females) of

Sprague Dawley rats at vital times, to the presence of oestrogen as well as changes in coagulation factors and ultrastructure. This design allowed for an association to be formed between cerebral ischaemia, inflammation and thrombotic potential.

Collectively the results strongly suggest that oestrogen is indeed neuroprotective through various actions including roles in the regulation of thrombosis and inflammation, targeting neural cells through the inhibition of apoptosis and exerting anti-inflammatory and antioxidant effects. It is evident that under the influence of oestrogen in cyclic females, there is reduced neural tissue injury as well as a lesser degree of inflammation evident in coagulation factor analysis and platelet activation morphology when compared to males and acyclic females. Oestrogen therefore exerts positive effects on the outcome of cerebral ischaemia through mechanisms which regulate inflammation, thrombosis and apoptosis. Furthermore it is unmistakeable that neural injury is closely shadowed, if not preceded, by inflammatory changes in the coagulation system, particularly manifested in platelet ultrastructure. It is therefore suggested that platelets may be used successfully to follow the progression of events of cerebral ischaemia and possibly assist in the assessment of treatment strategies and their effects on haemostasis.

This research advances the understanding that inflammation is evident soon after ischaemic insult and if such inflammation is not curbed, necrosis of platelets and more severe injury to neural tissue may follow. Therefore, the development of agents which not only target thrombosis, but also which control inflammation must be explored to advance treatment strategies. It is proposed that even before it is determined whether a stroke has been caused by thromboembolism or haemorrhage; it will be beneficial to immediately target inflammation in order to prevent most severe consequences in human patients.

Declaration

I, Wendy Jeannette van der Spuy, hereby declare that this research dissertation, entitled:

"Effects of Oestrogen on the Neural Tissue, Thrombotic and Inflammatory Profiles of Rats in Transient Experimental Cerebral Ischaemia"

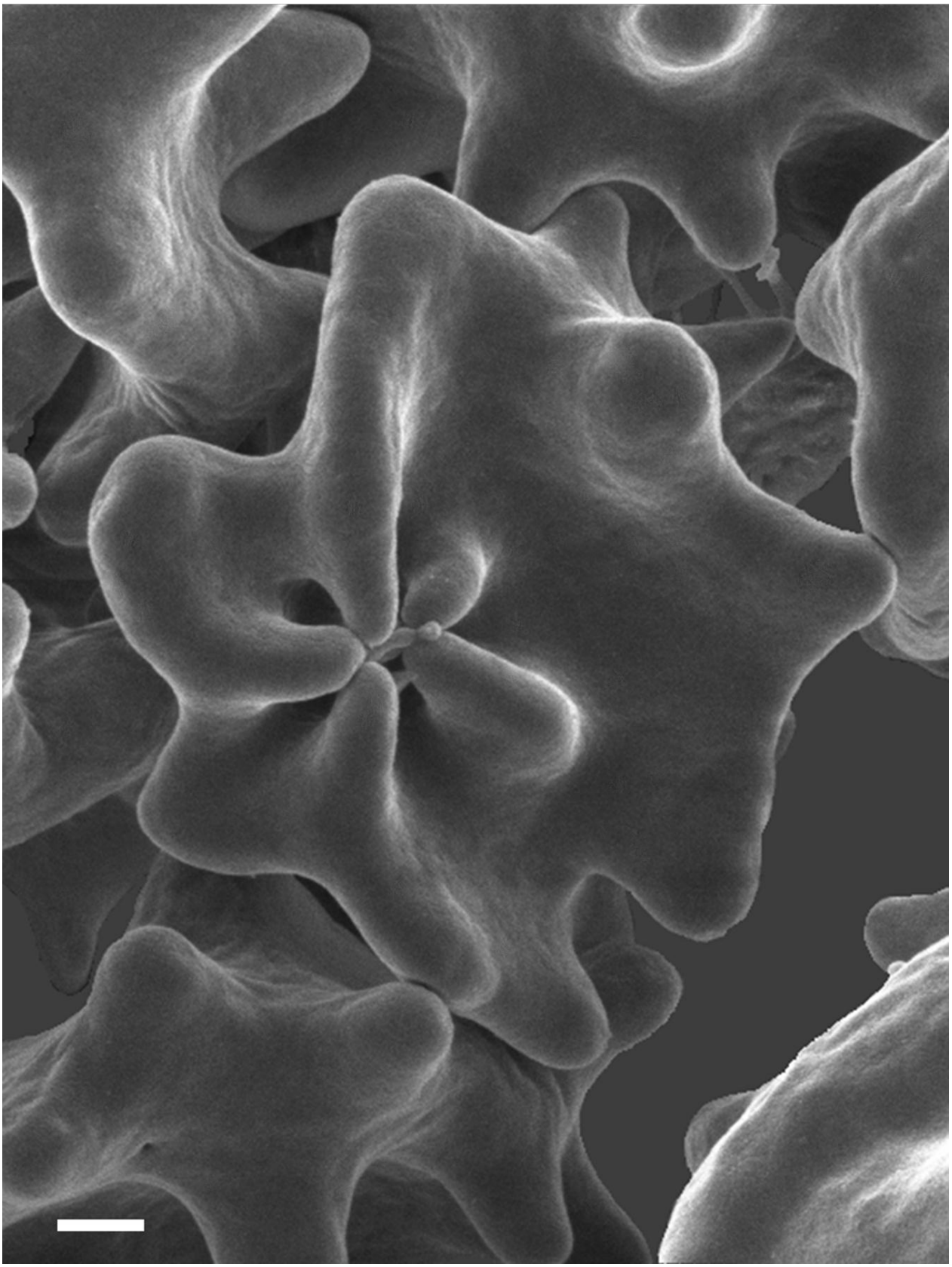
Which I herewith submit to the University of Pretoria for the degree of Philosophiae Doctor with Specialization in Anatomy: Cell Biology, is my own work and has not been presented for any degree at another University.

Signed: 

Date: 20 May 2013

Division Histology and Cell Biology, Department of Anatomy, Faculty of Health Sciences
University of Pretoria

South Africa
2013



Red blood cell with projections grasping an inclusion (Scale bar = 500nm)

Foreword

The foremost requirement for embarking on a journey such as this one must indeed be a level of insanity; yet to see what others have seen and perhaps to think in a way nobody else may have thought makes it quite a feat.

Words of thanks go to the following imperative entities and persons in my life:

God who foresaw the solution to every concern I had throughout this period of my life and career.

My family who stand by me, are proud of me, and love me unconditionally. My mother, who always asked about my progress and replied “that’s good” regardless of the answer. My baby sister Zoë, who worked as hard as I did during the endless weeks we were busy with rats, my admin lady and saving grace when I was sure to go mad in the lab - Love you lots Monkey. My love, Ross Wylie, who endured lengthy stories he didn’t understand, excitement and everything in between, for being my motivational angel.

My research support structure who offered expertise essential for achieving my goal. Professor Resia Pretorius, for allowing me to implement a study of my choosing and her valuable supervision. Her friendship and belief in my ability will always be greatly appreciated. Professor Marius Bosman, for his co-supervision and concepts related to inducing experimental cerebral ischaemia in the most effective and repeatable manner. Professor Megan Bester, for her accepted wisdom, input into and guidance with substantial aspects of my study, which were most valuable. Doctor Nanette Oberholzer, for her hours spent with me at the microscopy unit, track changes, motivation and immeasurable friendship - Baie lief vir jou my Maaikie.

The animal unit of La-Bio Research without whom no aspect of my animal study would have been possible. Doctor Daan and Mrs Bramie Goosen, to whom I am ever grateful for the early mornings and busy days as well as the excitement they shared with me once we realised we were functioning like a well-oiled machine when performing surgical procedures.

The Unit for Microscopy and Microanalysis where I am proud to be part of the furniture. Oom Chris van der Merwe, André Botha, Antoinette Lensink-Buys and Alan Hall, for their expertise, assistance and guidance in the microscopy lab.

This dissertation is dedicated to my father,

Thomas Arthur van der Spuy

(1925-2001)

Who instilled in me the hunger to pursue entirely that which interests me

Abbreviations, Symbols and Chemical Formulae

-	Hyphen indicating negative (before a number) or to (between numbers)
%	Percentage
&	And
/	Slash indicating per (between units)
:	Colon indicating to (when used in a ratio)
+	Positive or upregulate
<	Less-than
=	Is equal to
>	Greater-than
±	Plus-minus indicating the precision of an approximation
®	Registered trademark
°C	Degrees centigrade
µg	Microgram
µl	Microlitre
µm	Micrometre
10^9	One billion
17β-Oestradiol	17beta-isomer of oestradiol (major circulating oestrogen in humans)
Ad libitum	Without restraint
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance

AREC	Animal Research Ethics Committee
AUCC	Animal Use and Care Committee
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CCA	Common carotid artery
cm^3	Centimetre cubed
CSF	Cerebrospinal fluid
dH_2O	Distilled water
dl	Decilitre
DMD	Dense matted deposit
DMSO	Dimethylsulphoxide
e.g.	*Exempli gratia* (for example)
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
et al.	*Et alia* (and others)
EtOH	Ethanol
FA	Formaldehyde
FEG	Field emission gun
g	Gram
GA	Glutaraldehyde
GLM	General linear model statistical analysis
h	Hour

HBsAg	Hepatitis B surface antigen
HCl	Hydrochloride
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
i.e.	*Id est* (that is)
ICA	Internal carotid artery
In vitro	Experiment taking place in an artificial environment outside a living organism (within the glass)
In vivo	Experiment taking place in a natural environment within a living organism (within the living)
Inc.	Incorporated
IP	Intraperitoneal
kg	Kilogram
kV	Kilovolt (referring to acceleration voltage of electrons)
l	Litre
LSM	Fischer's least squares means statistical analysis
Lux	SI unit of illuminance and luminous emittance measuring luminous power per area
M	Molar
MCA	Middle cerebral artery
MF	Sexually mature intact or cyclic females
MF-0	Zero hour control or pre-ischaemic cyclic females
MF-2	Two hour post-reperfusion cyclic females
MF-24	Twenty-four hour post-reperfusion cyclic females

MF-48	Forty-eight hour post-reperfusion cyclic females
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
MM	Sexually mature males
MM-0	Zero hour control or pre-ischaemic males
MM-2	Two hour post-reperfusion males
MM-24	Twenty-four hour post-reperfusion males
MM-48	Forty-eight hour post-reperfusion males
Na_3PO_4	Sodium phosphate
nm	Nanometre
OsO_4	Osmium tetroxide
OVX	Sexually mature ovariectomised or acyclic females
OVX-0	Zero hour control or pre-ischaemic acyclic females
OVX-2	Two hour post-reperfusion acyclic females
OVX-24	Twenty-four hour post-reperfusion acyclic females
OVX-48	Forty-eight hour post-reperfusion acyclic females
PBS	Phosphate buffered saline
pH	Logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution
pmol	Picomol

PRP	Platelet-rich plasma
Pty Ltd.	Proprietary Limited
p-Value	Probability value
RBC	Red blood cell
RCF	Relative centrifugal force
rpm	Revolutions per minute
SANBS	South African National Blood Service
SANS	South African National Standard
SAS	Integrated system of software products used to perform statistical analysis
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
™	Trade mark
Tris	Tris(hydroxymethyl)aminomethane
TTC	2,3,5-Triphenyltetrazolium chloride
U	Unit
USA	United States of America
VA	Vertebral artery
VDS	Viscosity-based detection system

Table of Contents

List of Figures & Tables

Chapter 1: Introduction

Stroke by mechanism of thromboembolic cerebral ischemia is one of the foremost causes of disability and/or death worldwide (Braeuninger & Kleinschnitz 2009, Elkins & Johnston 2003). Current research is under consensus that there are sex-based differences in both the prevalence and presentation of stroke and thrombosis (Alkayed *et al.* 1998, Alkayed *et al.* 2000, Bailey *et al.* 2009, Braeuninger & Kleinschnitz 2009, Carswell *et al.* 2000, Drača 2009, Gibson *et al.* 2005, Jia *et al.* 2009, Liao *et al.* 2001, Mendelsohn & Karas 1999, Selvamani & Sohrabji 2010, Suzuki *et al.* 2009, Wong *et al.* 2008). Cerebral ischaemia triggers a series of events including inflammation (Brouns & De Deyn 2009, Gibson *et al.* 2005, Herd & Page 1994, Saenger & Christenson 2010, Wang *et al.* 2007), which is deeply interrelated with thrombosis; as not only can inflammation produce local thrombosis but thrombosis can also amplify inflammation (Myers & Wakefield 2005, Stewart *et al.* 1974). It is recognized, through research involving experimental animal models of cerebral ischemia that sex hormones, especially oestrogen, offer a degree of neuroprotection (Liao *et al.* 2001, Selvamani & Sohrabji 2010, Suzuki *et al.* 2009). Mechanisms of this neuroprotection may be linked to certain anti-inflammatory properties of oestrogen, as well as oestrogen's regulation of thrombosis through the lowering of coagulation factors, amongst others (Suzuki *et al.* 2009, Vegeto *et al.* 2008). It is also understood that sex hormones alter the function and morphology of platelets and fibrin networks (Bailey *et al.* 2009, Kadir *et al.* 1999, Leng *et al.* 2004, Mendelsohn & Karas 1999, Peters *et al.* 2002, Wong *et al.* 2008), and changes in their morphology offer one of the earliest validations of inflammation (Pretorius *et al.* 2009a, Pretorius *et al.* 2009b, Pretorius *et al.* 2009c, Pretorius *et al.* 2011a). Thus sex hormone levels, inflammatory processes and thrombotic mechanisms are profoundly interconnected in predicting the outcome and consequences of cerebral ischaemia.

Several experimental animal models exist to study the consequences and mechanisms of cerebral ischaemia (Braeuninger & Kleinschnitz 2009, Hoyte & Buchan 2009, Traystman 2003). To ascertain the role of oestrogen, it becomes necessary to not only compare factors in male and female animals, but also to compare these factors to females who have lost the protection rendered by oestrogen, i.e. ovariectomised and thus acyclic females. Experimental models provide an opportunity to investigate the interaction between oestrogen, thrombosis and inflammation under stringent and repeatable conditions. Understanding the mechanisms of these interactions may enable improved treatment strategies for patients presenting with

cerebral ischaemia. Because platelet and fibrin morphology changes in conditions like thrombosis and is associated with stroke, the question arises whether ultrastructural and morphological analyses of their interaction also have a place in contemporary medical research.

A modified experimental model, in which cerebral ischaemia was induced by two-vessel occlusion in a state of mild systemic hyperglycaemia rather than hypoxia and/or hypotension, was established in order to successfully evaluate the research objectives which governed this study:

1. Implementation of the animal model and induction of transient experimental cerebral ischaemia (animal model)
2. Analysis of cerebral tissue injury in order to quantify degenerative and/or regenerative changes within and between experimental groups (neural tissue injury)
3. Analysis of cerebral tissue protein content (neural tissue protein)
4. Analysis of plasma 17β-oestradiol levels (17β-oestradiol)
5. Analysis of blood coagulation factors and the ultrastructural changes of platelets (coagulation factors and coagulum)
6. Analysis of the ultrastructural changes of red blood cells and their interaction within a coagulum (red blood cell interaction)
7. Comprehensive correlation of colorimetric, chemical and ultrastructural analyses

This study was the first to employ an exclusively hyperglycaemic two-vessel occlusion model, in which the effect of oestrogen on cerebral ischaemia could be determined in one comprehensive experimental animal study. The research design served to establish the effect of oestrogen not only on ischaemic neural tissue injury, but also on the existence and progression of inflammation, studying the earliest coagulation processes through the examination of platelet ultrastructure and thrombotic interactions.

It was hypothesised that oestrogen would exhibit neuroprotective and anti-inflammatory properties, expecting that neural tissue injury and the related thombotic interactions at vital time points would differ between male, cyclic and acyclic female animals. The interpretation of this research may lead not only to a better understanding of the earliest pathologic processes involved in cerebral ischaemia; but also provide important information concerning the mechanisms involved in the relationship between cerebral ischaemia, inflammation, thrombosis and neuroprotection; together contributing to the innovation of treatment strategies.

Chapter 2: Literature Review

2.1. Introduction

The emergence of a larger aging population renders value to cerebral ischaemic research, as stroke is already the second leading cause of global disability and/or death (Braeuninger & Kleinschnitz 2009, Elkins & Johnston 2003). Thrombotic cerebral ischaemia accounts for more than half of all cases of stroke worldwide. It results from an occlusion of cerebral vasculature, reducing or preventing the supply of oxygen to the cerebrum, thus hampering metabolic demand and ultimately leading to death of brain tissue by ischaemic stroke.

Research suggests that there are sex-based differences in the prevalence and presentation of both stroke and thrombosis (Alkayed *et al.* 1998, Alkayed *et al.* 2000, Bailey *et al.* 2009, Braeuninger & Kleinschnitz 2009, Carswell *et al.* 2000, Drača 2009, Gibson *et al.* 2005, Jia *et al.* 2009, Liao *et al.* 2001, Mendelsohn & Karas 1999, Selvamani & Sohrabji 2008, Suzuki *et al.* 2009, Wong *et al.* 2008). Sex hormones not only alter procoagulant protein expression (Lowe *et al.* 2004) and the function of blood and vascular cells (Butenas & Mann 2002, Kadir *et al.* 1999, Leng *et al.* 2004), but differences in platelet function (Liao *et al.* 2001, Suzuki *et al.* 2009) and thrombosis activity (Bailey *et al.* 2009) have also been noted. Oestrogen is to a degree neuroprotective (Liao *et al.* 2001, Selvamani & Sohrabji 2010, Suzuki *et al.* 2009) in certain cases of induced cerebral ischaemia, since females appear to suffer less severe consequences of stroke, which includes less neural tissue injury than their male counterparts (McCullough & Hurn 2003, Suzuki *et al.* 2009). In the absence of ovarian hormone production at menopause, females are again at higher risk to strokes than their male counterparts and this risk continues to increase with age, as women have a longer life expectancy than men (Mitka 2006, Suzuki *et al.* 2009). Thus both sex and age play an important role in the occurrence of thrombotic events and the severity of neural injury subsequent to a stroke.

Platelets and fibrin play an important role in the normal coagulation process where they are involved in the maintenance of haemostasis (Herd & Page 1994). Their activation may be due to damage of a vessel wall or activation of the endothelium by chemicals, cytokines, and also inflammatory processes (Butenas & Mann 2002, Camera *et al.* 1999). Activated platelets synthesize/secrete inducers of platelet aggregation and adhere to the injured vessel wall as well

as aggregate to form a platelet-rich plug or thrombus, which secures haemostasis. This plug is then stabilized by fibrin formation as fibrinogen is activated by binding to activated platelets. Fibrinogen is the major plasma protein coagulation factor (Lowe *et al.* 2004) and though plasma levels thereof are decreased by oestrogen during the menstrual cycle, these levels are known to be higher in females than in males (Bailey *et al.* 2009). Although fibrin forms the core matrix of a thrombus, its structure depends also on the cellular elements embedded in its meshwork and the overall rate of coagulation reactions initiated by platelet aggregation (Wohner 2008). Morphological changes of fibrin networks may therefore occur due to several kinetic and modulating factors present in plasma. Because platelet and fibrin morphology is changed in conditions like thrombosis and associated with stroke (Pretorius *et al.* 2009a, Pretorius *et al.* 2009b, Pretorius *et al.* 2009c, Pretorius *et al.* 2011a), the question arises whether ultrastructural and morphological analyses thereof have a place in contemporary medical research and diagnosis.

A thrombotic event is associated with a change in haemostasis and cellular components that play a fundamental role in blood platelets and fibrin network formation. It is well known that thrombotic events are the most common cause of stroke and resultant cerebral ischaemia (Braeuninger & Kleinschnitz 2009). Furthermore, it is known that cerebral ischaemia triggers a cascade of inflammatory processes amongst others (Gibson *et al.* 2005, Herd & Page 1994, Wang *et al.* 2007). Inflammation again causes an alteration in platelet activation (Butenas & Mann 2002, Camera *et al.* 1999) and possibly further thrombotic events. It is suggested that oestrogen provides neuroprotection through certain anti-inflammatory mechanisms, amongst others (Suzuki *et al.* 2009, Vegeto *et al.* 2008). Platelets are thus central to both thrombosis and inflammation, intricately intertwining the two processes involved in promoting damage in cerebral ischaemia.

2.2. Cerebral Ischaemia

With two-thirds of deaths from stroke complications occurring in developing regions of the world, such as sub-Saharan Africa, stroke is the second leading cause of death worldwide (Braeuninger & Kleinschnitz 2009, Elkins & Johnston 2003, Wang *et al.* 2007). Stroke incidence and resultant cerebral ischaemia can be linked to coagulation processes. The cascade of blood coagulation is initiated when subendothelial tissue factor is exposed to the flow of blood subsequent to damage or activation of the vessel endothelium by chemicals, cytokines, or inflammatory processes (Butenas & Mann 2002). The formation of a thrombus at a site of vessel injury is thus a haemostatic process. A thrombotic event, however, is associated with a change in haemostasis and cellular components that play a fundamental role in blood platelet and fibrin network formation. Overactivity of any one component of the coagulation cascade can result in the formation of tight and rigid fibrin networks (Fatah *et al.* 1992), which can cause blockage of one or several cerebral blood vessels resulting in cerebral ischaemia.

Cerebral ischaemia is known to trigger a series of complex events (Figure 2.1), initiated with cerebral hypoperfusion and comprising bioenergetic failure of cellular components, excitotoxicity, oxidative stress, biphasic dysfunction of the blood-brain barrier (BBB), microvascular injury, haemostatic activation, inflammation, formation of oedema, as well as apoptosis and ultimate necrosis of neuronal, glial and endothelial cells. This cascade of events is dependent on variables such as onset and duration of ischaemia, effectiveness of reperfusion and resultant infarct size or tissue injury (Brouns & De Deyn 2009, Danton & Dietrich 2003, Gibson *et al.* 2005, Saenger & Christenson 2010).

BBB disruption in ischaemic stroke appears to be dependent on the response to and aggressiveness of reperfusion. Increased permeability of the BBB takes place within the first 24h of an ischaemic event, with further damage occurring 48-72h after ischaemia in the absence of sufficient reperfusion (Saenger & Christenson 2010). Inflammation itself is recognised as a key element of the pathological progression of ischaemic stroke. The destructive or beneficial nature of inflammation seems to be dependent on the severity of ischaemia. It is thus likely that early inflammatory responses may potentiate ischaemic injury, while late responses may be beneficial to recovery and repair of ischaemic lesions (Wang *et al.* 2007). Inflammation and thrombosis are interconnected as they are capable of effecting and amplifying one another (Libby & Simon 2001).

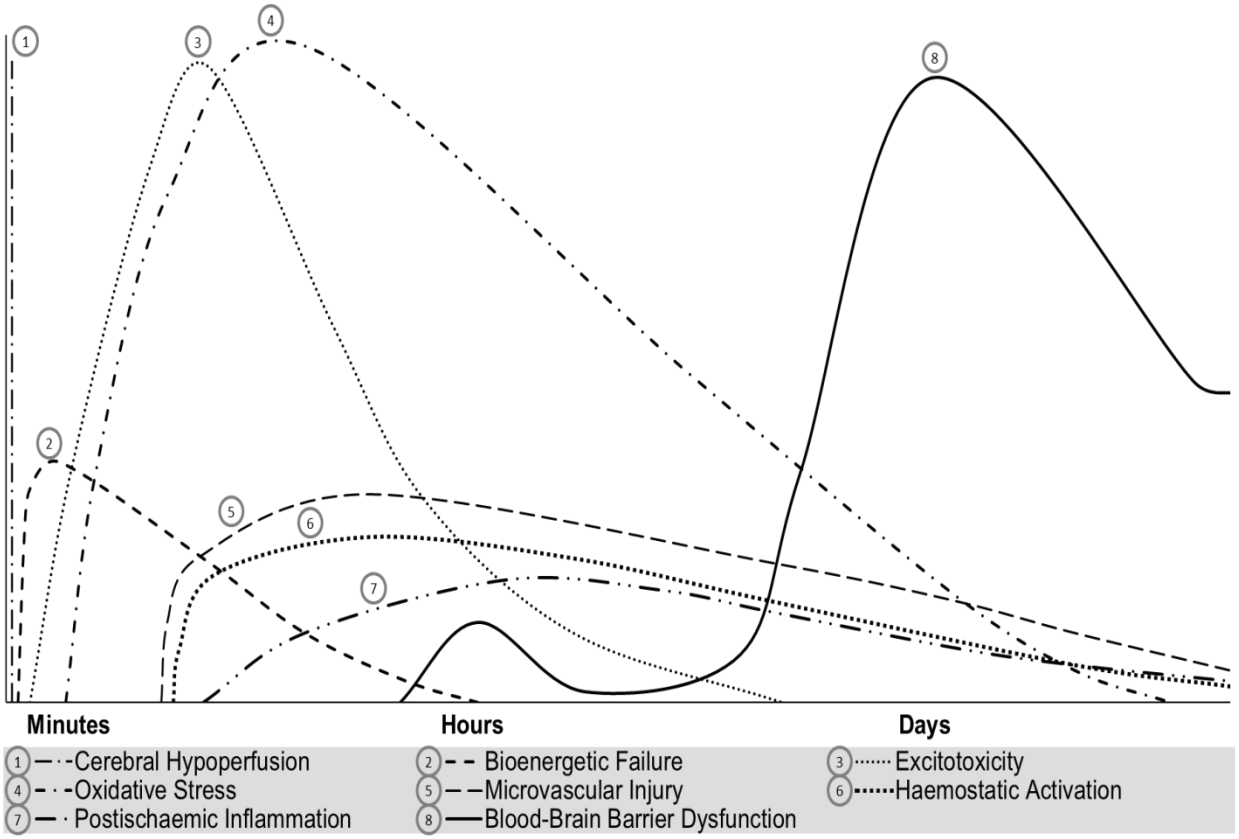


Figure 2.1. Sequence of events of the ischaemic cascade (adapted from Brouns & De Deyn 2009, Saenger & Christenson 2010). Initiated by cerebral hypoperfusion, the ischaemic cascade comprises bioenergetic failure of cellular components, excitotoxicity, oxidative stress, microvascular injury, haemostatic activation, post-ischaemic inflammation and biphasic blood-brain barrier dysfunction

2.3. Thrombosis

Thrombosis, the most common cause of stroke, is influenced by factors including endothelial injury, blood stasis or turbulent flow and hypercoagulability of blood (Myers & Wakefield 2005). Endothelial injury is the most common cause of haemostatic coagulation processes, and it is essential that these processes are better understood. Endothelial damage to vasculature initiates a local inflammatory response, promoting a state of prothrombosis that is driven by tissue factor, adhesion molecules, as well as pro-inflammatory cytokines and prothrombotic microparticles (Libby & Simon 2001). Various disease states are implicated in promoting tissue factor exposure within vascular walls to blood flow that leads to the initiation of non-haemostatic coagulation processes (Mackman 2004, Myers & Wakefield 2005). Thrombosis and inflammation are interrelated (Myers & Wakefield 2005, Stewart *et al.* 1974), since inflammation produces local thrombosis and thrombosis can amplify inflammation (Libby & Simon 2001). A thrombus, whether formed through haemostatic or non-haemostatic mechanisms, consists of platelets and fibrin as well as trapped red and white blood cells, which stabilize its structure.

Platelets and fibrin play an important role in the coagulation process where they are involved in the maintenance of haemostasis. Platelets have a lifespan of 8-12 days and, though devoid of a nucleus, possess many features of classical inflammatory cells. Like neutrophils, they can undergo chemotaxis (Zhang *et al.* 1993), phagocytose foreign particles, contain and release adhesive proteins, activate complement, interact with foreign bodies, alter vascular tone, enhance vascular permeability, as well as store and metabolize various vasoactive substances and release inflammatory mediators (Herd & Page 1994). Structurally, platelet surfaces consist of a typical bilayer membrane composed of lipids, proteins and carbohydrates. Surface glycoproteins are essential to their function and play a primary role in their adhesion to exposed subendothelial matrix proteins, interaction with thrombin and exposure of fibrinogen receptors to facilitate aggregation (Herd & Page 1994, Roth 1992). Internally, platelets are only capable of limited protein synthesis and few mitochondria are present that contribute to energy metabolism of the platelet. Additionally, a random cytoplasmic distribution of lysosomes, glycogen granules and peroxisomes are present (Herd & Page 1994). Following activation, platelets change shape from a discoid to a spherical form. This process is mediated by a contractile microtubular system, which is morphologically characterised by an extension of dendritic pseudopodia (Herd & Page 1994, White 1987).

Platelets are activated by a number of stimuli resulting in the expression and/or activation of surface receptors, secretion of vasoactive substances, adhesion, aggregation, and finally thrombus formation. The activation may be due to damage of the vascular wall, or activation of the endothelium by chemicals, cytokines and also inflammatory processes (Butenas & Mann 2002, Camera *et al.* 1999). Upon activation, platelets cover the exposed subendothelial matrix and mediate additional platelet and leukocyte recruitment through the release of microparticles that mediate local leukocyte-leukocyte and leukocyte-endothelial cell interactions (Wagner & Burger 2003); mechanisms which play a role in both thrombosis and inflammation. Platelets are essential in the initial stages of thrombus formation, because they adhere and aggregate at sites of vascular wall injury and then serve as a surface for coagulation reactions, the overall rate of which determines the final structure of fibrin (Wohner 2008). Thrombi thus form locally in a vessel when injury occurs or endothelial activation takes place as a haemostatic mechanism to repair the insult. Platelets, during adhesion to endothelium, are activated and release pro-inflammatory cytokines that further stimulate the endothelium (Wagner & Burger 2003, Weber & Springer 1997), promote haemoattraction of leukocytes, stimulate smooth muscle cell and fibroblast proliferation, and promote collagen synthesis; thus contributing directly to lesion progression and maturation (Ross 1985, Wagner & Burger 2003).

Fibrinogen is the major plasma protein coagulation factor and best-known precursor of fibrin. It plays an important role in platelet aggregation by linking activated platelets and, therefore, performing a key role in haemostasis and thrombosis. Activated platelets synthesize/secrete inducers of platelet aggregation, adhere to the injured vessel wall and aggregate to each other in order to form a platelet-rich plug which secures haemostasis. This plug is then stabilized by fibrin formation as fibrinogen is activated by binding to activated platelets. Thus not all circulating fibrinogen is functional or clottable. As the tissue repair process takes place, the fibrin plug is digested by fibrinolytic enzymes (Lowe *et al.* 2004). Low levels of plasma fibrinogen are thus associated with an increased risk for bleeding, since platelet aggregation as well as fibrin plug formation is impaired. Elevated fibrinogen levels, on the other hand, may well be associated with the risk of stroke (Danesh *et al.* 2005) as elevated fibrinogen synthesis is inclined to shift the haemostatic balance in favour of coagulation/thrombosis.

Interestingly, thrombi are found to form readily and rapidly in the complete absence of fibrinogen in animal models. These thrombi are however unstable and fail to resist shear stress, resulting in frequent thromboembolisation, with downstream vessel occlusion (Ni *et al.* 2000, Wagner &

Burger 2003). Fibrinogen/fibrin complexes are thus required to secure thrombus stability or anchorage to the site of injury. This stability is additionally dependent on fibrinogen/fibrin interaction with platelet integrin (a surface protein), which also slows down the growth of the thrombus (Hawiger 1995, Ni *et al.* 2000, Wagner & Burger 2003). Fibronectin is known to support platelet adhesion and distribution (Hynes 1990, Wagner & Burger 2003). However, deficiency of plasma fibronectin does not affect initial platelet adhesion (Sakai *et al.* 2001, Wagner & Burger 2003), but delays thrombus formation quite substantially as platelets are continuously shed. Therefore, fibronectin is an important mediator of platelet-platelet interactions within thrombi as they form and grow. This mediation takes place through the rapid binding of fibronectins to activated integrins, thus cross-linking platelets. Fibrin is then generated which anchors the growing thrombus to the site of vascular injury (Ni *et al.* 2000, Wagner & Burger 2003).

Platelet adhesion and activation is thus regulated by specific proteins on the platelet surface, with fibrinogen and fibronectin playing a fundamental role in the coagulation process (Lowe *et al.* 2004, Ni *et al.* 2000). Fibrin assembly (through the coagulation pathway and involvement of the platelets) from fibrinogen proceeds in a highly ordered fashion. Fibrin forms a network that functions to stabilize the primary platelet plug. Although fibrin forms the core matrix of a thrombus, its structure depends also on the cellular elements embedded in its meshwork. Morphological changes of fibrin networks may therefore occur due to several kinetic and modulating factors present in plasma. Consequently, fibrinolysis also plays an important role in haemostasis. Leukocyte-derived enzymes, such as elastase, influence fibrinolysis by direct digestion of fibrin or indirectly modulating it by partial degradation of zymogens and inhibitors of coagulation and fibrinolytic proteases (Wohner 2008).

Thrombosis research has shown that sex hormones have complex effects on vascular walls, coagulation proteins and platelets, all of which may alter thrombosis. In line with this, females have shown cyclic patterns in the levels of their coagulation proteins (e.g. fibrinogen), which correspond to menstrual cycle patterns (Bailey *et al.* 2009, Kadir *et al.* 1999) and additionally possess slightly higher fibrinogen levels than their male counterparts, though oestrogen is known to decrease fibrinogen plasma levels (Bailey *et al.* 2009, Mendelsohn & Karas 1999). There also seem to be sex-related differences in platelet function. It is known that isolated female platelets bind more fibrinogen and have a greater maximal aggregation extent than male platelet isolates; but this platelet reactivity is altered in ovariectomised females (Bailey *et al.*

2009, Leng *et al.* 2004). Males have been shown to have higher platelet counts and faster clotting times than their female counterparts, making them more susceptible to thrombosis. This is possibly due to the differences in growth hormone secretion between the sexes (pulsatile in males, sustained in females), which in turn influence protein production of coagulation and thrombosis regulators (Bailey *et al.* 2009, Wong *et al.* 2008). Isolates of male rat platelets have been shown to display greater maximal aggregation *in vitro* than platelets isolated from female rats. This aggregation is reduced in male rats that have undergone castration (Bailey *et al.* 2009, Emms & Lewis 1985). It has been noted that both megakaryocytes and platelets express the oestrogen receptor beta (ERβ) as well as the androgen receptor (Bailey *et al.* 2009, Jayachandran & Miller 2003), so it is almost certain that the sex hormones have an effect on thrombosis. Oestrogen is hypothesized to have a direct effect on platelet function, whereas androgen seems to regulate megakaryocyte biology and platelet production (Bailey *et al.* 2009, Peters *et al.* 2002).

The occurrence of thrombosis is akin to haemostasis in the wrong place, which results from local activation of platelets and coagulation and also from increased concentrations of plasma fibrinogen (Lowe *et al.* 2004). As previously mentioned, though oestrogen lowers fibrinogen levels, females still have elevated fibrinogen levels when compared to their male counterparts (Bailey *et al.* 2009, Mendelsohn & Karas 1999). These fibrinogen-lowering effects of oestrogen are ruled out in menopause, rendering fibrinogen levels even higher than in cyclic females (Lowe *et al.* 1997, Lowe *et al.* 2004). Many diseases like cancer, thrombotic disease, bleeding disorders, asthma and even conditions like HIV/AIDS are associated with changes in platelet and fibrin structure. Fibrin structure itself has been shown to play a role in the development of vascular complications (Pretorius *et al.* 2006, Pretorius *et al.* 2007).

Cerebral ischaemia may be a consequence of thrombosis when a change in haemostasis occurs, for example in the instance of a blood disorder or diminished blood flow (due to age or even normal thrombus formation), resulting in a local accumulation of coagulation factors and thus increasing platelet aggregation. These changes in haemostasis alter the coagulation cascade and result in the formation of rigid fibrin networks that do not digest as programmed. These rigid thrombi may cause ischaemia locally if they diminish or inhibit the blood flow in a vessel for an extended period, or pieces thereof may break free, forming thromboemboli, which may become lodged in another vessel where they can cause ischaemia by impedance of blood flow.

2.4. Inflammation

Inflammation is characterised by interactions between endothelial cells, platelets and leukocytes and causes endothelial activation, regardless of the mechanisms by which inflammation itself was activated. Endothelial activation sets off the cell adhesion cascade, which results in the adherence and aggregation of platelets, chemokine deposition by platelets on the activated endothelial surface, the expression of cell adhesion molecules by endothelial cells and platelets and, ultimately, activation of leukocytes. Chemokines activate leukocytes, and further binding to adhesion molecules mediate the process of leukocyte rolling (on the activated "sticky" endothelium), adhesion (through binding to fibrinogen) and transmigration into the subendothelial tissue (Butcher 1991, Diacovo *et al.* 1996, Konstantopoulos *et al.* 1998, Kuijper *et al.* 1996, Springer 1994, Wagner & Burger 2003, Weber & Springer 1997). Platelets are thus central to both thrombosis and inflammation.

The objective of inflammation is to recruit leukocytes rapidly to a site of vascular injury. Endothelial dysfunction or injury promotes activation of the coagulation cascade by exposure of tissue factor (Day *et al.* 2005, Gimbrone 1995, Myers & Wakefield 2005), as well as the activation of inflammatory processes (Altman 2003, Laursen *et al.* 2001, Myers & Wakefield 2005). Inflammatory mediators then promote coagulation through further elevation of tissue factor (Drake *et al.* 1989, Esmon 2003), which elevates fibrinogen synthesis and fibrinogen levels will consequently continue to rise under inflammatory conditions (Esmon 2003, Taylor *et al.* 1987), unless haemostatic factors counteract this. Tissue factor is a membrane-bound protein which functions as a procoagulant (Libby & Simon 2001), triggering thrombin generation, which then prompts activation of the coagulation cascade (Mann *et al.* 1998, Myers & Wakefield 2005, Nemerson 1988). Thrombin, in turn, amplifies the inflammatory response by activating the endothelium, resulting in the formation of more tissue factor (Esmon 2003, Miller *et al.* 1998, Pendurthi *et al.* 1997) and high levels of platelet-activating factor (Bar-Shavit *et al.* 1986, Esmon 2003), which is a neutrophil agonist (Esmon 2003, Lorant *et al.* 1991) enhancing leukocyte activation and adhesion as well as increasing inflammatory cytokines (André *et al.* 2002, Esmon 2003, Henn *et al.* 1998). Inflammatory cytokines have been shown to increase platelet reactivity, which increases thrombogenic potential (Burstein 1997, Esmon 2003), further linking inflammation and thrombosis.

Monocytes do not express tissue factor, unless stimulated by inflammatory mediators to transcribe the gene for tissue factor (Brand *et al.* 1991, Libby & Simon 2001, Wilcox *et al.* 1989), thus their recruitment and activation can lead to thrombogenesis. Activated monocytes, which express tissue factor on their surfaces (Rauch & Nemerson 2000), facilitate monocyte-platelet and monocyte-endothelial interactions through binding mechanisms of cell adhesion molecules (Myers & Wakefield 2005, Shebuski & Kilgore 2002, Wakefield *et al.* 1997). These interactions, driven by inflammatory mediators and tissue factor, lead to accelerated fibrin formation and deposition into a developing thrombus (Myers & Wakefield 2005, Shebuski & Kilgore 2002). Therefore, the specific interaction between cell adhesion molecules and their leukocyte receptors, is what stimulates fibrin formation (Goel & Diamond 2001, Myers & Wakefield 2005) and procoagulant microparticles, derived both from activated leukocytes and platelets, amplify the coagulation process (Frenette *et al.* 2000, Myers & Wakefield 2005). Like endothelial cells and activated monocytes, smooth muscle of blood vessels can express tissue factor when exposed through endothelial injury, thus also contributing to thrombogenesis (Libby & Simon 2001, Schecter *et al.* 1997). Besides production of procoagulant tissue factor, smooth muscle can also undergo inflammatory activation when exposed to thrombin and products of thrombosis (Kranzhöfer *et al.* 1996, Libby & Simon 2001), thus amplifying the inflammatory response and promoting systemic procoagulant effects due to increased fibrinogen levels in circulation (Libby & Simon 2001).

Hence, the progression of the inflammatory response, subsequent to a stimulus, reflects a balance between prothrombotic and anticoagulant activity. The ability of pro-inflammatory cytokines to downregulate antithrombotic proteins and upregulate prothrombotic proteins shifts this balance towards a procoagulant state (Myers & Wakefield 2005, ten Cate *et al.* 1997). In addition, these cytokines induce the immune defence mechanism and mediate leukocyte recruitment. Inflammatory cells are important to the process of thrombus recanalization and organization. Although it may seem intuitive that a decrease in inflammation will decrease thrombogenesis; once a clot forms, the presence of neutrophils is important for recanalization (Myers & Wakefield 2005, Varma *et al.* 2003). Accordingly, inflammation leads to an imbalance between the pro- and anticoagulant properties of endothelium that can lead to local stimulation of the coagulation cascade (Nathan 2002, Wagner & Burger 2003). Early inflammatory responses may consequently contribute to damage, whereas late or delayed inflammatory responses are necessary to facilitate repair.

As mentioned previously, cerebral ischaemia triggers inflammatory processes (Gibson *et al.* 2005, Herd & Page 1994, Wang *et al.* 2007). The first inflammatory cells which enter the brain subsequent to trauma are neutrophils, followed by monocytes and, later on, resident microglia, astrocytes and neurons are also activated (Morganti-Kossmann *et al.* 2001). Leukocytes (immediately) and microglia (a few hours post-injury) secrete pro-inflammatory cytokines and chemokines, the extent of this secretion playing detrimental roles in the pathophysiology of stroke (Morganti-Kossmann *et al.* 2001, Wang *et al.* 2007, Suzuki *et al.* 2009). Significant leukocyte influx into cerebral parenchyma and tissue remodelling are characteristics of cerebral ischaemia/reperfusion (Barone *et al.* 1995, Wang *et al.* 2007). Interestingly, infarct volume has been shown to be reduced significantly through the inhibition of neutrophil infiltration, as it is evident that neutrophils wield the most damage to ischaemic lesions once reperfusion is undertaken (Connolly *et al.* 1996, Guha & Mackman 2001, Wang *et al.* 2007).

In areas of ischaemia/reperfusion injury, platelets co-localise with leukocytes; an interaction linking haemostatic thrombotic and inflammatory responses (Libby & Simon 2001, Ostrovsky *et al.* 1998). The inflammatory reaction subsequent to cerebral ischaemia is characterised by neutrophil adherence to blood vessels 4-6h after the onset of ischaemia and their infiltration into the neural tissue with subsequent release of pro-inflammatory mediators, potentiating injury (Hallenbeck 1996, Wang *et al.* 2007) by resultant accumulation and activation of monocytes in the area of the lesion. Platelets promote accumulation of both neutrophils and monocytes at sites of injury. Neutrophil-platelet aggregates, in specific, influence cellular responses by inducing further leukocyte activation, enhancing cell-adhesion molecule expression and generating signals that promote platelet integrin (surface protein) activation and chemokine synthesis (Furman *et al.* 1998, Libby & Simon 2001, Ott *et al.* 1996). Chemokines stimulate cytoskeletal reorganisation of neutrophils and monocytes to facilitate their motility, proliferation of fibroblasts and astrocytes for glial scar formation, apoptosis and necrosis of neurons, as well as the phagocytic ability of macrophages and microglia to remove the debris of damaged tissue (Morganti-Kossmann *et al.* 2001).

2.5. Neuroprotection

Sex hormones target the central and peripheral nervous systems, affecting brain development and differentiation and influencing neuronal functions (Drača 2009, Manthey & Behl 2006). In humans, it is recognised that premenopausal or cyclic women present with a lower incidence of ischaemic stroke than men. This distinction is however no longer present when postmenopausal or acyclic women are compared to men. Furthermore, the ischaemic stroke risk increases in both sexes with age (Braeuninger & Kleinschnitz 2009, Gibson *et al.* 2005, Wolf 1990). Thus sex hormones must have a role in neuroprotection and the decline thereof with age.

Experimental animal studies in mammals have not only reported that young females present with smaller cerebral infarcts and thus less neural tissue injury than their male counterparts (Alkayed *et al.* 1998, Braeuninger & Kleinschnitz 2009), but also that high endogenous oestradiol levels during the oestrus cycle seem to correlate with smaller infarct size in females (Braeuninger & Kleinschnitz 2009, Carswell *et al.* 2000). This advantage is abolished in ovariectomised animals due to the loss of endogenous female sex hormones (Alkayed *et al.* 1998, Alkayed *et al.* 2000, Drača 2009, Gibson *et al.* 2005, Hawk *et al.* 1998, Liao *et al.* 2001, Park *et al.* 2006, Selvamani & Sohrabji 2010, Simpkins *et al.* 1997) and the consequences of cerebral ischaemia in aged animals are more severe than in young animals (Alkayed *et al.* 1998, Davis *et al.* 1995).

The female sex hormone 17β-oestradiol has been shown to be the principal circulating oestrogen protecting the brain from damage by reducing infarct size after experimental cerebral ischaemia, through attenuation of markers of apoptosis by activation of mediators of cell survival signalling pathways. It seems that, when administered several days before inducing cerebral ischaemia, physiological levels of oestradiol attenuate brain injury through the suppression of neuronal apoptosis and genomic actions by acting through mechanisms of the classical nuclear oestrogen receptors (Jia *et al.* 2009, Liao *et al.* 2001, Prewitt & Wilson 2007, Suzuki *et al.* 2009). This is however not the case with acute administration of 17β-oestradiol at the time of injury, as this does not reduce the extent of infarction (Dubal *et al.* 1998, Suzuki *et al.* 2009).

In studies of the neuroprotective extent of oestradiol, researchers have shown that a single high dose injection (1mg/kg) of 17β-oestradiol administered to male rats immediately before induction of experimental cerebral ischaemia was capable of reducing cortical tissue loss or injury; and

that castration of male rats, resulting in the loss of testosterone, did not alter these results (Toung *et al.* 1998). Moreover, injection of exogenous 17β-oestradiol was only neuroprotective in the male brain, revealing that endogenous oestrogen is sufficient to protect the female brain and that exogenous oestradiol had no additional protective effect (Toung *et al.* 1998). Thus the hypothesis that oestrogen is a major mediator of sex differences displayed in stroke is heavily strengthened (Drača 2009).

17β-Oestradiol salvages the brain from ischaemic injury, even enhancing recovery and reducing infarct size in ovariectomised (Selvamani & Sohrabji 2010, Simpkins *et al.* 1997) and reproductively senescent or aged females (Alkayed *et al.* 2000, Liao *et al.* 2001), as well as in male animals (Hawk *et al.* 1998). In the case of acyclic females, this is subject to administration of oestradiol at the onset of senescence or ovariectomy and not in older acyclic females (Bake & Sohrabji 2004, Selvamani & Sohrabji 2010, Suzuki *et al.* 2009). This seems to be due to the upregulation of oestrogen receptor alpha (ERα) close to the onset of senescence, in response to declining oestrogenic stimuli from the ovaries, inadvertently providing a substrate for exogenous oestrogen. However, in older acyclic females, the ERα is already downregulated and thus exogenous oestrogen becomes deleterious (Jezierski & Sohrabji 2000, Jezierski & Sohrabji 2001, Selvamani & Sohrabji 2010). Ischaemic injury itself has been found to increase the expression of ERα in the cerebral cortex, without influencing ERβ expression. Consequently, it is believed that it is this ERα re-expression after ischaemic injury that mediates 17β-oestradiol's profound neuroprotection against ischaemia (Dubal *et al.* 2001, Suzuki *et al.* 2007, Suzuki *et al.* 2009).

Cerebral ischaemia triggers a complex series of events, including excitotoxicity, inflammation, and formation of oedema, as well as apoptosis and necrosis (Danton & Dietrich 2003, Gibson *et al.* 2005, Saenger & Christenson 2010); all of which are reduced by oestradiol through free radical scavenger action amongst others (Demopoulos *et al.* 1972, Drača 2009, Gibson *et al.* 2005, Singer *et al.* 1996). Oestradiol seems to target neural cells by indirect transcriptional mechanisms as well as by direct mechanisms: stabilizing neurotransmission, inhibiting apoptosis, reducing cerebral oedema and exerting anti-inflammatory and antioxidant effects (Drača 2009, Manthey & Behl 2006). Figure 2.2 proposes further points at which oestradiol wields an influence in neuroprotection against cerebral ischaemic consequences.

Post-ischaemic inflammation strongly contributes to the extent of cerebral injury, and 17β-oestradiol may exert protection through anti-inflammatory (Figure 2.2) actions (Suzuki *et al.* 2009, Vegeto *et al.* 2008). In fact, the presence of initial neural inflammation is negatively correlated with serum oestradiol levels (Wang *et al.* 2007). The proposed anti-inflammatory action of oestradiol is strengthened by findings that 17β-oestradiol is neuroprotective when administered immediately upon ovariectomy, but not when administered after 10 weeks of hypoestrogenicity, demonstrating that a prolonged period of hypoestrogenicity disrupts not only the neuroprotective, but also the anti-inflammatory actions of oestradiol (Suzuki *et al.* 2007, Suzuki *et al.* 2009). The first cellular response in inflammation is the activation and accumulation of neutrophils (Morganti-Kossmann *et al.* 2001). It is evident in models of transient cerebral ischaemia that tissue injury is reduced significantly through the inhibition of neutrophil infiltration, as it is apparent that neutrophils wield the most damage to ischaemic lesions once reperfusion is undertaken (Connolly *et al.* 1996, Guha & Mackman 2001, Wang *et al.* 2007). It would seem that neutrophil accumulation is also negatively correlated with serum oestradiol levels (Liao *et al.* 2001), strengthening evidence for the neuroprotective role of the female sex hormone even more. The anti-inflammatory properties of 17β-oestradiol in the cerebral circulation thus influence the incidence, outcome, and severity of injury in stroke by attenuating ischaemia-induced inflammatory responses (Suzuki *et al.* 2009).

Not only does oestradiol exert protective anti-inflammatory actions subsequent to cerebral ischaemia, but it also has a role in thrombosis (Figure 2.2) regulation (Bailey *et al.* 2009, Wong *et al.* 2008), the alteration of which may result in cerebral ischaemia. Coagulation factors and proteins are lowered by the presence of oestrogen, though some factors (e.g. fibrinogen) are inherently higher in females than in males (Bailey *et al.* 2009, Mendelsohn & Karas 1999). There are, additionally, cyclic patterns in the presence of coagulation proteins which correspond to the menstrual cycle in females (Bailey *et al.* 2009, Kadir *et al.* 1999). Functionally, female platelet isolates – though capable of binding more fibrinogen and displaying a greater maximal aggregation extent than male platelet isolates (Bailey *et al.* 2009, Leng *et al.* 2004) – actually do not aggregate as quickly as the larger number of male platelets do, thus, to some degree, rendering females less susceptible to thrombosis. In the absence of oestradiol in acyclicity, female platelets are again more susceptible to thrombosis (Bailey *et al.* 2009, Wong *et al.* 2008). Platelets are indeed found to express ERβ, which is hypothesized to have a direct effect on platelet function (Bailey *et al.* 2009, Jayachandran & Miller 2003, Peters *et al.* 2002). It therefore becomes clear that there are not only sex-based differences in coagulation processes, but also

age-based differences. In a hypo-oestrogenic state, females not only have higher levels of coagulation factors, but also higher maximal platelet aggregation capabilities than males, rendering them more prone to thrombosis in an acyclic state.

Finally, it must be noted that oestradiol replacement is not universally neuroprotective. It has been suggested that the neuroprotective effects of oestrogen are more evident in transient than in permanent models of cerebral ischaemia (Macrae & Carswell 2006, Selvamani & Sohrabji 2010). This is suggested due to findings that in severe ischaemic injury, there are no sex differences in infarct size and also no reduction of the infarct with 17β-oestradiol administration (Selvamani & Sohrabji 2010, Vergouwen *et al.* 2000). Permanent ischaemia leads to severe metabolic impairment in the cerebral cortex, which results in necrosis of many neurons in the region within several hours following injury. Regions surrounding the core of ischaemia can be salvaged from apoptosis through the powerful neuroprotective action of 17β-oestradiol (Prewitt & Wilson 2007, Suzuki *et al.* 2009), but the effects of oestradiol at the ischaemic core are only visible in transient ischaemic models. Conclusively, 17β-oestradiol protects the brain through suppression of neuronal apoptosis during the initial 24h after injury, in part by suppressing the inflammatory response, and enhances neurogenesis within the first 96h after ischaemic stroke (Suzuki *et al.* 2009).

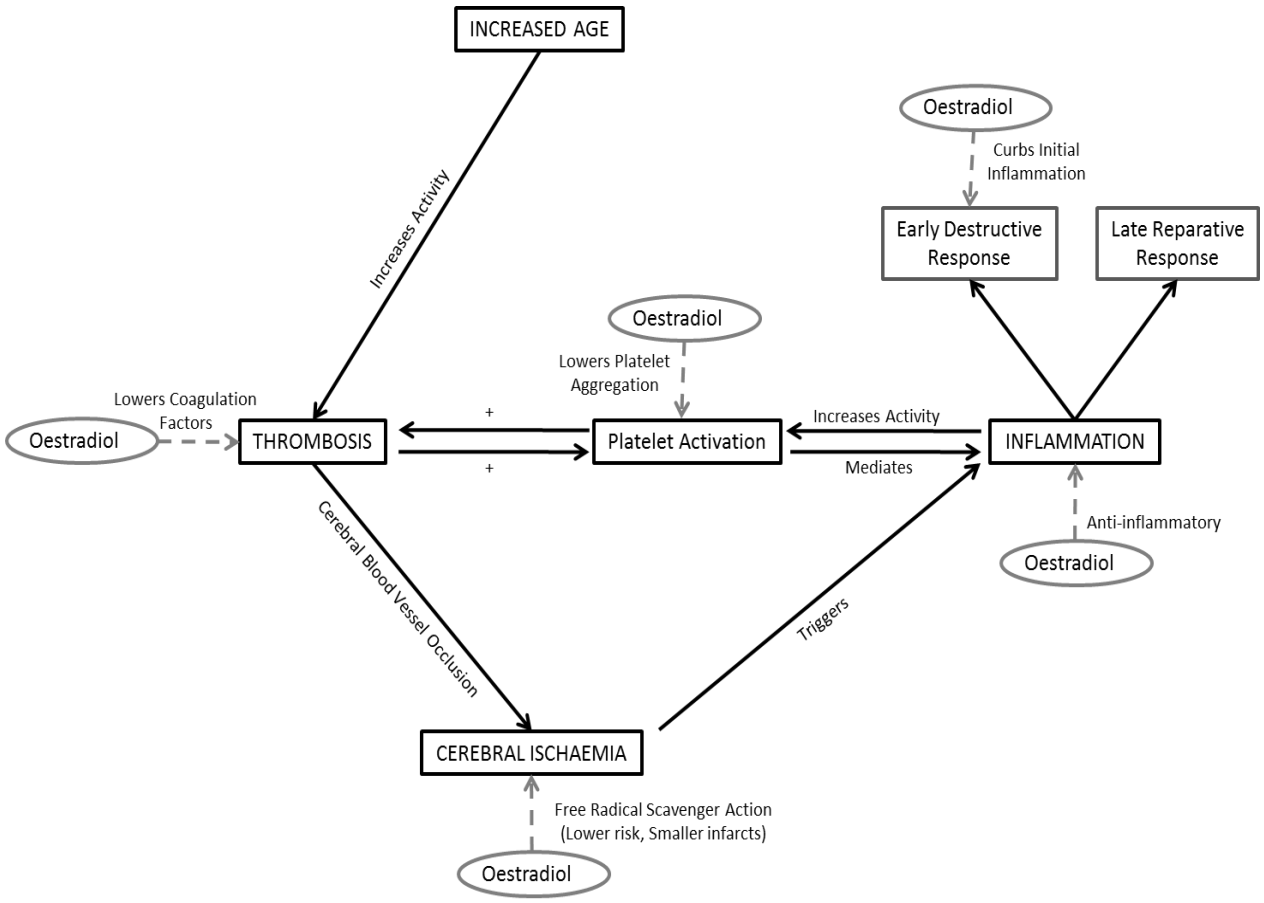


Figure 2.2. Possible points of oestradiol's influence in neuroprotection against cerebral ischaemia. Oestradiol affects thrombosis activity through the lowering of coagulation factors and platelet aggregation, displays anti-inflammatory mechanisms and lowers the negative consequences of cerebral infarction through free radical scavenger action

2.6. Experimental Cerebral Ischaemia

Focal and global cerebral ischaemia models are distinguishable. Global ischaemia is subdivided into complete and incomplete models, while focal ischaemia is divided into true focal and multifocal models. Complete global ischaemia models are regarded as models of circulatory failure. Therefore incomplete global or diffuse ischaemia models, which produce forebrain ischaemia and tissue injury, are preferred in cerebral ischaemia experiments. True focal and multifocal ischaemia models are differentiated according to infarct distribution. Focal ischaemia occurs as a result of occlusion of blood flow to a specific brain region, whereas multifocal ischaemia occurs as a result of occlusion of blood flow of a supplying artery, leading to a reduction of blood flow in multiple cerebral regions. Whether a focal or global model is implemented, cerebral ischaemia can be induced in either a permanent or transient manner (Braeuninger & Kleinschnitz 2009, Hoyte & Buchan 2009, Traystman 2003).

Primary and secondary neural damage are differentiated. Primary injury is classified as insults of either focal or diffuse nature. Secondary injury is set into motion at the time of insult or trauma, but may appear with a delay of several hours, or days, subsequent to the primary insult. Secondary neural injury is the result of a complex network of vascular, cellular and biochemical cascades leading to ischaemia and neurological deficit (Graham *et al.* 2000, Morganti-Kossman et al 2001).

2.6.1. Animal Models

Animal models of ischaemic stroke are classified according to a variety of factors including animal species, mechanisms and aetiology (intra- or extravascular) of occlusion, transient or permanent occlusion, the involvement of vascular territory as well as infarct distribution (Braeuninger & Kleinschnitz 2009, Traystman 2003). The Sprague Dawley rat (Ace Animals Inc. 2006, Harlan Laboratories Inc. 2008) is the outbred species of choice in stroke research (Chen *et al.* 2000, Liao *et al.* 2001, Selvamani & Sohrabji 2010) and is a general model for the study of human health and disease. Adult body weight is within the ranges of 250-300g for females and 450-520g for males. Breeding onset is between 65-100 days of age in both females and males, and the weight ranges correspond with these ages (Ace Animals Inc. 2006, Harlan Laboratories Inc. 2008). It is of utmost importance to note that, because human brains are gyrencephalic and

small rodents are lissencephalic, they differ in neural metabolic rate and mechanism. In terms of treatment development research, rats and mice require higher doses (mg/kg) to produce similar effects as in larger mammals. Therefore effective doses determined in rodent stroke models cannot merely be extrapolated to treatment in the human condition, even if adjusted for body weight (Braeuninger & Kleinschnitz 2009, McCann & Ricaurte 2001).

2.6.2. Models of Experimental Cerebral Ischaemia

Various mechanisms of stroke induction are available including those which require craniotomy and others which do not require this maximally invasive technique. Models requiring craniotomy are traumatic and do not closely mimic human stroke conditions. The existing models of experimental cerebral ischaemia are shown in Figure 2.3, and some optimal models are discussed briefly.

Focal models, including middle cerebral artery occlusion by intraluminal thread and blood clot emboli models are most similar to the clinical situation in humans, as they do not require further stress of craniotomy (reviewed in Braeuninger & Kleinschnitz 2009). Models of embolic cerebral ischaemia do offer a certain pathophysiological relevance, as vessel occlusion by a thromboembolus is the most frequent cause of ischaemic stroke in humans, although a common complication of this model is intracerebral and subarachnoid haemorrhage (Braeuninger & Kleinschnitz 2009, Zhang *et al.* 1997). The intraluminal thread middle cerebral artery occlusion model produces focal infarction in an artery supplying a large cerebral territory, although this model is also complicated by subarachnoid haemorrhage (Schmid-Elsaesser *et al.* 1998, Tsuchiya *et al.* 2003) and ipsilateral retinal injury (Steele *et al.* 2008) and thus visual dysfunction (Braeuninger & Kleinschnitz 2009, Tsuchiya *et al.* 2003). The least invasive model seems to be the photochemical thrombosis model, in which a cortical lesion is induced by focal irradiation of the skull after systemic injection of a photosensitive dye. Advantages of this model are the high reproducibility of lesion location and size, as well as the simplicity of the technique (Braeuninger & Kleinschnitz 2009, Que *et al.* 1999). However, this model does not reflect vascular-ischaemic brain injury or stroke, as platelet activation and coagulation are not instrumental to its induction (Braeuninger & Kleinschnitz 2009, Kleinschnitz *et al.* 2007).

Incomplete global models, including four-vessel occlusion (transient bilateral common carotid artery and permanent bilateral vertebral artery occlusion) and two-vessel occlusion (transient bilateral common carotid artery occlusion combined with systemic hypotension or hypoxia) are most preferred in studies of diffuse ischaemia. These models are relevant in ischaemia caused by disease states such as diabetes and atherosclerosis, as well as in traumatic brain injury. The models both produce high-grade forebrain ischaemia, but can be complicated by post-ischaemic seizures following extended ischaemic periods (Ginsberg & Busto 1989). Both two- and four-vessel occlusion produce oligemia throughout the forebrain, including the cortex, striatum and hippocampus, with blood flow to the brainstem being preserved in the two-vessel occlusion model (Hoyte & Buchan 2009, McBean & Kelly 1998). Damage produced by two-vessel occlusion insult is similar in size and location to that produced by four-vessel occlusion, although fewer respiratory problems are evident in the two-vessel occlusion model. The two-vessel occlusion model is further preferred over the four-vessel occlusion model due to the simplicity of the surgical technique and thus the achievement of repeatability (McBean & Kelly 1998). The major advantages of incomplete global over focal ischaemic models are that the duration of ischaemia is a great deal shorter and achievement of repeatability is more successful. Two-vessel occlusion without hypotension or hypoxia is neither sufficient to reduce cerebral blood flow beneath the ischaemic threshold, nor to upset the brain's energy state enough to produce quantifiable cell death (Eklöf & Siesjö 1972, Eklöf & Siesjö 1973, McBean & Kelly 1998).

It is clearly understood that hyperglycaemia intensifies the outcome of cerebral ischaemia in both focal and global cerebral ischaemic models (Dietrich *et al.* 1993, Pulsinelli *et al.* 1982). It is believed that enhanced neural tissue damage, subsequent to hyperglycaemic cerebral ischaemia, is a consequence of elevated lactate production, which leads to neuronal and/or astrocytic acidosis (Dietrich *et al.* 1993, Siesjö 1988). Moderate pre-ischaemic hyperglycaemia enhances neuronal and glial injury and has been shown to significantly aggravate the BBB and accentuate oedema, thus worsening the consequences of transient forebrain ischaemia, potentially through increasing cerebrovascular permeability by affecting endothelial integrity (Dietrich *et al.* 1993, Pulsinelli *et al.* 1982). Purely hyperglycaemic models have however not been employed in research, thus the two-vessel occlusion model, which is unsuccessful when employed with exclusion of hypotension and/or hypoxia, is ideal for modification to a hyperglycaemic model.

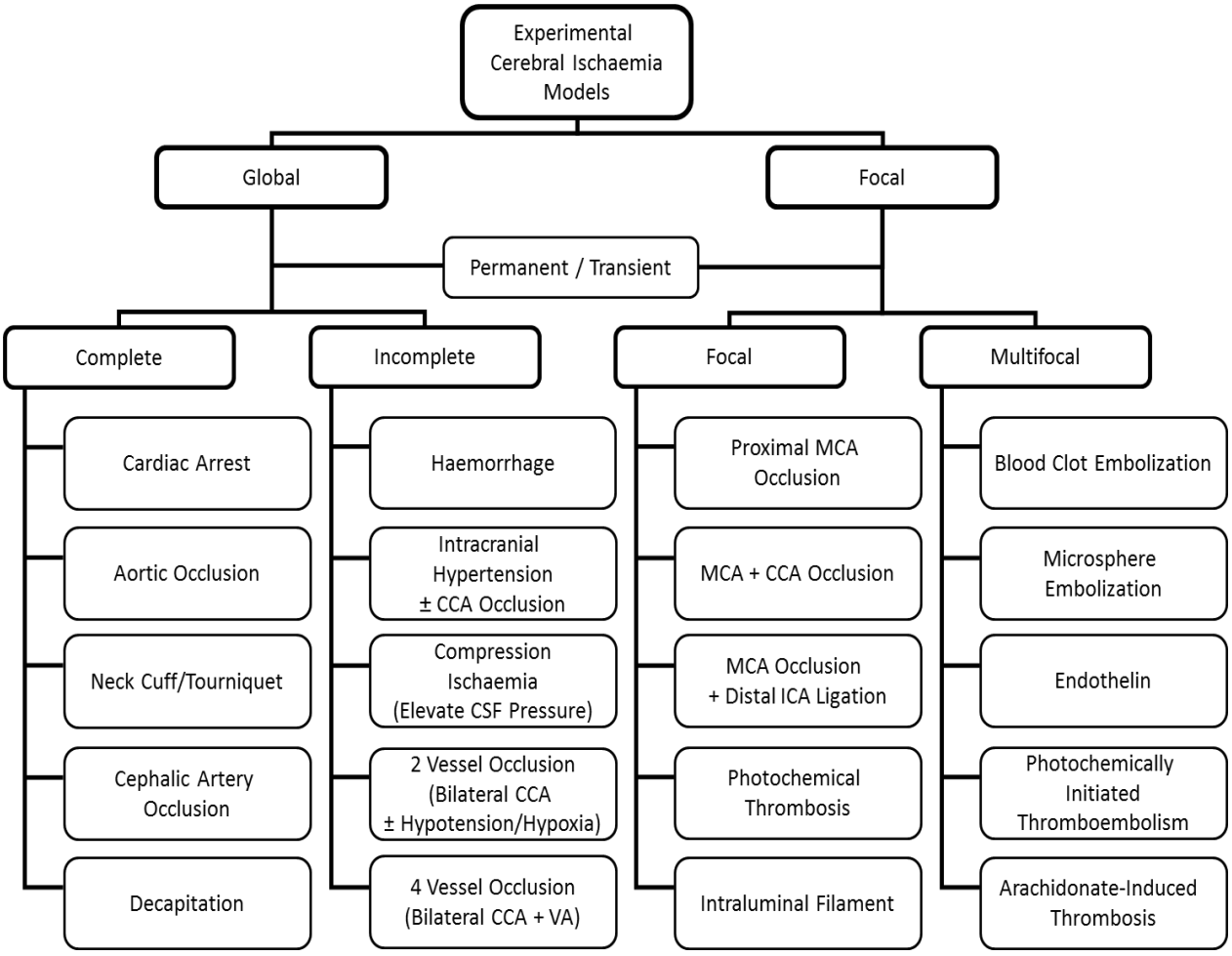


Figure 2.3. Existing models of experimental cerebral ischaemia. Models are divided into global and focal mechanisms of cerebral ischaemia and may be induced in a permanent or transient manner. CCA: common carotid artery, CSF: cerebrospinal fluid, VA: vertebral artery, MCA: middle cerebral artery, ICA: internal carotid artery (adapted from Braeuninger & Kleinschnitz 2009, Chu *et al.* 2008, Ginsberg & Busto 1989, Hoyte & Buchan 2009, Traystman 2003)

Chapter 3: Implementation of the Animal Model

3.1. Introduction

Animal models of cerebral ischaemia are classified according to factors such as animal species, mechanisms and aetiology (intra- or extra-vascular) of occlusion, transient or permanent occlusion, the involvement of vascular territory, as well as infarct distribution (Braeuninger & Kleinschnitz 2009, Traystman 2003). The readily available Sprague Dawley rat (Ace Animals Inc. 2006, Harlan Laboratories Inc. 2008) is the outbred species of choice in stroke research (Chen *et al.* 2000, Liao *et al.* 2001, Selvamani & Sohrabji 2010). The strain was established by Robert Dawley in 1925 and is used widely in the field of neuroscience (Harlan Laboratories Inc. 2008). For this study, adult Sprague Dawley rats were utilised to afford compatibility with international research standards. Furthermore, the specific ischaemic model that was established is a modification to the classical two-vessel occlusion model discussed in Chapter 2, which in the present study served to produce forebrain ischaemia through inducing mild systemic hyperglycaemia prior to surgically employing two-vessel occlusion in exclusion of hypotension and/or hypoxia. Hyperglycaemic two-vessel occlusion was performed in a transient manner, allowing for quantification of ischaemic injury subsequent to varying periods of reperfusion. Implementation of the animal model, including induction of transient experimental cerebral ischaemia is discussed here in detail.

3.2. Implementation of the Sprague Dawley Rat Model

A total of 79 age-matched, outbred Sprague Dawley rats, consisting of 26 males and 53 females were allocated for the purposes of this study. Animals were allowed to acclimatise for five days prior to the commencement of any surgical procedures employed in the study. Animals were accommodated at the laboratory animal facility of La-Bio Research (Tshwane University of Technology, Pretoria, South Africa). Rats were maintained in a micro-barrier unit consisting of individually ventilated cages. The physical size of these cages was in accordance with European standards and allowed for the animals to conduct their species-specific behaviour. The animal room temperature was maintained between 19-23°C with a humidity level of 45-75%. A 12h day/night cycle was a constant in the animal unit and light intensity was kept between 70-

100Lux. The animals were provided with suitable food and water *ad libitum*. Food, water and bedding were autoclaved before use in the cages, as per micro-barrier unit requirements.

One male and two female groups were allotted (Figure 3.1): sexually mature males (MM), sexually mature intact or cyclic females (MF) and sexually mature ovariectomised or acyclic females (OVX). One group of female rats (OVX) were anaesthetised and underwent bilateral ovariectomy two weeks prior to commencement of the experimental study itself. The three groups were then further divided into subgroups for scheduled termination phases (0h, 2h, 24h and 48h) before undergoing transient experimental cerebral ischaemia. These subgroups (six animals in each) consisted of 0h or pre-ischaemic controls, 2h post-reperfusion for immediate damage determination, 24h post-reperfusion for determination of maximal tissue injury/compromisation (Jia *et al.* 2009), and 48h post-reperfusion for analysis around the onset of possible regeneration (Gibson *et al.* 2005).

For the experimental study, test groups were anaesthetised and underwent transient experimental cerebral ischaemia by hyperglycaemic two-vessel occlusion. Ischaemia was induced for a period of 15min and the occlusion (microsurgical vessel clips) was then removed to allow for reperfusion. Test groups of six animals each per group were terminated 2h, 24h, and 48h post-reperfusion. Control groups (0h pre-ischaemic termination subgroups) did not undergo experimental cerebral ischaemia. Animal mass data prior to experimental ischaemia (Figure 3.2) shows that acyclic females gained weight between bilateral ovariectomy and hyperglycaemic two-vessel occlusion and males were considerably larger than all females, but because animals were age-matched (sexually mature) this was not of consequence to further analysis.

In order to study the ultrastructural progression of ischaemia and correlate this with ischaemic tissue injury of the brain and/or regeneration of lesions, six animals per group was the absolute minimum statistically allowed for each time phase under study, thus necessitating the use of 72 animals. Due to ovariectomy and experimental cerebral ischaemia procedures, it was necessary to account for a possible 10% loss of animals due to prolonged anaesthesia exposure, thus an additional seven animals were included [2 males (2 MM) and 5 females (2 MF + 3 OVX)]. In each of the three groups under study; results essentially included six 0h pre-ischaemia control animals, six animals at 2h post-reperfusion, six animals at 24h post-reperfusion and six animals at 48h post-reperfusion.

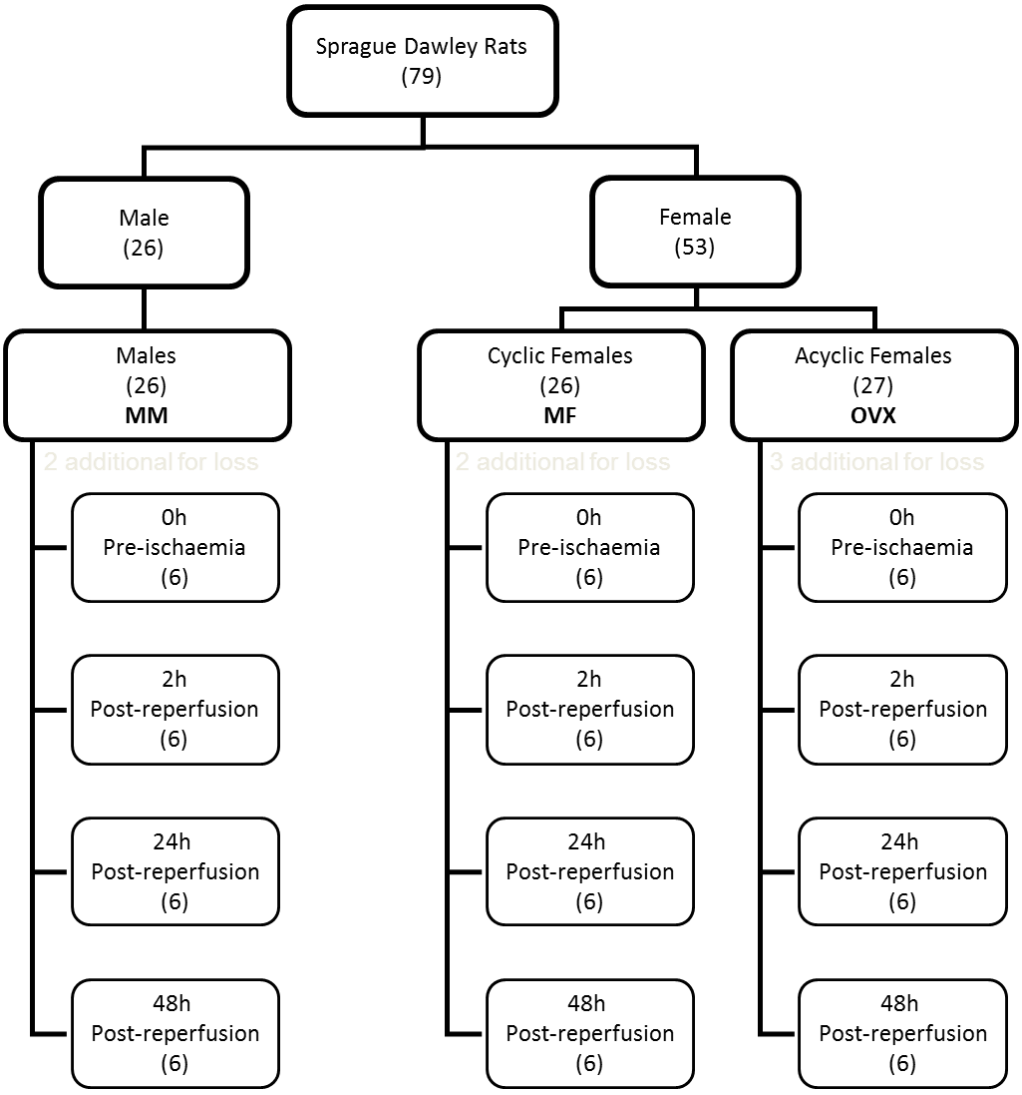


Figure 3.1. Experimental study design. Three groups consisting of one male and two female groups were allotted. Each group was then allocated additional animals to compensate for loss should surgical procedures prove fatal and divided into four subgroups of six animals each for time-based termination

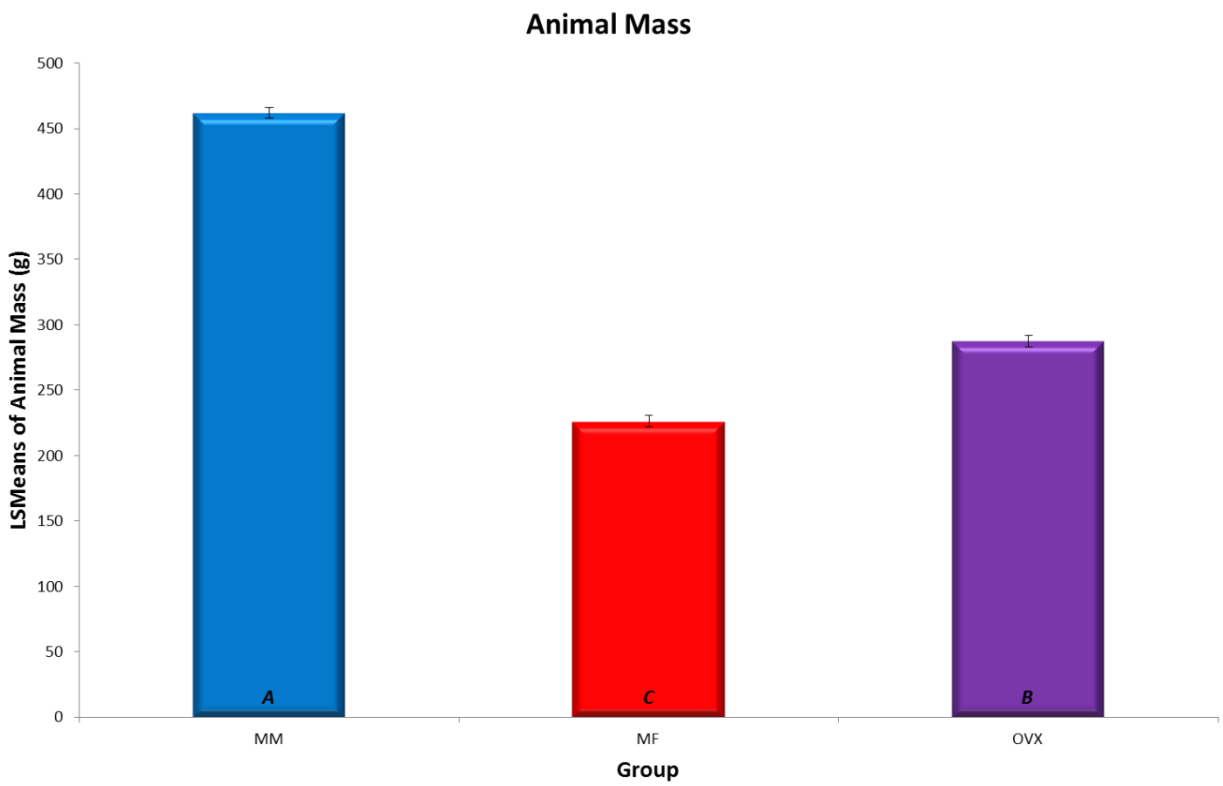


Figure 3.2. Animal mass analysis where group effect is accounted for. Termination times were determined to have no significant effect within groups, warranting group-wise differentiation only. Italicised letters of different value at base of bars indicate statistical significance at a confidence level equal or better than 95%. Variability within this model was 96% and the p-value obtained was <0.0001. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females. Because animals were age-matched, body mass was of no consequence to further analysis in the study.

3.3. Surgical Procedures

Surgical procedures were all performed under general anaesthesia. A Ketamine/Xylazine combination of anaesthetic and sedative-analgesic provided proper anaesthesia and analgesia for the surgical procedures without negatively affecting blood pressure, which was vital for success of the study. Ovariectomy can be performed one (Jia *et al.* 2009) to three weeks (Selvamani & Sohrabji 2010) prior to induction of experimental cerebral ischaemia. Bilateral ovariectomy was thus performed on 27 female rats of group OVX, two weeks prior to experimental cerebral ischaemic procedures. Mild hyperglycaemia is known to attenuate cerebral ischaemia in both focal and global experimental models (Dietrich *et al.* 1993, Pulsinelli *et al.* 1982). Ketamine/Xylazine anaesthesia is proven to cause acute hyperglycaemia in fed but not fasted Sprague Dawley rats, with glucose levels remaining elevated for approximately 3h post-anaesthesia (Saha *et al.* 2005). The classical two-vessel occlusion model performed in rats is accompanied by either hypoxia or hypotension, to attenuate cerebral ischaemia by preventing collateral blood supply to the forebrain during the procedure (Eklöf & Siesjö 1972, Eklöf & Siesjö 1973, Hoyte & Buchan 2009, McBean & Kelly 1998). To assess the effects of systemic hyperglycaemia in exclusivity, a modified two-vessel occlusion model was employed, anaesthetising fed-state rats with a Ketamine/Xylazine combination, without inducing hypoxia or hypotension. Transient cerebral ischaemia was thus induced by hyperglycaemic two-vessel occlusion in spontaneously breathing rats. Experimental cerebral ischaemia was therefore performed in all but the 18 control animals of the 0h or pre-ischaemic termination subgroups.

3.3.1. Bilateral Ovariectomy

Bilateral ovariectomy of 27 female rats (27 OVX) was performed two weeks prior to experimental cerebral ischaemia. A fasted animal was anaesthetised with a Ketamine/Xylazine combination (Ketamine 100mg/kg IP and Xylazine 10mg/kg IP) and placed in a dorsal recumbent position with its tail pointed towards the surgeon. The ventral abdominal area (surgical site) was shaved and swabbed with 70% alcohol. A 2-3cm ventral midline skin incision was made halfway between the sternum and vaginal opening and a single incision made through the linea alba into the peritoneal cavity. The left ovary and oviduct were exteriorised through the opening, a haemostat clamped around the uterine vasculature between the oviduct and uterus and the ovary and part of the oviduct was removed with a single cut through the oviduct near the ovary.

The haemostat was removed and the remaining tissue replaced into the peritoneal cavity. The right ovary was then removed by repeating the procedure. Muscle and skin incisions were sutured with 3-0 Vycril and the analgesics, Temgesic (0.3mg/ml at 0.1ml/100g body weight twice a day) and Carprofen (5mg/kg once a day) were administered for 2-3 days.

3.3.2. Transient Experimental Cerebral Ischaemia

Induction of transient experimental cerebral ischaemia, by hyperglycaemic two-vessel occlusion, in 61 of the 79 animals (all surviving, except six animals from each group, belonging to the 0h or pre-ischaemic termination subgroups). A fed-state animal was anaesthetised with Ketamine/Xylazine combination (Ketamine 100mg/kg IP and Xylazine 10mg/kg IP) and temperature probes were inserted rectally. Warming pads were used to maintain the animal's body temperature at 37°C (±0.5°C). A tail prick was made to measure blood glucose levels for confirmation of hyperglycaemia (>150mg/dl) using an Accu-Chek® Performa Nano blood glucose meter (Dischem, Pretoria, South Africa). Once hyperglycaemia was confirmed, induction of experimental cerebral ischaemia was commenced in spontaneously breathing rats, with the aid of an operating microscope. Bilateral common carotid arteries were exposed through a midline neck incision and carefully dissected free from surrounding fascia and the adjacent vagus nerve. Left and then right arteries were occluded using Schwarz microsurgical vessel clips with slightly angled jaws (Surgical Tools Inc., Virginia, USA) within 1min of each other. Transient experimental cerebral ischaemia was induced for 15min after the second artery was clamped, after which the microsurgical vessel clips were removed and reperfusion allowed to take place. Carotid arteries were inspected to ensure return of good pulsations before closing the neck incision with a resorbable suture. Animals were allowed to survive for maximum of 48h with free access to food and water.

3.4. Monitoring and Termination Procedures

Animals were observed for signs of abnormal behaviour, by qualified personnel, on a daily basis for the duration of the study. Monitoring factors logged included, but was not limited to, weight, habitus, appetite, respiratory pattern and locomotive behaviour. Provision was made to record any unexpected events observed (A score sheet is provided in Table 3.1). If at any time animals were found to undergo undue discomfort or pain, they would be terminated immediately and replaced with an animal accounted for as “additional for loss” in Figure 3.1. After each of the surgical procedures, rats were monitored for any signs of infection, pain or discomfort and exclusion criteria for the duration of the study. At the conclusion of each scheduled phase, animals were anaesthetised with Ketamine (100mg/kg IP) and terminated by cardiac puncture. Blood was collected separately for each animal for chemical and ultrastructural analysis. Whole brains were also collected individually from each animal for analysis of neural tissue injury subsequent to ischaemia and for protein content determination.

The Sprague Dawley rat model proved ideal for the experimental procedures employed. Animals allocated for possible loss due to fatality of surgical procedures were sufficient in number for compensation thereof and ensured that six animals per subgroup could be analysed at scheduled termination intervals. No animals died subsequent to reperfusion after induction of cerebral ischaemia, nor did any of the animals suffer excessive weight loss or discomfort during waking and survival periods. The results presented in the chapters to follow, indicate that this model was successfully implemented.

Table 3.1. Monitoring log book discomfort and pain score sheet. Animals were monitored specifically for weight loss, behavioural and cognitive alterations

Discomfort and Pain Score Sheet						
Subgroup				**Animal**		
Initial Weight (g)					Page	
Date						
Day						
Time						
Observation from a Distance						
Fed						
Active						
Scratching						
Anxiety						
Pinched Face						
Ruffled Coat						
*Diarrhoea (0-3)						
Observation on Handling						
Not Inquisitive & Alert						
Not Feeding/Drinking						
Vocalization upon Gentle Palpitation						
Body Weight (g)						
Body Temperature (°C)						
Pale or Sunken Eyes						
Dehydrated						
**Condition (4-1)						
Nothing Abnormal						
Other Notes						
Signature						

√	Done, doing or present
X	Not doing, not seen or absent
*	0 = normal; 1 = loose faeces in cage; 2 = pools of faeces in cage; 3 = running out on handling; +m = mucus; +b = blood
**	4 = normal; 1 = emaciated

3.5. Fluid and Tissue Collection

At the conclusion of each phase, whole blood was collected in citrate-containing tubes (citrate-to-plasma ratio of 1:9) to prevent coagulation, separately for each individual animal for analysis of 17β-oestradiol levels, fibrinogen levels and platelet count determination. For coagulum preparation, an equal portion of collected blood from each animal was pooled in a separate tube for each subgroup for ultrastructural analysis. Whole brains were collected individually from each animal for analysis of neural tissue injury and protein content determination. Materials and methods are discussed under the relevant sections to follow.

3.6. Statistical Considerations

The study compared the three groups of rats (MM, MF and OVX), in which transient experimental cerebral ischaemia was induced, with respect to variables elucidated in following chapters within four termination subgroups (times 0h, 2h, 24h, 48h) for each group.

3.6.1. Sample Size

The study was conducted as a two-factor design with the main independent factors being groups of experimental cerebral ischaemia (three levels – with sex and presence/absence of circulating oestradiol nested within group information) and termination time (four levels – one pre-ischaemia and three post-reperfusion) with six replicates. By convention, sample size is regarded sufficient if in the analysis of variance (ANOVA) the error degrees of freedom exceeds 30. In this experiment the error degrees of freedom was anticipated to be 60 by the Hochberg method of calculation.

3.6.2. Data Analysis

Data processing and analysis was performed using SAS® (Version 9.3 running on a desktop computer on Windows XP service pack 3). In a multiple ANOVA group effect, time effect and interaction between group and time was assessed. If differences between the levels of group and/or time were detected using the General Linear Model (GLM) procedure, specific differences

were tested for using Fisher's Least Squares Means (LSM) to assess pair-wise differences. Data summary for the outcomes report LSmeans and standard error at 95% confidence intervals. Of particular interest was comparison of the mature males (MM) and acyclic females (OVX) to the cyclic females (MF). Testing was performed at the 0.05 level of significance.

3.7. Ethical Considerations

All experimental procedures were carried out in strict accordance with the requirements of the South African National Standard (SANS 10386:2008) pertaining to the care and use of animals for scientific purposes. Ethical clearance was obtained from the University of Pretoria's Animal Use and Care Committee (AUCC), for registration purposes (Ethical clearance no. AUCC h011-11). Additionally, the Animal Research Ethics Committee (AREC) of the Tshwane University of Technology, where the animal unit operates, verified the accordance of the above ethical clearance with accepted scientific practices (Ethical clearance no. AREC 2011/06/007) and confirmed that the animal facility of La-Bio Research operates within the standards and rules of the South African National Standard (SANS 10386:2008).

Chapter 4: Analysis of Neural Tissue Injury

4.1. Introduction

Experimental cerebral ischaemia was induced using a modified hyperglycaemic two-vessel occlusion model, occluding bilateral common carotid arteries in a state of acute systemic hyperglycaemia induced by anaesthetising fed-state rats with a combination of Ketamine/Xylazine. It is known that moderate pre-ischaemic hyperglycaemia enhances neural tissue injury as the blood-brain barrier (BBB) is significantly aggravated and oedema accentuated, thus worsening the consequences of transient ischaemia (Dietrich *et al.* 1993, Pulsinelli *et al.* 1982). Hence, in a modified model of two-vessel occlusion with mild hyperglycaemia, measurable neural tissue damage was expected. 17β-Oestradiol is an established neuroprotective agent, protecting the brain through the suppression of neuronal apoptosis during the initial 24h after ischaemic injury, in part by suppressing the inflammatory response, and enhancing neurogenesis within the first 96h after such ischaemic injury (Suzuki *et al.* 2009). With regards to oestradiol's suppression of the inflammatory response, it is also known that the presence of initial neural inflammation is negatively correlated with serum oestradiol levels (Wang *et al.* 2007). Since both sex and age are known to play an important role in cerebral ischaemia and the severity of neural injury subsequent to ischaemia, the presence or absence of oestradiol in allocated groups was expected to yield valuable information. Accordingly, it was expected that cyclic female animals would present with less neural tissue damage than male and acyclic female animals to which the same method of experimental cerebral ischaemia was applied.

Cerebral tissue injury analysis is important in determination of the severity of induced ischaemia and can be quantified through various techniques, the staining of mitochondria being a preferred method. Between experimental groups, it is an indicator of hormonal and possible thrombotic and inflammatory influences on the severity of ischaemia; within groups, it is an indicator of hormonal and possible thrombotic and inflammatory influences on the efficacy of regeneration after ischaemia. Tissue injury is widely analysed through the staining of fresh neural tissue sections with 2,3,5-triphenyltetrazolium chloride (TTC), a marker for mitochondrial function (Bederson *et al.* 1986), which normally colourless, is reduced by succinate dehydrogenase in mitochondria of surviving tissue to a red formazan product (Preston & Webster 2000). Thus the areas which do stain are unaffected by ischaemia and the areas which remain unstained are

areas of marked infarction or tissue injury. Serial photographs are ordinarily taken of the neural sections and the unstained areas physically measured to calculate the volume of tissue injury. However, with the hyperglycaemic two-vessel occlusion model employed in this study, unstained neural tissue is sparse (Figure 4.1), as expected in an adult model of mild incomplete global cerebral ischaemia. Clear areas of tissue damage could thus not be confidently measured, necessitating the employment of an alternative method for neural tissue injury determination. Further research revealed that the TTC stain can be extracted from stained neural sections (Preston & Webster 2000, Xue *et al.* 2004) with an ethanol/dimethylsulphoxide (EtOH/DMSO) solvent. The absorbance of the extract is then measured and the values for experimental animals and their matched controls are then entered into an equation to determine tissue injury.

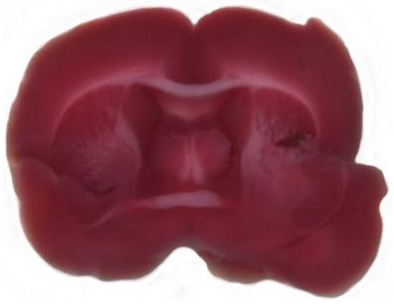


Figure 4.1. Coronal section of neural tissue, stained with TTC, indicating the sparseness of unstained neural areas which are of consequence in the employed model of transient cerebral ischaemia. Clear areas of tissue damage can therefore not be confidently measured, necessitating an alternative method of tissue injury determination

4.2. Materials & Methods

4.2.1. Colorimetric Analysis of Neural Tissue Injury

Whole brains were removed from each individual animal at the termination intervals defined in Chapter 3 (0h, 2h, 24h and 48h). Upon removal, the cerebellum and olfactory bulbs of each brain were carefully dissected off and discarded. The cerebrum was then weighed and the volume determined before being orientated in an adult rat coronal brain matrix (Wirsam Scientific & Precision Equipment Pty Ltd., Johannesburg, South Africa) (Figure 4.2) submerged in chilled 0.1M phosphate buffered saline (PBS) and sectioned into 2mm coronal sections. To quantify ischaemic damage, sections were stained in a flat-bottomed, sealed container with 0.2% TTC

(Sigma-Aldrich®, Kempton Park, South Africa) in 0.1M PBS at a pH of 7.40 in a laboratory with temperature regulated at 19-23°C. The period for staining was optimised at 3.5h to ensure both clear demarcation of viable versus ischaemic tissue and for penetration of the stain through a 2mm section. During staining, the container was gently agitated every 5min to ensure even distribution of stain throughout the container (Joshi *et al.* 2004).

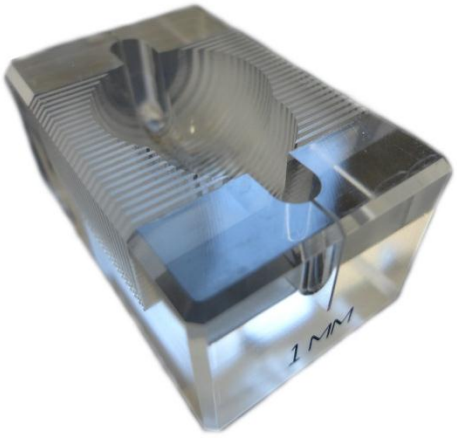


Figure 4.2. Brain matrix used to make 2mm coronal slices of cerebrum. To ensure evenly sliced sections, several blades were positioned in series in every second slot and a single slicing action was employed. Blades were removed individually and neural sections then transferred directly to the staining container

After 3.5h, the TTC solution was removed from the container and the sections washed in two changes of 0.1M PBS with duration of 1min each. A solvent of 50:50 EtOH/DMSO (Sladowski *et al.* 1993) was added to the container at 20ml/g of tissue (Xue *et al.* 2004) and allowed to extract the TTC formazan product from the tissue for 25h in a dark cupboard. This period of extraction was determined sufficient to dissolve and redistribute the tissue formazan throughout the contents of the container in the laboratory. At the conclusion of the 25h extraction, the container was briefly shaken and a small amount of the extract drawn out for absorbance measurement of each sample (Preston & Webster 2000, Xue *et al.* 2004). Six repeats per brain of 200µl per well in a 96-well plate – 10 times dilutions of extract (20µl) in EtOH/DMSO solvent (180µl) – were measured with a BioTek® Epoch Microplate Spectrophotometer (Analytical & Diagnostic Products, Roodepoort, South Africa) set at the optimal wavelength of 485nm (Figure 4.3).

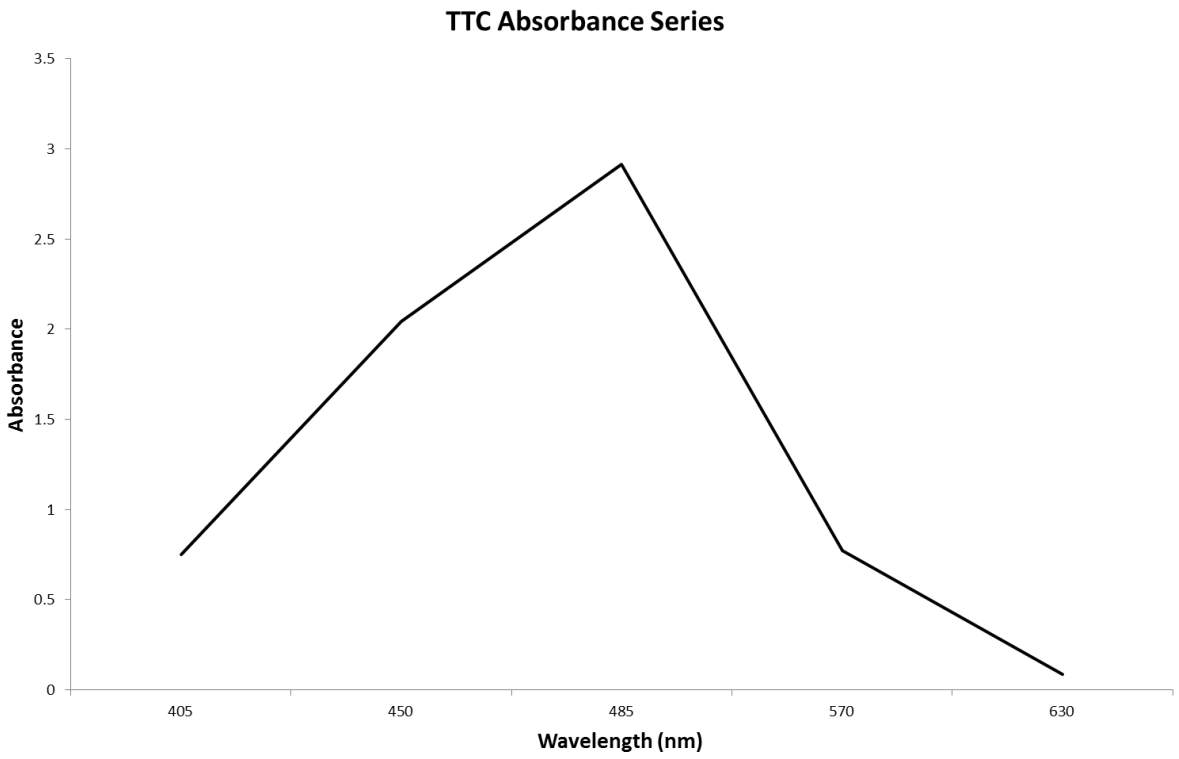


Figure 4.3. Extracted TTC absorbance series. Optimal absorbance wavelength for measurement of TTC extracted from neural tissue with EtOH/DMSO was determined to be 485nm

More intense absorbance (higher value) is indicative of greater mitochondrial viability and less intense absorbance (lower value) is indicative of mitochondrial loss of viability due to cerebral ischaemia. The percentage of tissue injury for each experimental animal in the group was calculated against each control animal within their respective group and statistical analyses performed. The percentage of tissue injury was calculated using the equation (Preston & Webster 2000, Xue *et al.* 2004):

$$\% \text{ Tissue Injury} = 100 \times [1-(\text{Absorbance}_{\text{Injury}}/\text{Absorbance}_{\text{Control}})]$$

4.3. Results & Discussion

Experimental cerebral ischaemia was successfully induced utilising the hyperglycaemic two-vessel occlusion model. Tissue injury was evident in all experimental animals when compared to group-matched control animals. Statistical analysis was performed using classes of group and time as the information related to gender and oestradiol levels are nested within the groups. Variability within the tissue injury model was calculated to be 63% and the p-value obtained for the model as a whole was <0.0001. Groups are again as follows: males (MM), cyclic females (MF) and acyclic females (OVX). Each group was subdivided into termination times: 2h, 24h and 48h post-reperfusion which were compared to their relevant controls: 0h pre-ischaemia to determine tissue injury.

In Figure 4.4, where the interaction between group and time ($p<0.0001$) were taken into account, it is clear that at 2h post-reperfusion, neural tissue injury in MM-2 (21.045%) is significantly higher than that in MF-2 (14.798%) which is also significantly higher that the injury in OVX-2 (11.621%). Importantly at 24h post-reperfusion, there were no significant differences in tissue injury found between MM-24 (21.348%) and OVX-24 (22.864%), and both were significantly higher in percentage injury than MF-24 (2.780%), validating the hypothesis that oestrogen does indeed play a role in tissue survival subsequent to ischaemic injury at the point when maximal injury is expected. By 48h post-reperfusion, the same trend as with the 2h subgroups is seen, in that MM-48 (29.299%) displays significantly more injury than MF-48 (10.071%) which is also significantly higher in injury percentage than OVX-48 (5.911%). MM-48 displays the highest level of neural tissue injury amongst all group-time comparisons and MF-24 displays the lowest level of tissue injury, closely followed by OVX-48.

When only group ($p<0.0001$) effect was accounted for in statistical analysis (Figure 4.5) it was found that MM (23.897%) displays significantly more neural tissue injury overall than OVX (13.466%) which in turn also displays significantly more neural tissue injury overall than MF (9.217%). Therefore, though the lack of oestrogen in acyclic females definitely lessens neuroprotection, there is still some degree of protection from the insult of mild cerebral ischaemic injury when compared to males. In the statistical model where only time ($p=0.6827$) effect was accounted for, no significant differences were identified, indicating that the pattern of injury across groups at specific time points were not significantly different from one another.

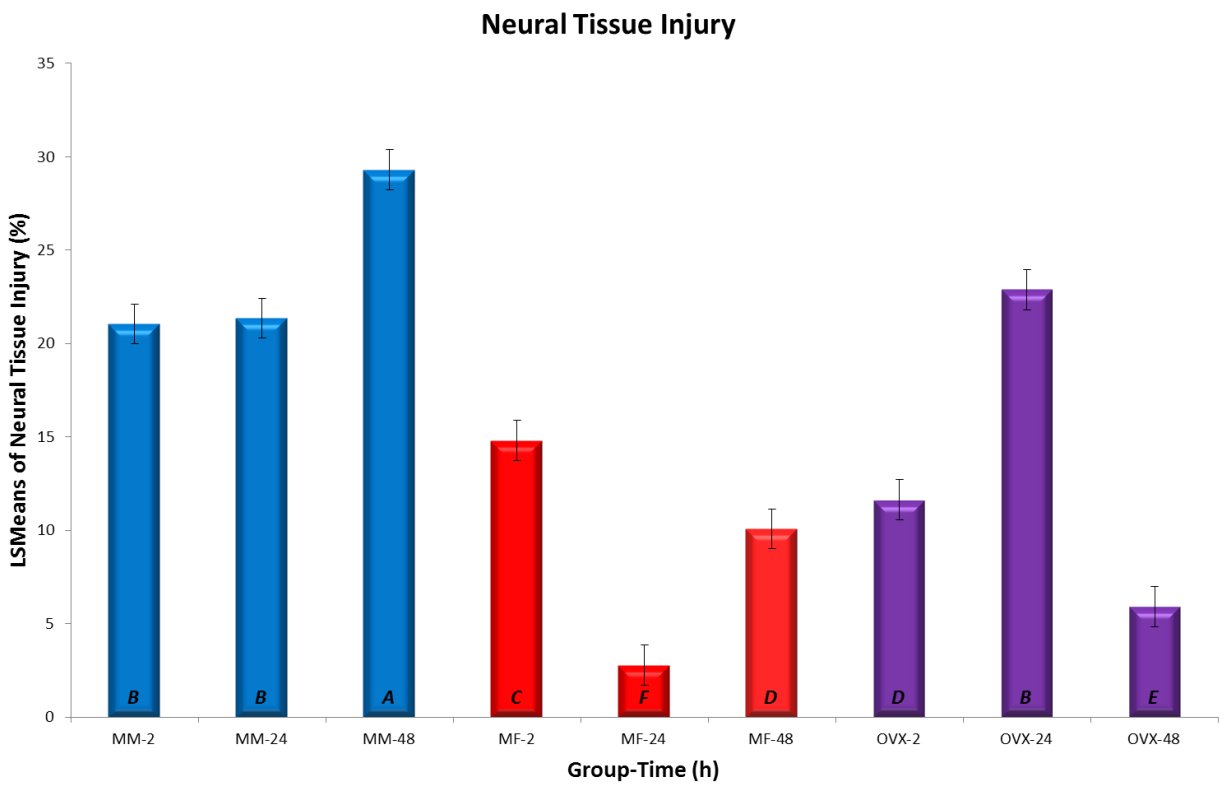


Figure 4.4. Percentage neural tissue injury analysis where group and time interaction is accounted for. Absorbance values per animal in each subgroup were used to calculate neural tissue injury against absorbance values of animals allocated as controls to that specific group. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 63% and the p-value obtained was <0.0001. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females. Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

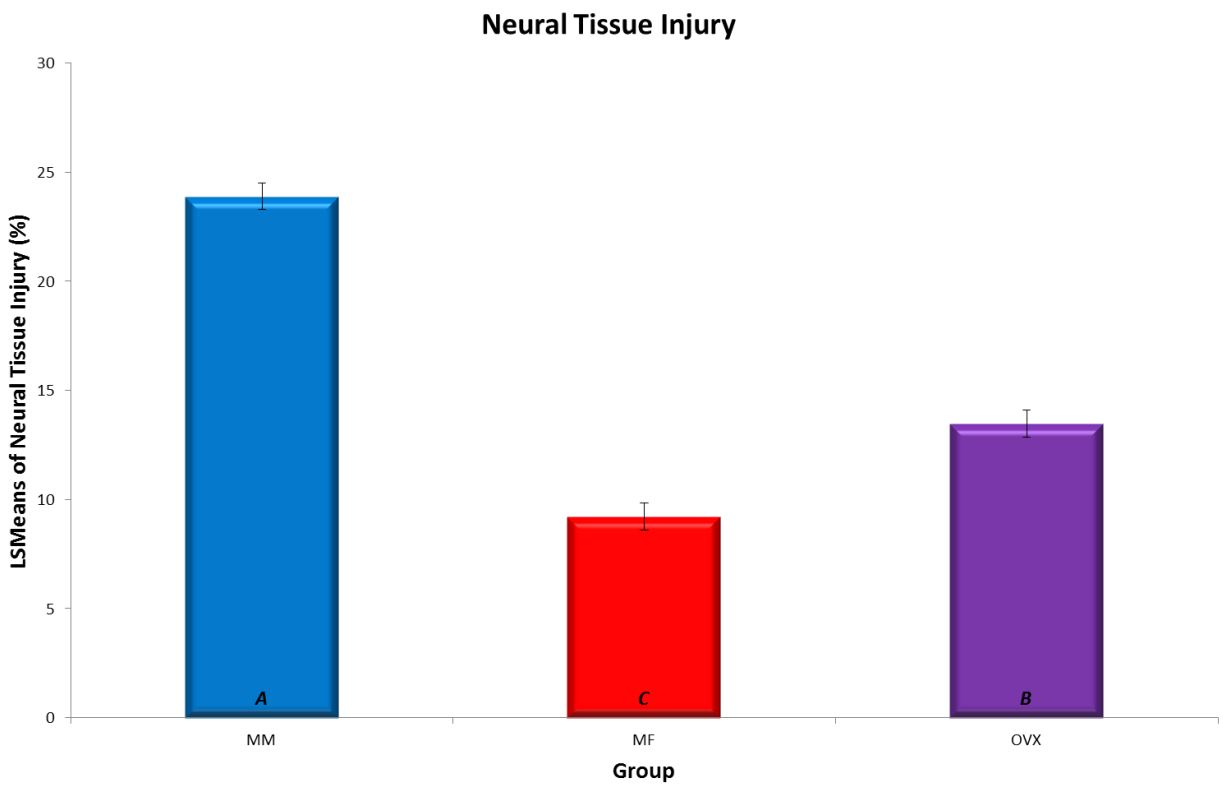


Figure 4.5. Percentage neural tissue injury analysis where group effect is accounted for. Italicised letters of different value at the base of bars indicate statistical significance at a confidence level equal or better than 95%. Variability within the model was calculated to be 63% and the p-value obtained was <0.0001. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females

The ischaemic cascade elucidated in Chapter 2 leads us to expect a series of events subsequent to cerebral ischaemia. Oxidative stress peaks rapidly after cerebral hypoperfusion and lowers progressively within hours. Haemostatic activation takes place within minutes and thus inflammation should be evident very early. Inflammation indeed is shown to peak early in the cascade and progressively lessens after a number of hours, with minimal inflammation still present at a few days. Dysfunction of the BBB takes place in two phases, the second being substantially higher than the first: the initial peak is evident within hours of ischaemia and the second peak, which is apparent within days, is most likely the point where secondary neural tissue injury becomes apparent.

From the neural tissue injury results in Figure 4.4, it can therefore be deduced that subsequent to cerebral ischaemia male neural tissue progressively deteriorates from 2h to 48h due to the absence of oestrogen and thus an inability to curb the inflammatory response and suppress apoptosis by the 24h point. Cyclic female neural tissue presents with initial or primary injury at 2h though less damage is apparent than in males of the same subgroup. By 24h regeneration variables seem to be in play, with oestrogen possibly compensating in some way to curb the peak in inflammation and initiate recovery by stalling apoptosis. At 48h, the second and highest peak of BBB dysfunction is conceivably in play, thus secondary neural injury becomes evident in cyclic females. Acyclic females seem to display a smaller degree of initial injury than cyclic females, which could be due to the inherent state of inflammation found in menopausal females – termed thrombotic preparedness, which is accompanied by impaired fibrinolytic potential (Gharacholou & Becker 2009, Pretorius *et al.* 2010) and changes the haemostatic profile from normal – thus the inflammatory shock of ischaemic injury to the haemostatic system may be lessened. By 24h, acyclic females present with greatest neural tissue injury, due to the absence of normal oestrogen levels and therefore the inability to suppress apoptosis and initiate recovery. Tissue injury in acyclic females is slightly more severe than that displayed in males at the same time. At 48h however, the neural tissue of acyclic females seems to have recovered to a great extent, almost returning to control levels. It is conceivable that the return to a state of “normal” thrombotic preparedness in acyclic females lessens the effect of the second peak of BBB dysfunction, resulting in less secondary injury than evidenced in cyclic females at the same time. Figure 4.5 supports this to some extent as acyclic females display significantly less overall neural tissue injury than males, thus without the neuroprotective ability of oestrogen acyclic females are still protected to an extent from the consequences of mild insult through mechanisms which may be linked to inherent inflammation.

Chapter 5: Analysis of Neural Tissue Protein Content

5.1. Introduction

Sex hormones influence brain development, differentiation and neuronal functions (Drača 2009, Manthey & Behl 2006). Intact, premenopausal or cyclic females present with a lower incidence of ischaemic stroke than males; but ovariectomised, postmenopausal or acyclic females are more prone to ischaemic stroke than males. It is also accepted that ischaemic stroke risk increases in both sexes with age (Braeuninger & Kleinschnitz 2009, Gibson *et al.* 2005, Wolf 1990) and that sex hormones must have a role in neuroprotection and the decline thereof with age. Analysis of neural tissue protein content may serve to strengthen data related to neural tissue injury at different time points, providing information related to neural degeneration and later regeneration.

Administration of oestrogen prior to experimental cerebral ischaemia is known to protect males from ischaemic injury (Hawk *et al.* 1998). Oestrogen also protects acyclic females from ischaemic injury when administered upon ovariectomy (Alkayed *et al.* 2000, Bake & Sohrabji 2004, Liao *et al.* 2001, Selvamani & Sohrabji 2010, Simpkins *et al.* 1997, Suzuki *et al.* 2009), but does not protect older acyclic females who have been hypo-oestrogenic for a period (Bake & Sohrabji 2004, Selvamani & Sohrabji 2010, Suzuki *et al.* 2009) nor cyclic females further than endogenous oestrogen is capable (Toung *et al.* 1998). Ischaemic injury has been found to increase oestrogen receptor alpha (ERα) in the cerebral cortex without influencing oestrogen receptor beta (ERβ) expression and this is thought to be one of the mediators of oestrogen's neuroprotection against ischaemia (Dubal *et al.* 2001, Suzuki *et al.* 2007, Suzuki *et al.* 2009). In older acyclic females ERα is already downregulated (Jezierski & Sohrabji 2000, Jezierski & Sohrabji 2001, Selvamani & Sohrabji 2010) but these receptors must be present in much the same concentration in both males and cyclic females for neuroprotection to be evident in research results of exogenous oestrogen administration.

Approximately 8% of the rat brain is of protein composition, the majority (up to 78%) is made up of water and the remaining 14% is constituted of carbohydrate, lipid, organic and inorganic substances (McIlwain & Bachelard 1985). Protein itself yields both a soluble cytoplasmic fraction and an insoluble nuclear fraction. ERα is predominantly a cytoplasmic receptor but is also found

to localize in mitochondria and nuclei both in their unbound and oestradiol-bound states in several tissue types (Ivanova *et al.* 2010). Because ERα is principally a cytoplasmic protein, it is essentially a soluble protein, therefore quantification of soluble proteins and not insoluble or total protein would be sufficient to provide information regarding oestrogen's role in neural tissue insult, evidence of ERα down- and/or upregulation upon insult and the possible downregulation of protection seen in acyclic females. There are, of course, many soluble proteins in addition to ERα present in the neural tissue of animals. Cerebral ischaemic insult will therefore cause an upregulation of some proteins and a downregulation of others, but the quantification of overall soluble neural tissue protein will provide an indication of whether males and females differ significantly in protein content before and/or after cerebral ischaemia. Furthermore, if proteins such as ERα are downregulated after ovariectomy in acyclic females, it is of interest to determine whether the protein expression of ERα, for example, is still upregulated by ischaemia; evidencing a protein content difference between acyclic females, cyclic females and males.

Researchers have found total protein content of neural tissue to be identical in young and adult animals, whereas in aged animals there are considerable increases (Nagy et al. 1981). As animals age, their number of neurons is reduced, protein synthesis and turnover (Kristofiková et al. 1991, Nagy 1985) as well as water content decrease (Boer et al. 1984, Kiyota 1959, Kristofiková et al. 1991). The increase in the total protein content of older animals may be due to the decrease in the water content because neural tissue protein content is expressed with respect to wet weight of the tissue (Kiyota 1959, Kristofiková et al. 1991). The age-matching of animals in this study does however serve to account for impediments in the analysis of brain protein content which could be experienced by for instance using sexually senescent and thus older females rather than age-matched, ovariectomised ones.

5.2. Materials & Methods

Upon whole brain removal from each individual animal at the termination intervals described in Chapter 3 (0h, 2h, 24h and 48h), cerebellums and olfactory bulbs were carefully dissected off and discarded (Figure 5.1). The cerebrums were then individually weighed and their volume determined before sectioning them for neural tissue injury determination. This allowed for brain density determination of individual animals. Brains were subsequently sliced and stained with 2,3,5-triphenyltetrazolium chloride (TTC) which was later extracted with an ethanol/dimethylsulfoxide (EtOH/DMSO) reagent as elucidated in Chapter 4 for colorimetric

determination of mitochondrial function and thus calculation of neural tissue injury. Subsequent to extraction, the tissue was individually homogenised by fine chopping with a sharp scalpel and 1g thereof vortexed with and then frozen in a lysis buffer consisting of 1ml 0.1M tris(hydroxymethyl)aminomethane/hydrochloride (Tris/HCl) containing 0.001% sodium dodecyl sulphate (SDS) at a pH of 8.00. The samples, collected in separate vials for each animal, were stored at -20°C prior to analysis.

Figure 5.1. Dissection prior to weighing and determining the volume of relevant neural tissue. Whole brains were removed, cerebellum and olfactory bulbs dissected off, cerebrum weighed and volume determined for density calculation

For purposes of differentiating male, cyclic and acyclic female soluble protein content prior and subsequent to experimental cerebral ischaemia, analysis of previously frozen neural tissue was undertaken using the Bradford method – a protein assay widely used for rapidly processing large numbers of samples. Since the developed protein-dye complex has a high extinction coefficient, the method ensures great sensitivity in protein measurement. The binding of dye to protein takes place rapidly (within 5min) and the dye-protein complex remains dispersed in solution for up to an hour, thus requiring less critical assay timing than alternative methods (Bradford 1976, Duhamel et al. 1981). The commercially available Bio-Rad protein assay is based on the Bradford dye-binding method (Bradford 1976) and involves a simple and accurate colorimetric assay process for determining the concentration of total solubilised protein. It is a dye-binding assay in which a differential colour change of Coomassie brilliant blue G-250 dye occurs in response to protein concentration variations. The assay involves the addition of the acidic dye to a protein standard and a protein sample, with subsequent absorbance measurement using a spectrophotometer. A standard curve must be generated to provide a relative measurement of protein concentration. Standard curves are generated for the protein

standard as well as for particular reagents added to the protein sample (e.g. solubilising reagents and detergents) to ascertain whether they interfere with the assay. Should a significant difference in dye colour be observed between the protein standard and the reagent, correction for the reagent is recommended to yield accurate results of protein content within the protein sample of interest (Bio-Rad Laboratories 2012, Friedenauer & Berlet 1989).

5.2.1. Cerebral Tissue Protein Content Analysis

For analysis, samples were thawed, the 15ml tubes filled up to the 10ml mark with 10% SDS, vortexed and placed in a 37°C waterbath to solubilise overnight. Subsequent to examination of the solute, samples were transferred to 50ml tubes, filled up to the 15ml mark with distilled water (dH_2O) and reconstituted in the larger surface area tube. These were placed into a 60°C waterbath to further solubilise the tissue for protein determination. Soluble protein content was then determined using the Bradford method, with bovine serum albumin (BSA) as standard. The Bio-Rad Protein Assay Kit II was used, which includes dye reagent concentrate and lyophilized BSA standard (Bio-Rad Laboratories Pty Ltd., Parklands, South Africa).

The dye reagent was prepared by diluting one part of the dye reagent concentrate with four parts of dH_2O. Dilutions of the BSA protein standard were made to provide a relative measurement of protein concentration. The lyophilized BSA standard of 1.29mg/ml was reconstituted in a 20 times dilution with dH_2O and mixed until dissolved to obtain a stock BSA standard. To compensate for interference, relevant controls were used to avoid over- or underestimation of protein content. To compensate for overestimation of protein content, it was necessary to generate values for a solvent-dye blank; whereas to rule out underestimation, factors (e.g. detergents) within the sample which may reduce dye binding – possibly through competition with the dye for protein-binding – must be identified (Compton & Jones 1985, Friedenauer & Berlet 1989).

Serial dilutions of the stock BSA standard (equal to 100µl in combination with dH_2O) were used to generate a standard curve by adding dye reagent (50µl) to each well and determining the absorbance values of these. A standard curve was also generated for serial dilutions of SDS to provide relevant control for protein concentration measurement. There is however a threshold (approximately 40µg/ml of reagent) below which SDS-dye does not develop colour on its own

and responses of the method to the protein in solution with SDS is equalised, and this maximal sub-threshold amount must be empirically determined for each lot of SDS (Duhamel et al. 1981). After generation of standard curves (with BSA-dye and SDS-dye in triplicate) and accounting for interference compensation, triplicates of each solubilised protein sample (5µl) was aliquoted into a 96-well plate with corresponding volumes of dH_2O (95µl) and dye reagent (50µl), to a total of 150µl in each well. Sample absorbance was then read at a wavelength of 630nm using a BioTek® Epoch Microplate Spectrophotometer (Analytical & Diagnostic Products, Roodepoort, South Africa). Protein concentrations of the neural tissue protein samples were calculated using the slope value from the standard curve (Figure 5.2) which was determined to be 4.9554. The protein concentration is calculated by solving for *x*, where *y* is the absorbance reading obtained from each of the protein samples, *m* is the slope of the standard curve and *b* is zero as this would be the y-intercept of the line when entered into the equation:

$$y = mx + b$$

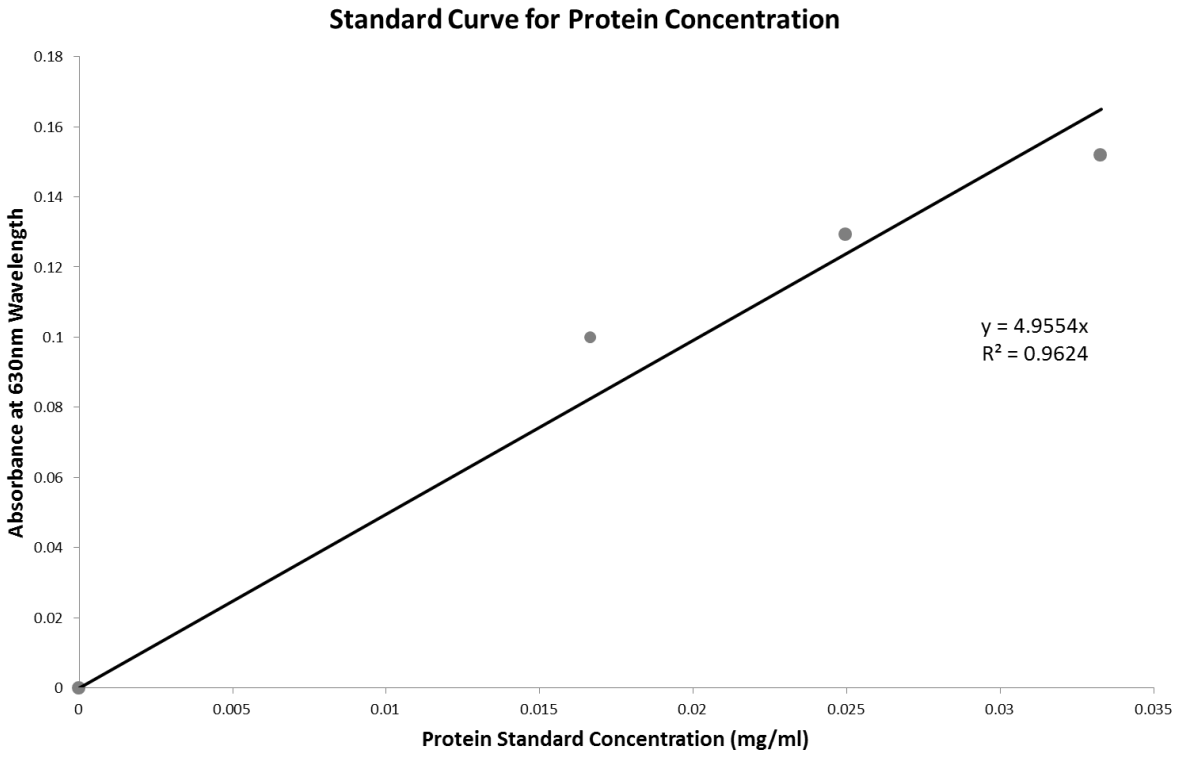


Figure 5.2. Standard curve for protein concentration calculation of neural tissue samples. The curve was generated for BSA concentrations with SDS added for interference compensation purposes. Variability was calculated as 96% and the slope to be used for calculation of protein sample concentrations was determined to be 4.9554

5.3. Results & Discussion

Brain density for each animal was quantified to determine whether it would have any effects on neural tissue protein analysis at a later stage. Soluble neural tissue protein was successfully extracted and quantified for each animal. Statistical analysis was performed using classes of group and time as the information related to gender and oestradiol presence are nested within the groups. Variability within the protein model was calculated to be 21% and the p-value obtained for the model as a whole was 0.2125. Groups are again as follows: males (MM), cyclic females (MF) and acyclic females (OVX). Each group was subdivided into termination times: 0h pre-ischaemia, 2h, 24h and 48h post-reperfusion.

With regards to brain density, statistical analysis revealed that MF-0 was significantly higher in density than all other group and time ($p=0.0002$) interaction pairings, rendering MF (0.936g/cm^2) significantly higher in brain density than MM (0.825g/cm^2) and OVX (0.778g/cm^2) when only group ($p=0.0067$) effect was accounted for. However, there was no distinct pattern in brain density alteration within groups at stipulated termination times. In Figure 5.3, it is shown that when only time ($p=0.0057$) effect was accounted for statistically, brains at 2h (0.725g/cm^2) post-reperfusion were significantly lower in brain density than all other termination times (0h 0.929g/cm^2, 24h 0.856g/cm^2, 48h 0.875g/cm^2). This may be due to higher water content manifested as oedema (increasing the tissue volume therefore decreasing its density) which would be evident early on subsequent to ischaemic insult and present when inflammation is at a peak.

The differences in soluble neural tissue protein concentration (Figure 5.4) were not specifically significant when group and time ($p=0.4559$) interaction was accounted for. The increase in protein content subsequent to ischaemia could thus not be confirmed and the loss of neural tissue due to injury cannot be associated with neural tissue protein concentrations. When only time ($p=0.6828$) effect was accounted for, no time-dependent significance was found for the model. However in Figure 5.5, when only group ($p=0.0264$) effect was accounted for, OVX (0.0151mg/ml) displayed significantly lower protein content than MM (0.0163mg/ml) and MF (0.0164mg/ml). This confirms that soluble protein is present in much the same concentration in males and cyclic females of similar age and also that the overall protein and perhaps ERα receptors are downregulated in acyclic females following ovariectomy.

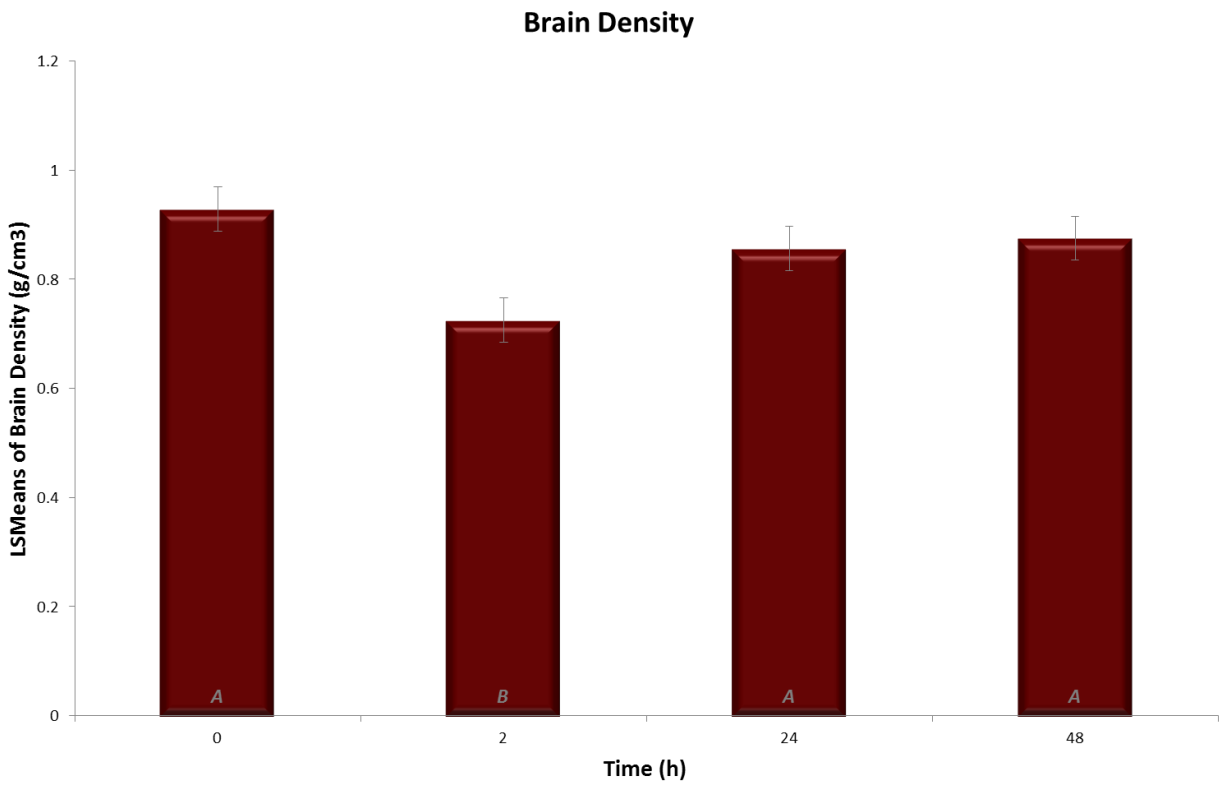


Figure 5.3. Brain density analysis where time effect is accounted for. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 49% and the p-value obtained was 0.0057. Time 0 = 0h control or pre-ischaemia, Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

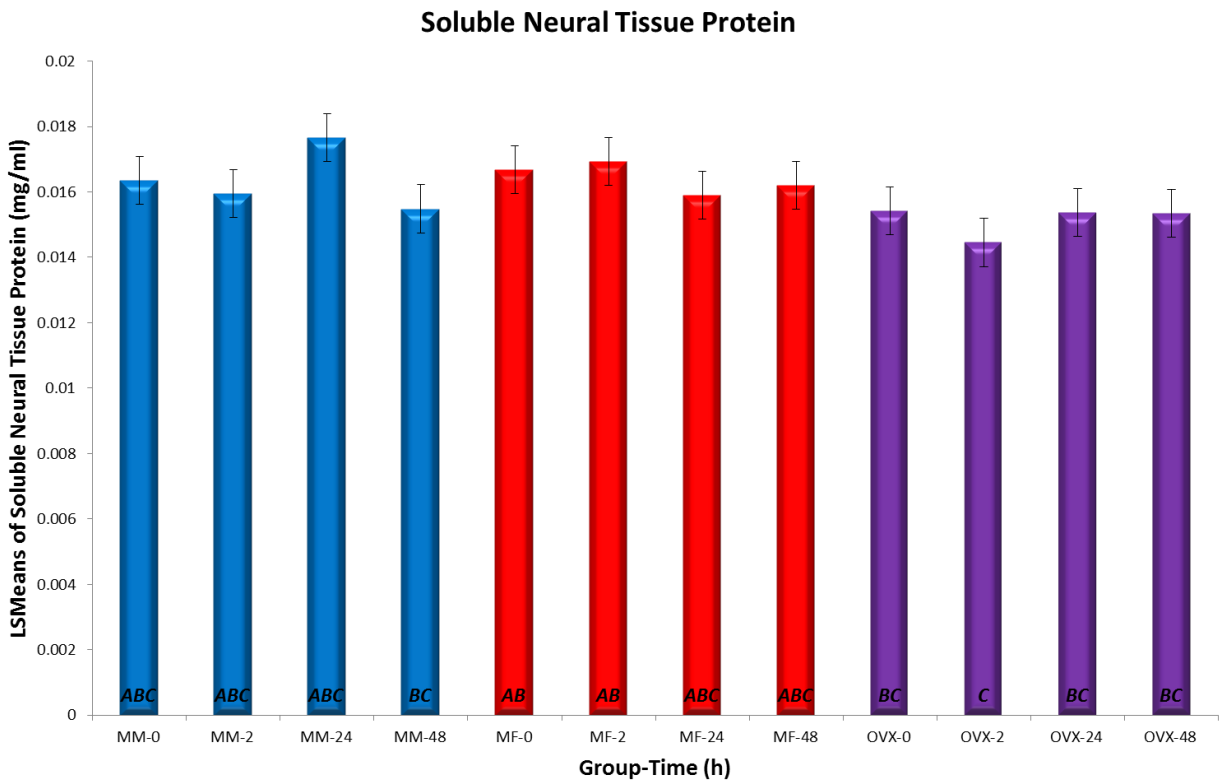


Figure 5.4. Soluble neural tissue protein analysis where group and time interaction is accounted for. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 21% and the p-value obtained was 0.4559. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females. Time 0 = 0h control or pre-ischaemia, Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

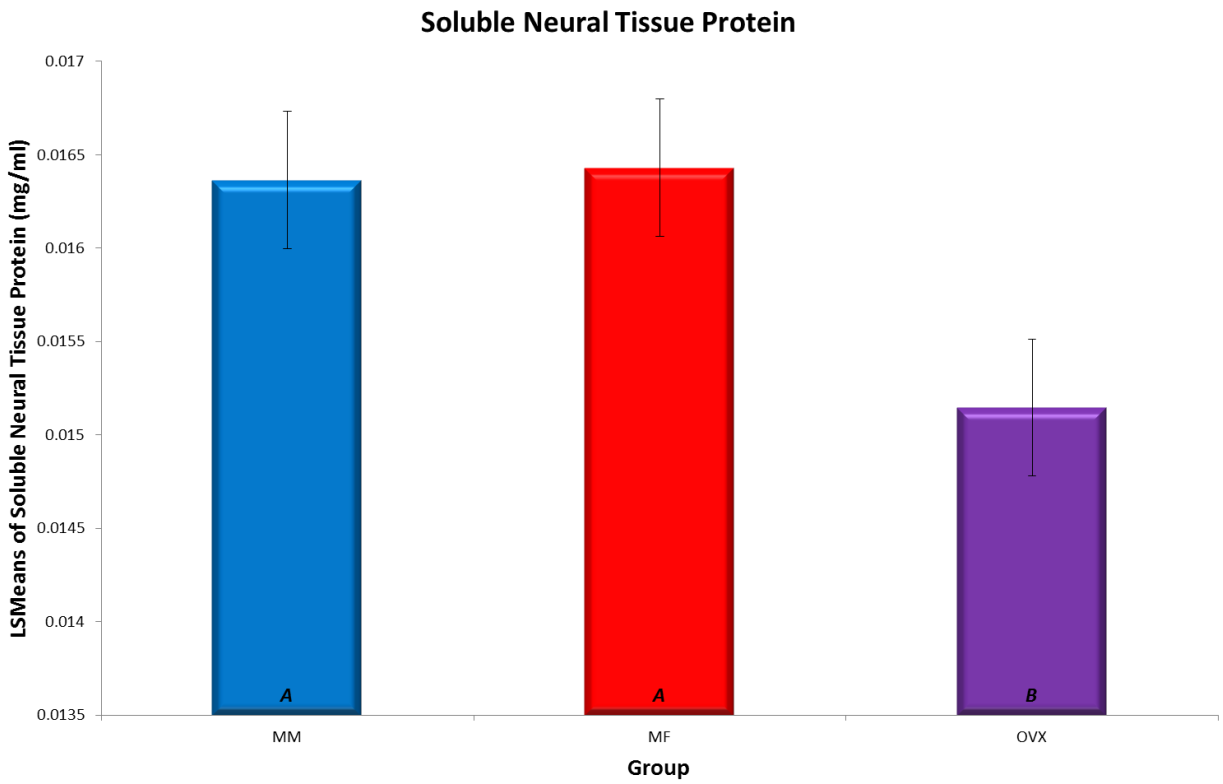


Figure 5.5. Soluble neural tissue protein analysis where group effect is accounted for. Time effect was determined to have no significant effect between groups. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 21% and the p-value obtained was 0.0264. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females

Correlation analysis between brain density and soluble protein revealed an insignificant correlation; therefore brain density had no effect on neural tissue protein content, or vice versa. Males and cyclic females were found to have similar protein levels overall (Figure 5.5), thus affording strength to the reasoning that proteins including ERα must be present in much the same concentration in both males and females for hormone treatment to protect males from ischaemic injury as endogenous oestrogen protects cyclic females. Protein in acyclic females was shown to be significantly downregulated (Figure 5.5), a consequence of ovariectomy, when compared to males and cyclic females. This affords strength to results of research by other groups who showed that ERα, specifically, is downregulated subsequent to ovariectomy. Males and females were, however, not found to differ significantly in protein content before and/or after cerebral ischaemia, thus upregulation of proteins such as ERα subsequent to ischaemic injury was not clearly evident. Correlation analysis between soluble neural tissue protein and neural tissue injury results, on the other hand, revealed a significantly negative correlation; therefore the level of neural tissue injury is inversely proportional to soluble neural tissue protein levels. This may serve to indirectly confirm that neural proteins, including ERα, are upregulated when neural injury occurs.

Chapter 6: Analysis of 17β-Oestradiol Levels

6.1. Introduction

Research suggests that cyclic females present with smaller cerebral infarcts and thus lower levels of neural tissue injury than their male counterparts (Alkayed *et al.* 1998, Braeuninger & Kleinschnitz 2009). Additionally, high endogenous levels of oestrogen during the oestrus cycle have been shown to correlate with lesser degrees of neural tissue injury in females (Braeuninger & Kleinschnitz 2009, Carswell *et al.* 2000). In ovariectomised or acyclic females however, this advantage over males is abolished due to the loss of endogenous oestrogen, and consequences are thus more severe than in sexually young animals (Alkayed *et al.* 1998, Alkayed *et al.* 2000, Davis *et al.* 1995, Drača 2009, Gibson *et al.* 2005, Hawk *et al.* 1998, Liao *et al.* 2001, Park *et al.* 2006, Selvamani & Sohrabji 2010, Simpkins *et al.* 1997).

17β-Oestradiol is the principal circulating oestrogen which offers protection from ischaemic damage. Acting through mechanisms of the classical nuclear oestrogen receptors, physiological levels of oestradiol mitigate brain injury by reducing neural tissue injury after experimental cerebral ischaemia through the attenuation of apoptosis markers (Jia *et al.* 2009, Liao *et al.* 2001, Prewitt & Wilson 2007, Suzuki *et al.* 2009). Ischaemic injury has been found to increase the expression of oestrogen receptor alpha (ERα) in the cerebral cortex without influencing oestrogen receptor beta (ERβ) expression. Thus it is believed that ERα re-expression after ischaemic injury mediates the profound neuroprotection by 17β-oestradiol against ischaemic consequences (Dubal *et al.* 2001, Suzuki *et al.* 2007, Suzuki *et al.* 2009). In essence, oestradiol targets neural cells by mechanisms which serve to stabilise neurotransmission, inhibit apoptosis, reduce cerebral oedema and exert anti-inflammatory and antioxidant effects (Drača 2009, Manthey & Behl 2006).

It is well understood that initial post-ischaemic inflammation strongly contributes to the extent of cerebral injury, and that 17β-oestradiol may exert protection through anti-inflammatory actions since the presence of initial inflammation is negatively correlated with serum oestradiol levels (Suzuki *et al.* 2009, Vegeto *et al.* 2008, Wang *et al.* 2007). Inflammation produces local thrombosis and thrombosis can amplify inflammation, thus these processes are deeply interconnected (Libby & Simon 2001, Myers & Wakefield 2005, Stewart *et al.* 1974). Oestradiol

is known to lower coagulation factors and proteins, thus also encompassing a role in thrombosis regulation (Bailey *et al.* 2009, Wong *et al.* 2008), the alteration of which may result in cerebral ischaemia or the increased severity thereof.

Chemical analysis of plasma 17β-oestradiol levels is necessary to ascertain hormonal differences between the groups of experimental animals which may have influenced the progression of factors related to neural tissue injury in Chapter 4.

6.2. Materials & Methods

Laboratory animal references record standard rat plasma volume ranges from 3.1-3.9% of blood volume, and haematocrit ranges from 34-57% of packed cell volume (Kohn & Clifford 2002, Probst *et al.* 2006, Ringler & Dabich 1979). Sprague Dawley rats are shown to present a serum yield average ranging from 33-66%, thus standing at approximately 50% of blood volume (Probst *et al.* 2006). Consequently, 1ml of whole blood drawn from a Sprague Dawley rat should yield approximately 500µl of plasma.

Approximately 4ml of whole blood was collected from each rat via cardiac puncture upon termination. For every 100µl of blood drawn from an animal, 11µl of sodium citrate was added to prevent the blood from clotting naturally. The blood was kept at 4°C for a period of no more than 2h (Liao *et al.* 2001) prior to being sent for analyses. Chemical analysis was undertaken at Ampath (Ampath Pathology Laboratory Support Services, Pretoria, South Africa) as they are an accredited laboratory. At least 200µl of serum was required for 17β-oestradiol analysis by chemiluminescence method using a Beckman Coulter UniCel® DxI 800 Immunoassay System. Chemiluminescence technology employs the generation of electromagnetic radiation in the form of light by the release of energy from a chemical reaction. Statistical analysis was performed subsequent to 17β-oestradiol analysis.

6.3. Results & Discussion

Due to gross error at the laboratory support service, the blood of 36 animals was discarded upon transfer from the haematology laboratory to the serology laboratory for 17β-oestradiol analysis. Pooled blood (for ultrastructural analysis) was then sent for analysis to determine one 17β-oestradiol value for each group, however these did not reflect the means of groups for which 17β-oestradiol values were fully available and could not be utilised for statistical analysis due to the lack of mean and standard deviation variable availability. The generation of probable 17β-oestradiol values was attempted from brain colorimetry values by linear, quadratic and other means of correlation; but these could not be unequivocally predicted for the animals.

Groups are again as follows: males (MM), cyclic females (MF) and acyclic females (OVX). Each group was subdivided into termination times: 0h pre-ischaemia, 2h, 24h and 48h post-reperfusion. Owing to this irretrievable loss, the results of 17β-oestradiol analysis could only be statistically compared within termination subgroups of OVX in entirety (0-48h) with class of time only. Variability within this model was calculated to be 65% and the p-value obtained was 0.0018. Furthermore, statistical analysis was performed with class of group for time 0h across MM, MF and OVX. Variability within this model was calculated to be 58% and the p-value obtained was 0.0014. Furthermore, pooled blood analysis values are reported although only for qualitative purposes.

Termination subgroups of OVX provided statistical data of group and time (p=0.0018) interaction for acyclic females which showed the 17β-oestradiol levels (Figure 6.1) for OVX-0 (56.333pmol/l) and OVX-48 (51.333pmol/l) to be jointly significantly lower than 17β-oestradiol levels for OVX-2 (68.167pmol/l) and OVX-24 (72.833pmol/l). These results offer evidence that 17β-oestradiol is upregulated in circulation upon ischaemic insult at times where initial (2h) and maximal (24h) injury are expected, conceivably to be taken up by receptors in the neural tissue which function in neuroprotective mechanisms. When analysing group and time interactions (p=0.0014) across 0h termination subgroups of males, cyclic and acyclic females (Figure 6.2), it was found that MF-0 (169.833pmol/l) displayed significantly higher 17β-oestradiol levels than both MM-0 (68.667pmol/l) and OVX-0 (56.333pmol/l) and that the latter were insignificant of each other. This confirms that upon ovariectomy, acyclic females should to a degree be more susceptible to ischaemic insult consequences than males and that upon insult; injury should be potentiated in acyclic females if 17β-oestradiol is indeed largely neuroprotective.

When plotting the single 17β-oestradiol values of pooled blood for each group and time subset (Figure 6.3) only results of qualitative or display value could be reported as it was not possible to perform quantitative statistical analysis. MM and OVX are shown to be correlative in terms of 17β-oestradiol levels as seen in Figure 6.2 and MF displays higher 17β-oestradiol values overall. Furthermore, when looking solely at acyclic females, values follow the same trend as in Figure 6.1 with OVX-2 (80pmol/l) and OVX-24 (91pmol/l) slightly higher than OVX-0 (79pmol/l) and OVX-48 (68pmol/l). Males also display higher 17β-oestradiol values at MM-2 (97pmol/l) and MM-24 (80pmol/l), though slight, than MM-0 (75pmol/l) and MM-48 (78pmol/l). This may offer further confirmation that age-matched males and acyclic females are comparable in neuroprotective potential. Cyclic females display a similar trend to the other groups, with MF-0 (138pmol/l) and MF-48 (113pmol/l) being at similar levels and both being lower than MF-2 (208pmol/l). MF-24 (87pmol/l) however is not at comparable levels to MF-2 as in other groups when relating 2h and 24h oestradiol levels, but is instead greatly lower than all time-points for the cyclic females.

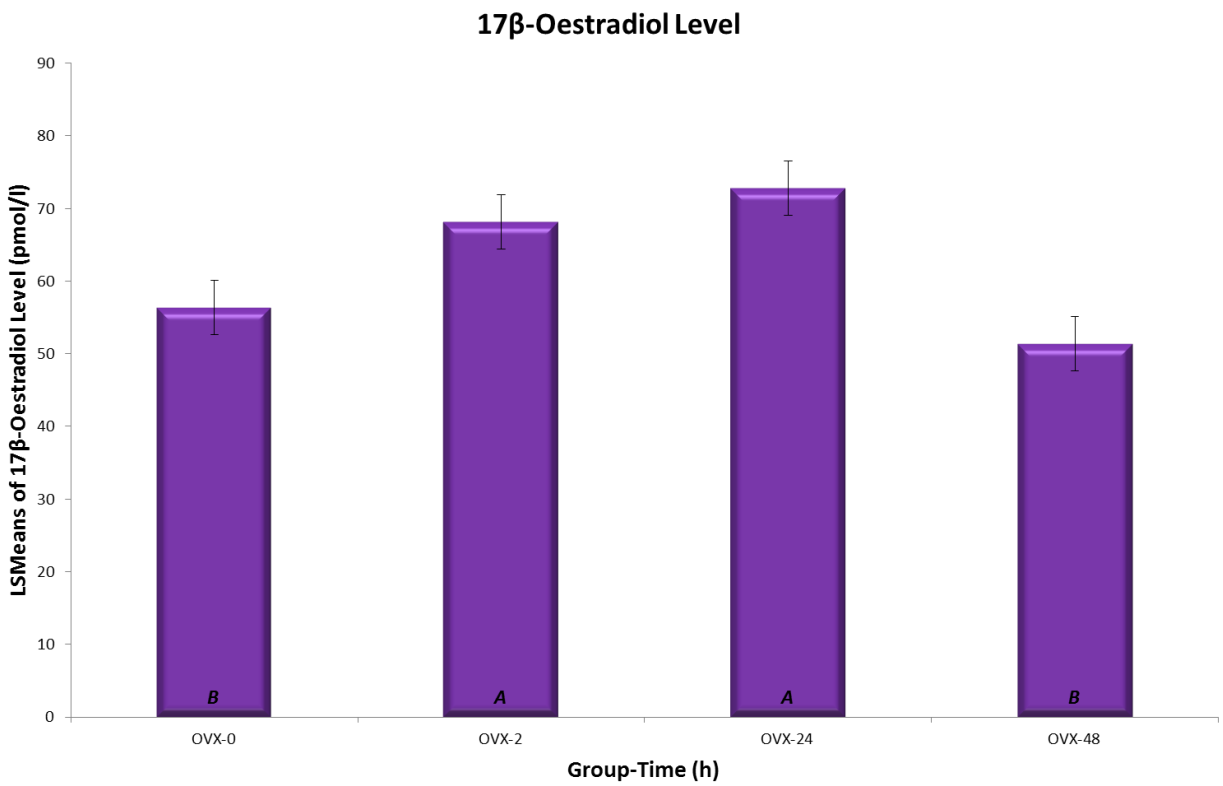


Figure 6.1. 17β-Oestradiol analysis where the group and time interaction in acyclic females of group OVX is accounted for. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 65% and the p-value obtained was 0.0018. Group OVX = Acyclic females. Time 0 = 0h control or pre-ischaemia, Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

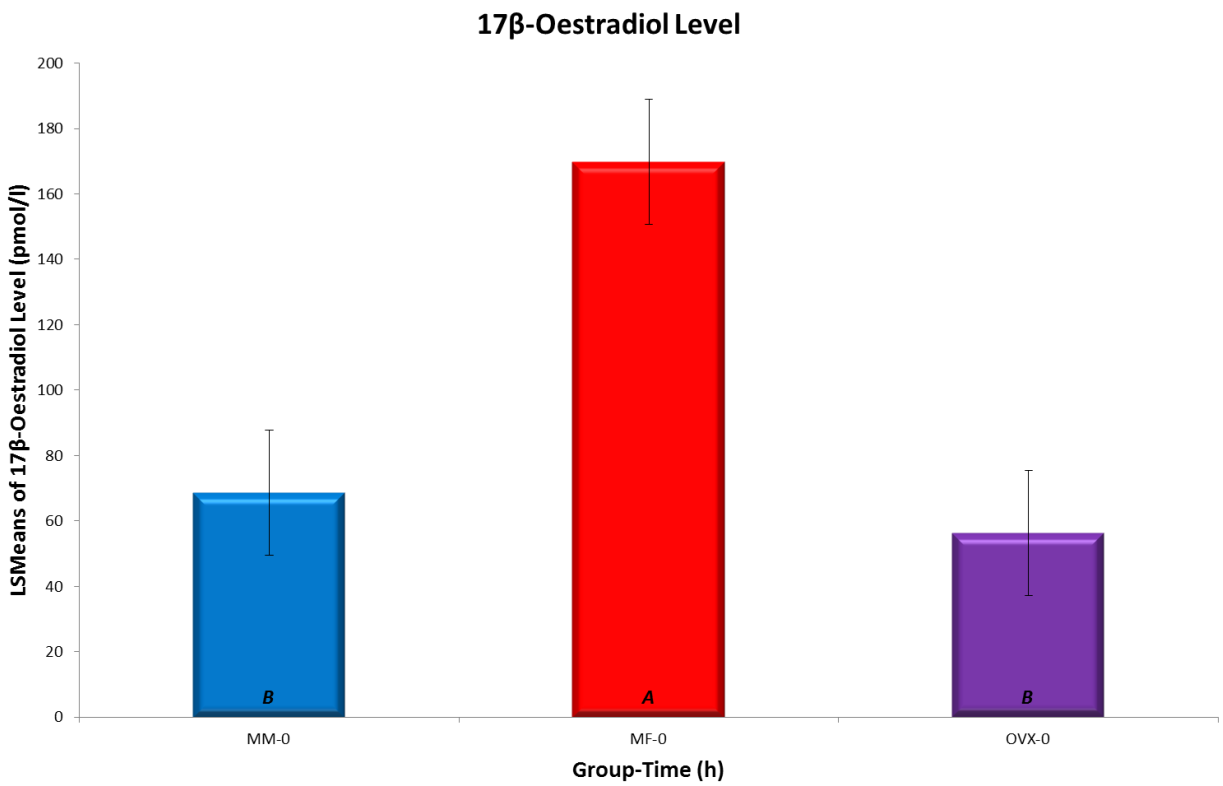


Figure 6.2. 17β-Oestradiol analysis where the group and time interaction is accounted for at 0h pre-ischaemia for each group. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 58% and the p-value obtained was 0.0014. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females

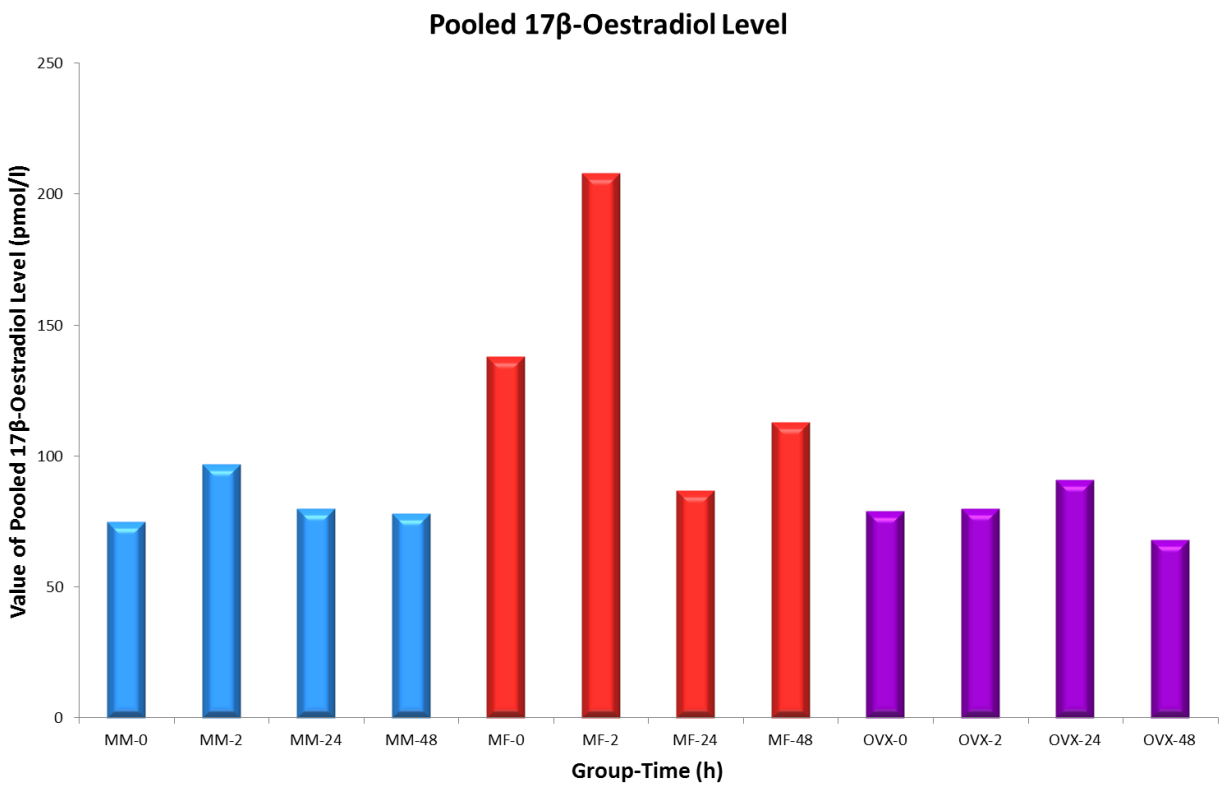


Figure 6.3. Pooled 17β-Oestradiol values where the group and time subsets are displayed. No statistical analysis could be performed as only one value was available per group; therefore no error bars are indicated. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females. Time 0 = 0h control or pre-ischaemia, Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

Overall it would seem that circulating 17β-oestradiol is increased across all groups upon primary injury at 2h (Figures 6.1 and 6.3). In cyclic females, 17β-oestradiol levels are at their lowest for the group at MF-24 and this corresponds to the point where the least neural tissue injury is evident. This may reflect that much circulating oestrogen has bound to ERα in the brain at this time to act in neuroprotection. The neural tissue injury versus 17β-oestradiol level follows the same pattern in acyclic females, where OVX-48 displays the lowest neural tissue injury and lowest 17β-oestradiol levels for that group. In males however, the pattern differs slightly as the lowest 17β-oestradiol level (besides control levels) correlates to the time when maximum neural tissue injury is evident in this group. This may indicate late-stage uptake of oestradiol in the brain to initiate systemic recovery but not necessarily neural regeneration.

Inclusive correlation analysis between oestrogen levels and neural tissue injury revealed a significant negative correlation; therefore the level of neural tissue injury is inversely proportional to oestradiol levels in circulation, confirming that oestrogen is neuroprotective in that high levels of 17β-oestradiol correspond to lesser neural tissue damage overall. Correlation analysis between oestrogen levels and soluble neural tissue protein revealed an insignificant positive correlation; therefore one can assume that circulating oestrogen does not directly affect the presence of soluble neural proteins including oestrogen receptors in the brain, but neural tissue injury indeed does as elucidated by correlation in Chapter 5.

Chapter 7: Analysis of Coagulation Factors and Coagula

7.1. Introduction

Many conditions, including inflammation, thrombotic disease, bleeding disorders, asthma, heart disease, cancer and even HIV/AIDS have been associated with ultrastructural changes in platelet and fibrin structure (Pretorius *et al.* 2009a, Pretorius *et al.* 2009b, Pretorius *et al.* 2009c, Pretorius *et al.* 2011a). Research has demonstrated that ultrastructural analysis is valuable in broadening the knowledge of disease patterns and suggests that information gained through the use of these techniques may enhance treatment regimens (Pretorius *et al.* 2008, Pretorius *et al.* 2010).

Platelets and fibrin play an important role in the process of coagulation through the maintenance of haemostasis. Structurally, platelet surfaces consist of a typical bilayer membrane composed of lipids, proteins and carbohydrates. Surface glycoproteins are essential to their function and play a primary role in their adhesion to exposed subendothelial matrix proteins, interaction with thrombin and exposure of fibrinogen receptors to facilitate aggregation (Herd & Page 1994, Roth 1992). Following activation, platelets change shape, a process mediated by a contractile microtubular system and morphologically characterised by an extension of dendritic pseudopodia (Herd & Page 1994, White 1987). Activated platelets secrete inducers of platelet aggregation, adhere to a site of injury and aggregate to each other in order to form a platelet-rich plug which secures haemostasis. This plug is then stabilized by the formation of fibrin as fibrinogen (its precursor) is activated by binding to activated platelets (Danesh *et al.* 2005, Lowe *et al.* 2004). Fibrin assembly then proceeds in a highly ordered fashion, forming a network which functions to stabilize the primary platelet plug (Wohner 2008).

Cerebral ischaemia is associated with parameters of altered blood coagulation which may lead to the occlusion of blood vessels; including increased thrombin activity, elevated fibrinogen levels, altered fibrin network ultrastructure, increased platelet counts and even resistance to fibrinolysis (Gailani & Renné 2007, Gaston *et al.*1971, Pretorius *et al.* 2011a, Todd *et al.* 1973, Undas *et al.* 2009). Haemostatic coagulation requires mediated cleavage of fibrinogen to fibrin; the ultimate fibrin structure dependent on the concentration of thrombin present during this process of thrombus formation. Non-haemostatic or abnormal coagulation, associated with

bleeding and/or thrombosis, is in large extent, due to abnormal thrombin generation patterns (Muszbek *et al.* 2008, Wolberg 2007). Increased thrombin activity on platelet surfaces has indeed been linked to altered fibrin network architecture (Wolberg & Campbell 2008). Fibrinolysis of a thrombus is just as important as formation thereof and, is a result of the combination of both regulated enzymatic activity and physical properties of the fibrin scaffold itself (Weisel & Litvinov 2008).

Both formation and fibrinolysis of a thrombus are dependent on a diversity of structural, biological, physical, and chemical properties depending on the conditions, rate and nature of this formation and fibrinolysis respectively (Weisel 2007, Weisel & Litvinov 2008). In healthy or control individuals, the experimental formation of thrombi is dependent on thrombin mediated conversion of fibrinogen to fibrin (Wolberg 2007); however in individuals who have suffered cerebral ischaemia, it has been evidenced that factors already present in blood plasma (possibly abnormal concentrations of thrombin), without the addition of exogenous thrombin was capable of rendering the spontaneous formation of dense matted deposits (DMDs) within fibrin networks (Lipinksi *et al.* 2012). Additionally, normal platelets display smooth membranes, with open canalicular system channel pores visible on their surfaces; whereas the platelets of individuals who have experienced cerebral ischaemia display more pseudopodia (morphological evidence of activation) than do platelets of control individuals (Pretorius *et al.* 2011a, Pretorius *et al.* 2011b). The rate of thrombus lysis appears to be faster for thrombi of major fibre (larger diameter) constituent than those of minor fibre (smaller diameter) constituent (Weisel & Litvinov 2008). Minor fibre thrombi, which are more compact (Fatah *et al.* 1996), with longer lysing processes may therefore have a greater chance of causing thrombotic events (Ajjan *et al.* 2008, Alzahrani & Ajjan 2010, Fatah *et al.* 1996, Pretorius *et al.* 2011a). This finding is strengthened by evidence that increased thrombin levels produce smaller fibre diameters and thus networks that are more tightly packed in cases of cerebral ischaemia (Pretorius *et al.* 2011a, Wolberg 2007).

As individuals' age, changes in vasculature, haemostasis, endothelium, platelets, coagulation and fibrinolytic factors occur (Franchini 2006). With increasing age, individuals have been found to display heightened coagulation enzyme activity (including fibrinogen), accompanied by signs of enhanced fibrin formation as well as secondary hyperfibrinolysis (Mari *et al.* 2008). Increased fibrin turnover may be associated with age-related increases in endothelial disturbance and the prevalence of atherosclerosis (Lee *et al.* 1995). It is suggested that platelet transmembrane signalling or secondary messenger accumulation may have an influence on altered platelet

activity due to higher platelet phospholipid content (Bastyr *et al.* 1990, Franchini 2006). These changes in haemostatic profile with increased age seem to be independent of sex (Hume 1961). The impaired fibrinolytic potential evident with increased age, termed thrombotic preparedness, is out of proportion to the physiological needs of an aging individual (Gharacholou & Becker 2009, Pretorius *et al.* 2010).

Upon stimulation, platelets release factors contained in their alpha granules which facilitate aggregation. The ultrastructural morphology of platelets themselves among young versus aged individuals has not been a focus point in research as yet. The ultrastructure of fibrin networks among aged versus young individuals, however, is where differences have been evidenced. It seems that with increasing age major fibre diameter is reduced and arrangement becomes more sparse than in younger individuals. Additionally, minor fibres seem to be more prevalent in older individuals, and at irregular intervals are found to clump together, forming minor fibre lattices and/or DMDs. This may be due to suggested enhanced fibrin formation and heightened coagulation enzyme activity (Pretorius *et al.* 2010). Enhanced fibrin formation is to an extent indicative of enhanced platelet aggregation, therefore in thrombotic preparedness, platelets are expected to be hyper-reactive and display activated morphology even in pre-ischaemic controls.

Hyperglycaemia, which defines diabetes when chronic, represents a high risk of thrombosis (Malý 2010) and is a known risk factor for and potentiator of cerebral ischaemia. Evidence suggests that increased activation of prothrombotic coagulation factors, together with decreased fibrinolysis, and an increased thrombotic tendency due to platelet hyper-reactivity, leads to this increased risk for thrombosis (Alzahrani & Ajjan 2010, Grant 2005). Platelets of chronically hyperglycaemic individuals have been characterised by dysregulation of numerous signalling pathways, resulting in intensified activation, adhesion and aggregation. The state of hyperglycaemia is associated with systemic inflammation, higher platelet activation and elevated coagulation markers (Ferreiro *et al.* 2010).

7.2. Materials & Methods

This chapter documents platelet counts and fibrinogen levels, which are expected to be elevated subsequent to cerebral ischaemia and, analyses the ultrastructural morphology of platelets to ascertain levels of activation which can be linked to inflammation and the ischaemic cascade. Approximately 4ml of whole blood was collected from each rat upon termination, via cardiac puncture. For every 100µl of blood drawn from an animal, 11µl of sodium citrate was added to prevent the blood from clotting naturally. Separately, 0.25ml from individual animals of each subgroup was pooled for ultrastructural analysis. The blood was kept at 4°C for a period of no more than 2h (Liao *et al.* 2001) prior to analyses.

7.2.1. Platelet Counts and Fibrinogen Level Analysis

Chemical analysis was undertaken at Ampath (Ampath Pathology Laboratory Support Services, Pretoria, South Africa) as they are an accredited laboratory. The ratio of citrate-to-plasma required for blood collection is 1:9. Whole blood of 250µl volume was required for performance of platelet counts using the Sysmex XE-2100™ Automated Hematology System which employs a traditional impedance (resistance) method. The volume of blood required for the fibrinogen level analysis was 150µl of citrated plasma. Fibrinogen analysis was performed with the Stago STA-R Evolution® Expert Series Hemostasis System which employs an electro-mechanical method of clot detection - monitoring increases in plasma viscosity - known as a viscosity-based detection system (VDS). Results of individual animal's platelet counts and fibrinogen levels were statistically compared within and between groups.

7.2.2. Platelet Coagula Preparation

Platelet aggregates can be optimally studied by the preparation of a platelet-rich plasma (PRP) smear or experimental thrombus from which reacted coagulation proteins have been removed by washing processes (Pretorius *et al.* 2011a). PRP was acquired by centrifuging the subgroup specific pooled whole blood at 1250rpm (maximum RCF = 17.523 x g; 1250g) for 5min. For investigation of platelet morphology, 20µl of PRP was mixed on a 0.2µm pore size Microsep polycarb membrane disc of 13mm diameter (Microsep, Johannesburg, South Africa), to obtain platelet aggregates. A repeatable method was employed to ensure consistency throughout

preparation of the membranes: mixing taking place on the edge of the membrane and spreading of the mixture was performed by drawing a small spatula across the membrane thrice (middle and both sides). These membranes were then placed in a Petri dish on filter paper dampened with 0.075M sodium phosphate (Na_3PO_4) buffer to create a humid environment and placed in an incubator at 37ºC for 2min to enhance cohesion. This was followed by a washing process (to remove any trapped blood proteins) in which the membranes with the coagula are bathed in 0.075M Na_3PO_4 buffer and shaken for 20min (Pretorius *et al.* 2006, Pretorius *et al.* 2007, Pretorius *et al.* 2011a).

Washed platelet aggregates were fixed in 2.5% formaldehyde/glutaraldehyde (FA/GA) for 20min. Each membrane was then rinsed thrice in 0.075M Na_3PO_4 buffer for 2min each before secondary fixation in 1% osmium tetraoxide (OsO_4) for 20min. The samples were again rinsed thrice with 0.075M Na_3PO_4 buffer for 2min each and dehydrated serially in 30%, 50%, 70%, 90% and three times absolute ethanol (EtOH), also for 2min in each change. Procedures for scanning electron microscopy (SEM), a technique whereby the external morphology of cells and structures can be studied, were completed by critical point drying of the membranes, mounting these on aluminium stubs using double-sided carbon tape, coating by carbon evaporation for conductivity and examination with a ZEISS ULTRA plus FEG Scanning Electron Microscope (Carl Zeiss Pty Ltd., Randburg, South Africa), with accelerating voltage set at 1kV for high quality surface analysis. Platelets were imaged with the In-lens secondary electron detector.

Preparations were also made to study the ultrastructural morphology of fibrin networks (using the same methods as for platelet coagulum preparation, with the addition of an equal amount of human thrombin to the plasma prior to mixing on the membrane) but due to the nature of the newly supplied membrane batch (previously supplied by Merck Millipore as MF-Millipore Membrane of 0.22µm pore size and 13mm diameter), upon ultrastructural analysis, there were too few areas free of non-biological inclusions to confidently study fibrin morphology. Fibrin network morphology could thus not be analysed and has been excluded from the dissertation.

7.3. Results & Discussion

Thrombosis and inflammation are deeply integrated (Libby & Simon 2001). Thrombotic events are associated with a change in haemostasis and cellular components that play a fundamental role in blood platelet and fibrin network formation (Fatah *et al.* 1992). Inflammation is recognised as a key element of the pathological progression of ischaemic stroke, and inflammation may be evident in the altered ultrastructure of platelets before notable changes are evident in chemical analyses. It is likely that early inflammatory responses may potentiate ischaemic injury, while late responses may be beneficial to the recovery and repair of ischaemic lesions (Wang *et al.* 2007). There is a noted difference between the sexes and with increased age where factors of thrombosis and inflammation are under discussion. Female platelet isolates, though capable of binding more fibrinogen and displaying a greater extent of aggregation than male platelet isolates (Bailey *et al.* 2009, Leng *et al.* 2004), actually do not aggregate as quickly as the larger number of male platelets do, thus, to some degree, rendering females less susceptible to thrombosis. In the absence of oestradiol, upon ovariectomy, acyclic female platelets are more susceptible to thrombosis than in cyclic female platelets (Bailey *et al.* 2009, Wong *et al.* 2008) due to their hyper-reactivity. Platelets express the oestrogen receptor beta (ERβ), which is hypothesized to have a direct effect on platelet function (Bailey *et al.* 2009, Jayachandran & Miller 2003, Peters *et al.* 2002).

7.3.1. Platelet Counts and Fibrinogen Levels

Males are known to have higher platelet counts and faster clotting times than females (Bailey *et al.* 2009, Wong *et al.* 2008) and since platelets are known to express ERβ (Bailey *et al.* 2009, Jayachandran & Miller 2003), this may tender evidence that the presence of oestrogen in circulation not only lowers platelet counts but also the clotting ability of platelets. In a human study of patients with acute cerebral ischaemia, platelet counts were shown to be significantly lower in ischaemic patients than in controls in the initial phases of ischaemia. The authors suggested that in the earliest phases of cerebral ischaemia, plasma fibrinogen levels may condition the extent of platelet accumulation in the area affected by ischaemia. They thus confirmed *in vivo* evidence that the platelet aggregation process is dependent on fibrinogen concentration (D'Erasmo et al. 1993, Pretorius et al. 2011a). During later phases of cerebral ischaemia platelet counts again increase (Gailani & Renné 2007, Gaston *et al.*1971, Pretorius *et*

al. 2011a, Todd *et al.* 1973, Undas *et al.* 2009). Inflammatory mediators elevate fibrinogen synthesis, levels of fibrinogen continue to rise under inflammatory conditions (Esmon 2003, Taylor *et al.* 1987) and elevated fibrinogen levels are associated with heightened stroke risk (Danesh *et al.* 2005). Oestradiol averts the presence of initial neural inflammation (Wang *et al.* 2007) and also lowers fibrinogen levels, though females are known to have higher fibrinogen levels than their male counterparts, therefore the lack of oestradiol in acyclic females could render these subjects with the highest fibrinogen levels (Bailey *et al.* 2009, Mendelsohn & Karas 1999).

Platelet counts and fibrinogen levels for each animal were quantified to determine the effect of experimental cerebral ischaemia on these and later their effect on the extent of neural tissue injury. Statistical analysis was performed using classes of group and time as information pertaining to gender and oestradiol are nested within the groups. Variability within the platelet count and fibrinogen levels models were calculated to be 65% and 75% respectively and the p-value obtained independently for both models was <0.0001. Groups are again as follows: males (MM), cyclic females (MF) and acyclic females (OVX). Each group was subdivided into termination times: 0h pre-ischaemia, 2h, 24h and 48h post-reperfusion.

Statistical analysis of platelet counts, taking into account group and time (p=0.0001) interaction (Figure 7.1) show that at 0h pre-ischaemia, MM-0 ($624.600x10^9$/l) and OVX-0 ($568.833x10^9$/l) display significantly higher platelet counts than MF-0 ($202.833x10^9$/l), which confirms evidence that the presence of oestradiol in high levels (as in cyclic females) lowers platelet counts. At 2h post-reperfusion, both MM-2 ($253.833x10^9$/l) and OVX-2 ($323.800x10^9$/l) correspond with research findings that platelet counts are lower than normal in the initial phases of cerebral ischaemia. MF-2 ($294.333x10^9$/l) however displays a slight increase in platelet counts when compared to its 0h counterpart, but these counts are still significantly lower than male and acyclic female controls. In later post-reperfusion phases (24h and 48h), platelet counts are shown to increase in MM (24h $375.500x10^9$/l, 48h $267.333x10^9$/l) and OVX (24h $473.600x10^9$/l, 48h $648.333x10^9$/l) from the levels evident at 2h. Conversely, in MF (24h $255.500x10^9$/l, 48h $233.833x10^9$/l), the levels are seen to progressively decrease once again to control levels. Only in OVX-48 do platelet counts increase to a point higher than that evident at 24h in acyclic females.

Analysis performed on platelet counts, taking only group ($p<0.0001$) effect into account confirmed that platelet counts were significantly higher in OVX ($503.642x10^9$/l) than in MM ($380.317x10^9$/l) and MF ($246.625x10^9$/l) and also that MM displayed significantly higher platelet counts than MF. This corresponds fully with literature. Finally, to ascertain the link between platelet count alteration and period after ischaemic injury (Figure 7.2), statistical analysis was performed taking only time ($p=0.0042$) effect into consideration. Platelet counts at 0h ($465.422x10^9$/l) and 48h ($383.167x10^9$/l) were significantly higher than at 2h ($290.656x10^9$/l) but not 24h ($368.200x10^9$/l), with 2h and 24h not significantly different from each other. These results confirm that in the earliest phases of injury subsequent to ischaemic insult, platelet counts are significantly lowered in circulation due to aggregation in the formation of coagula. By 24h, platelet counts are still low but incline towards slowly returning to normal levels through increasing in number again.

When analysing fibrinogen levels with group and time ($p=0.0166$) interaction considered (Figure 7.3), MM and OVX follow the same trend with low levels early on (MM-0 3.230g/l, OVX-0 2.770g/l), a dip at 2h (MM-2 2.762g/l, OVX-2 2.383g/l) during the initial phases of ischaemic injury, then reaching a significant peak by 24h (MM-24 7.498g/l, OVX-24 5.496g/l) and lowering again at 48h (MM-48 5.410g/l, OVX-48 4.150g/l) though still significantly higher than levels at 0h and 2h. MF (0h 2.173g/l, 2h 1.433g/l) only differs from MM and OVX at later stages, where levels at 24h (3.300g/l) are lower (not significantly though) than at 48h (3.815g/l). Overall, when only group ($p<0.0001$) effect is accounted for, MF (2.680g/l) is shown to have significantly lower fibrinogen levels than OVX (3.700g/l) and MM (4.725g/l), which is expected as oestrogen is known to lower fibrinogen levels, although research suggests that cyclic females should still display higher fibrinogen levels than males. Furthermore, MM displays significantly higher fibrinogen levels than OVX, again differing from literature which suggests that fibrinogen levels may be higher in acyclic females than in males.

To establish a link between fibrinogen levels and time subsequent to ischaemic insult, analysis was performed taking only time ($p<0.0001$) effect into account (Figure 7.4). Fibrinogen levels at 0h (2.724g/l) and 2h (2.193g/l) are jointly significantly the lowest, levels at 24h (5.431g/l) are significantly the highest and levels at 48h (4.458g/l) are also significantly lower than at 24h. This shows that in the initial phases of cerebral ischaemia, fibrinogen levels decrease from normal and then increase significantly in later phases subsequent to ischaemic injury.

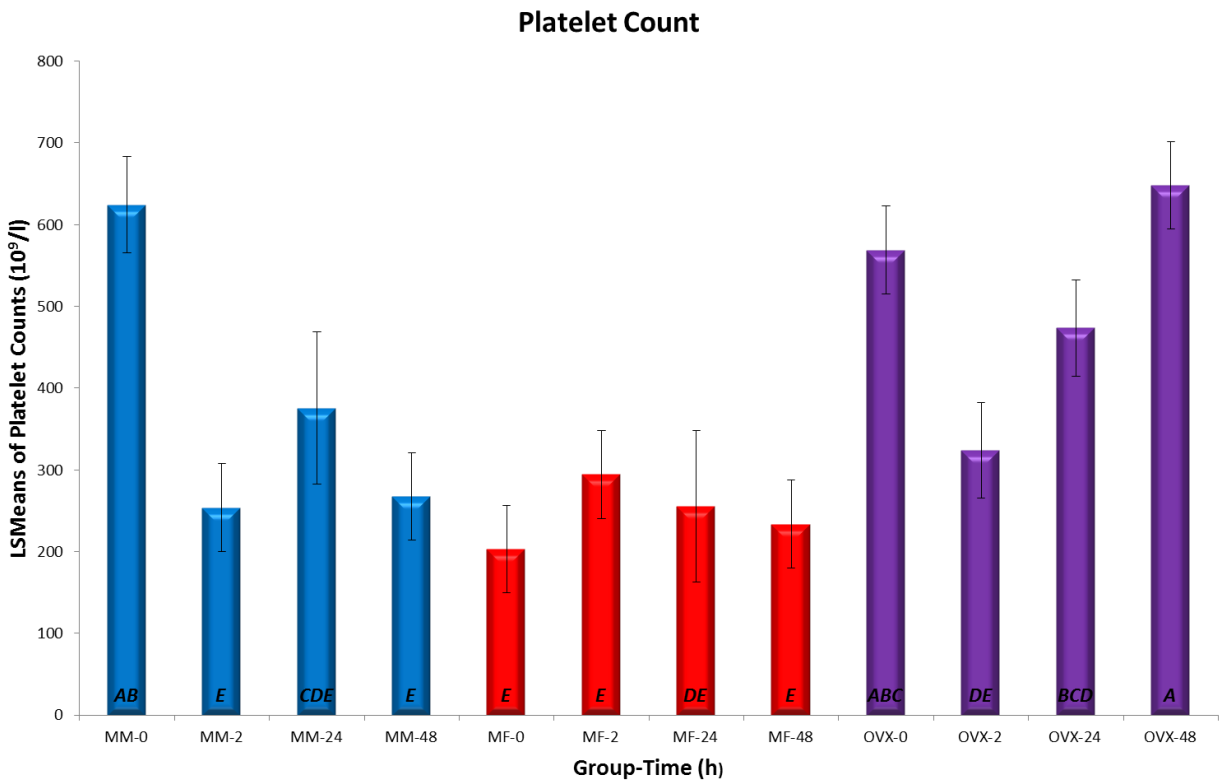


Figure 7.1. Platelet count analysis where the group and time interaction is accounted for. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 65% and the p-value obtained was 0.0001. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females. Time 0 = 0h control or pre-ischaemia, Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

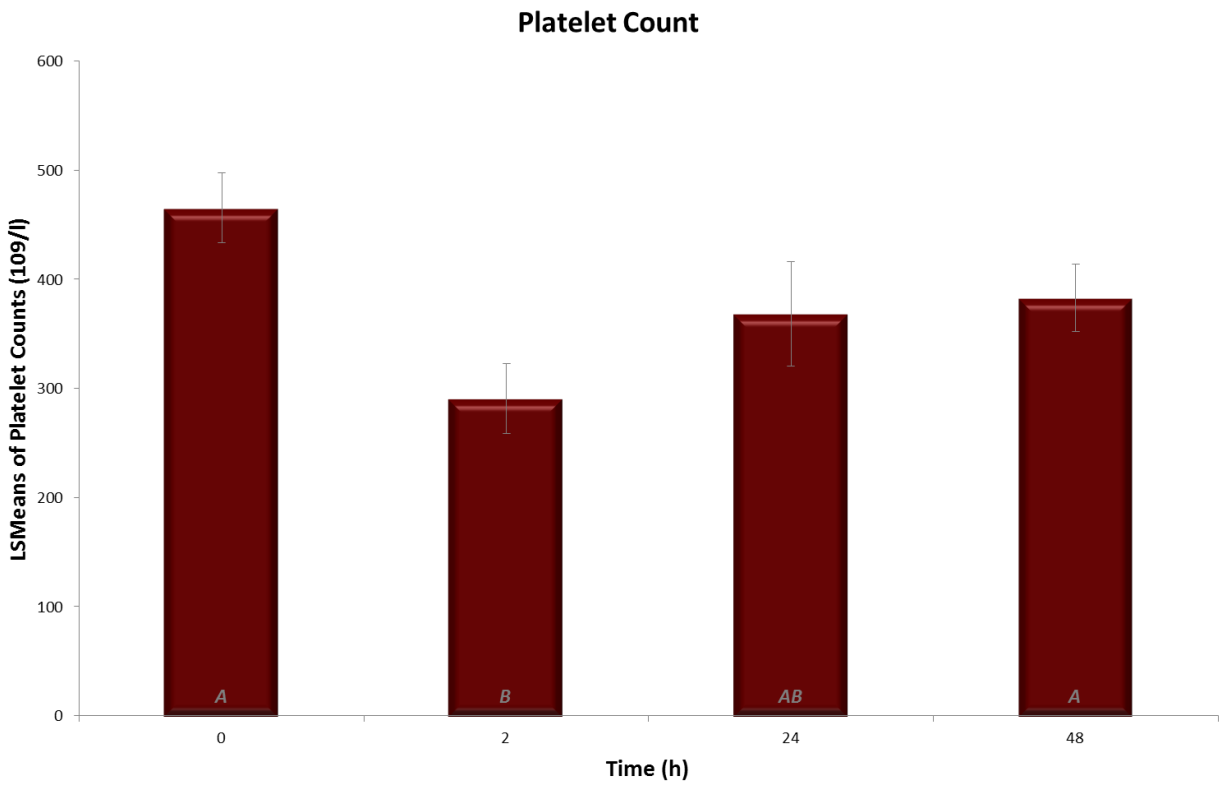


Figure 7.2. Platelet count analysis where time effect is accounted for. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 65% and the p-value obtained was 0.0042. Time 0 = 0h control or pre-ischaemia, Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

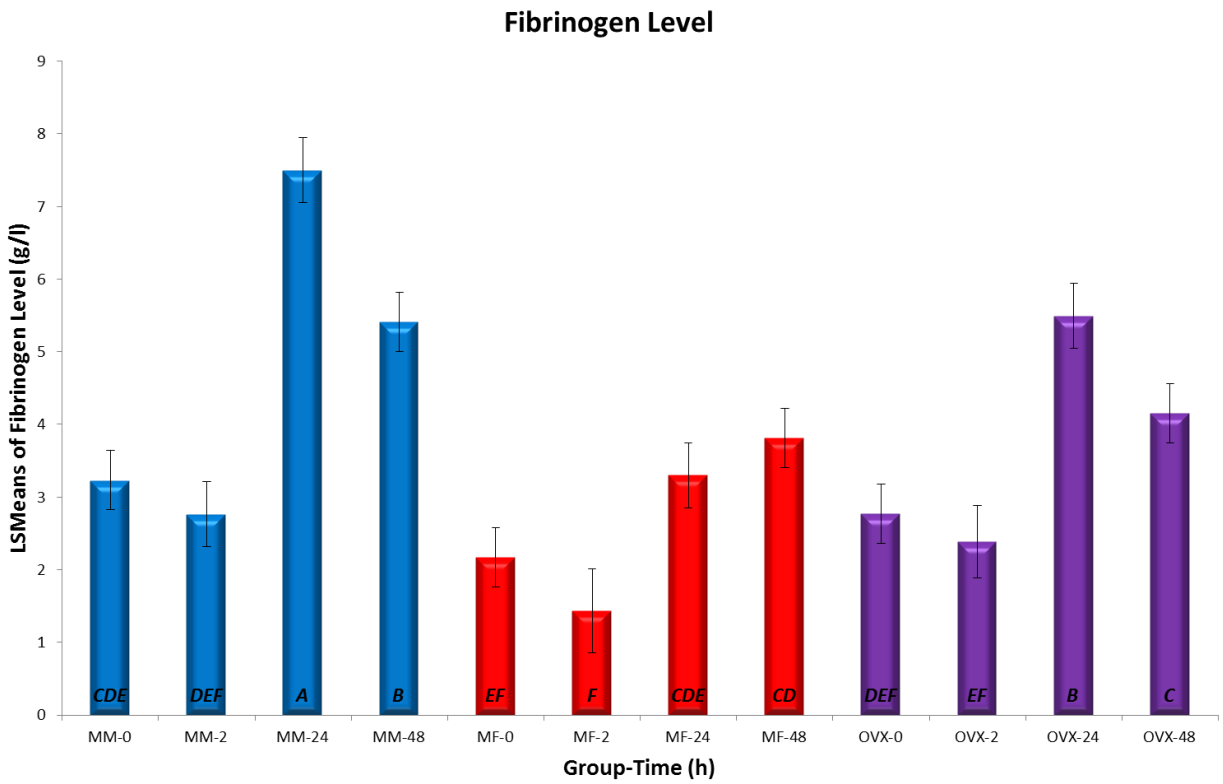


Figure 7.3. Fibrinogen level analysis where the group and time interaction is accounted for. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 75% and the p-value obtained was 0.0166. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females. Time 0 = 0h control or pre-ischaemia, Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

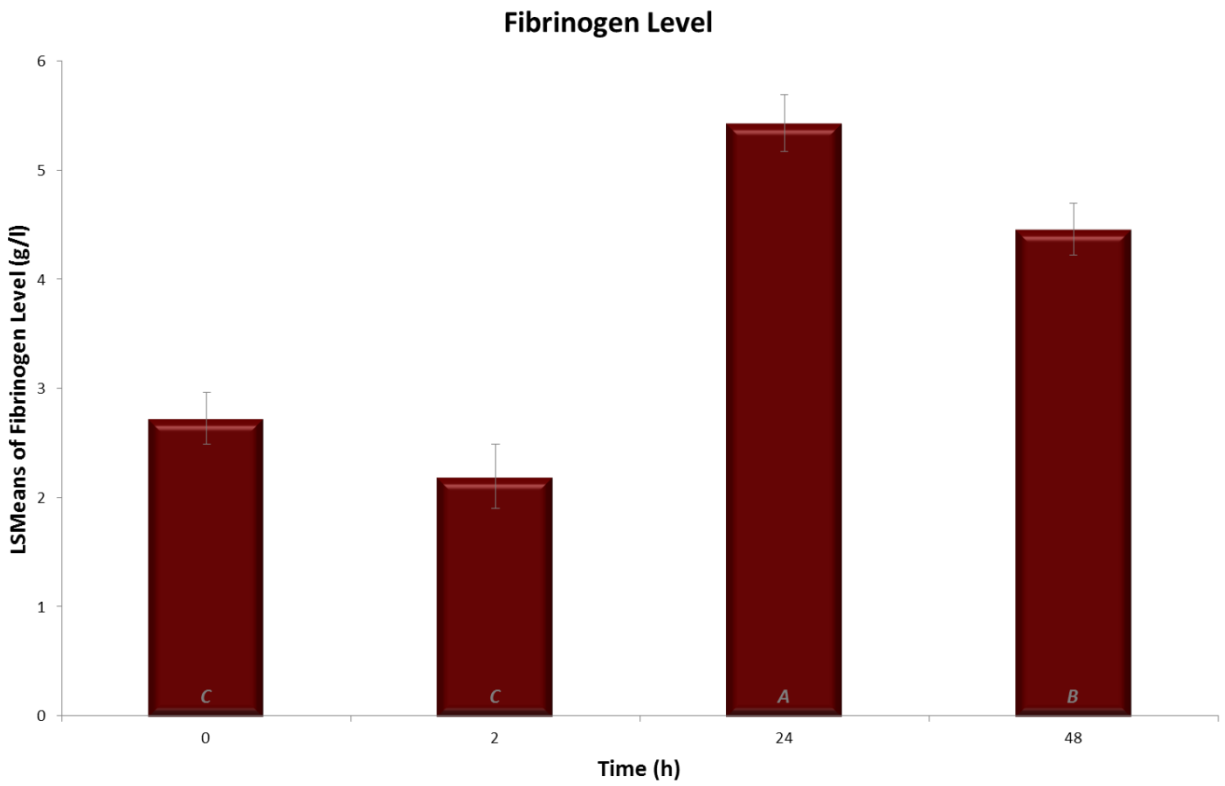


Figure 7.4. Fibrinogen level analysis where time effect is accounted for. Italicised letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95%. Variability within the model was calculated to be 75% and the p-value obtained was <0.0001. Time 0 = 0h control or pre-ischaemia, Time 2 = 2h post-reperfusion, Time 24 = 24h post-reperfusion, Time 48 = 48h post-reperfusion

Together, these findings confirm that fibrinogen levels are elevated subsequent to cerebral ischaemia (Figure 7.4), though not immediately and also that platelet counts are initially lowered and then gradually increase (Figure 7.2) through the phases of ischaemia. Results confirm that platelet counts are highest in acyclic females and higher in males than in cyclic females. In contrast to suggestions in research though, acyclic females were not found to present with higher fibrinogen levels than males. In fact, males were shown to have the highest fibrinogen levels, followed by acyclic females who had higher levels than cyclic females. This serves to confirm that oestrogen indeed lowers fibrinogen levels and platelet counts. Correlation analysis between oestrogen levels and platelet counts as well as fibrinogen levels revealed significant negative correlations; therefore the presence of oestrogen definitely lowers both platelet counts and fibrinogen levels as well as reactivity. Correlation between platelet counts and fibrinogen levels however showed an insignificant positive correlation, therefore though higher fibrinogen levels correspond to higher platelet counts, it cannot be confirmed by current data that this relation is significant. The question of whether oestrogen lowers platelet reactivity will be tackled through the analysis of platelet morphology. If platelets are shown to be more activated at specific times, it can again be correlated to fibrinogen levels as platelets which are activated to aggregate will be linked to elevated fibrinogen levels as fibrin formation is increased for coagulation purposes.

7.3.2. Ultrastructural Platelet Morphology

Control platelets (in healthy individuals) are known to display smooth membranes with open canalicular system channel pores visible on their surfaces. Upon activation, platelets change shape to a spherical form and begin to extend dendritic pseudopodia (Herd & Page 1994, White 1987). It is now known that the mechanisms of thrombosis and inflammation are deeply unified, with platelets central to both processes, thus a higher level of inflammation will be evident in the morphological display of more extensive pseudopodia from the main body of the platelet. Through the extension of multiple pseudopodia, more connections can be made between platelets thus resulting in larger aggregates and more connections can be made with fibrin networks resulting in the formation of more rigid and larger thrombi.

The study employed a hyperglycaemic two-vessel occlusion model, with induced mild systemic hyperglycaemia lasting up to 3h post-anaesthesia. Hyperglycaemia is known to render platelets hyper-reactive and is associated with systemic inflammation, thus worsening the outcome of

cerebral ischaemia. Furthermore, led by the ischaemic cascade, inflammation (visible in platelet activation) is expected to be evident early after ischaemic insult. Consequently it is expected that at 2h post-reperfusion, platelets will display a multitude of pseudopodia due to the shock from both experimental cerebral ischaemia and the hyperglycaemia under which it is induced. It is also anticipated that dysfunction of the blood-brain barrier (BBB) will be evident in the morphology of platelets, rendering them highly activated, displaying extensive pseudopodia. Furthermore, the morphology of platelets should change in such a way as to reflect further the consequences of the ischaemic insult itself.

Multiple micrographs for each subgroup were studied and a representative micrograph selected to signify each in the figures to follow. Groups are again as follows: males (MM), cyclic females (MF) and acyclic females (OVX); with each group subdivided into termination times: 0h pre-ischaemia, 2h, 24h and 48h post-reperfusion.

Figure 7.5.A-D shows representative platelets for MM in the four termination subgroups. Platelets for pre-ischaemic MM-0, display typical minimal pseudopodia formation and smooth membrane surfaces (Figure 7.5.A). At 2h post-reperfusion, MM-2 platelets all present with extensive formation of pseudopodia – typically seen in activated platelet states. This is to be expected, as the insult would have triggered the inflammatory response and the coagulation process would now be exposed to the initial stages of equilibrium disturbances (Figure 7.5.B). By 24h post-reperfusion in MM-24, the impact of the insult has a profound effect on the coagulation system and platelet swelling is visible with breaks in the membrane and reduced pseudopodia formation compared to 2h after insult (Figure 7.5.C). These morphological changes – swelling and membrane breakage – are typical of necrosis and were present in 70% of MM-24 platelets. Interestingly, necrotic platelets were also previously noted in humans 24h after stroke (Pretorius *et al.* 2012). Figure 7.5.D shows a platelet for 48h post-reperfusion in MM-48. Here it appears that the coagulation systems response has stabilised and that the recovery process has been commenced. Platelets are no longer necrotic in appearance, although the contours of the membrane and the extent of pseudopodia formation still suggest an activated state. We do not suggest that necrosis is reversible, but rather that the coagulation system recovers in such a way that necrotic platelets have been cleared and that platelets of activated morphology are once again visible.

Figure 7.6.A-D shows representative platelets for animals from group MF. The typical control platelet at 0h for MF-0 is similar to that of the MM-0, with some pseudopodia present and a smooth membrane surface (Figure 7.6.A). Platelets for MF-2, at 2h post-reperfusion also show extensive pseudopodia formation due to initial inflammation (Figure 7.6.B) – noted in the activated platelet state of the males. Figure 7.6.C shows a platelet from MF-24, 24h post-reperfusion, with platelets already returning to a pre-inflammatory state without many pseudopodia, similar to group-matched controls. Interestingly, no necrotic platelet ultrastructure was noted in MF. This suggests that oestrogen may indeed have a protective effect as previously suggested in the literature, exerting anti-inflammatory effects and delaying apoptosis not only in neural tissue but also at the level of blood haemostasis. At 48h post-reperfusion in MF-48, platelets again appear more activated, but not necrotic (Figure 7.6.D). This morphology is similar again to MM-48. This re-activation of platelets in MF-48 may possibly be due to the second phase of BBB disruption which again changes the balance in haemostasis; thus evidencing that male platelets which at 48h were also still in an activated state due to this very reason, though they were reverting to normal morphology when compared to the necrotic platelets in MM-24.

Representative platelets are shown in Figure 7.7.A-D for animals of group OVX. Here, OVX-0 before ischaemic insult shows more extensive pseudopodia formation than seen in the typical morphology noted in both MM-0 and MF-0 controls (Figure 7.7.A). This could be due to the state of thrombotic preparedness (inherent inflammation due to heightened fibrinogen activity and altered platelet activity caused by increased signalling) previously seen in menopausal women. Platelets from 2h post-reperfusion in OVX-2 presented with less pseudopodia than MM-2 and MF-2 (Figure 7.7.B). This could be due to the fact that with the presence of higher levels of inflammation after cerebral ischaemia, the state of thrombotic preparedness is soothed to some extent. Platelets at 24h post-reperfusion in OVX-24 showed platelet swelling with membrane breaks and reduced pseudopodia formation. This ultrastructure is similar to that of MM-24 and is typical of necrosis (Figure 7.7.C), which in OVX-24 was evident in 60% of platelets. At 48h after the insult, platelets from OVX-48 show a degree of recovery – similar to males at this time – platelets no longer have a necrotic appearance, although extensive pseudopodia formation once again suggests that the second phase of BBB disruption is in play (Figure 7.7.D), challenging the balance in haemostasis as in males and cyclic females at 48h. There is also a possible reversion to morphology typical of thrombotic preparedness at this time.

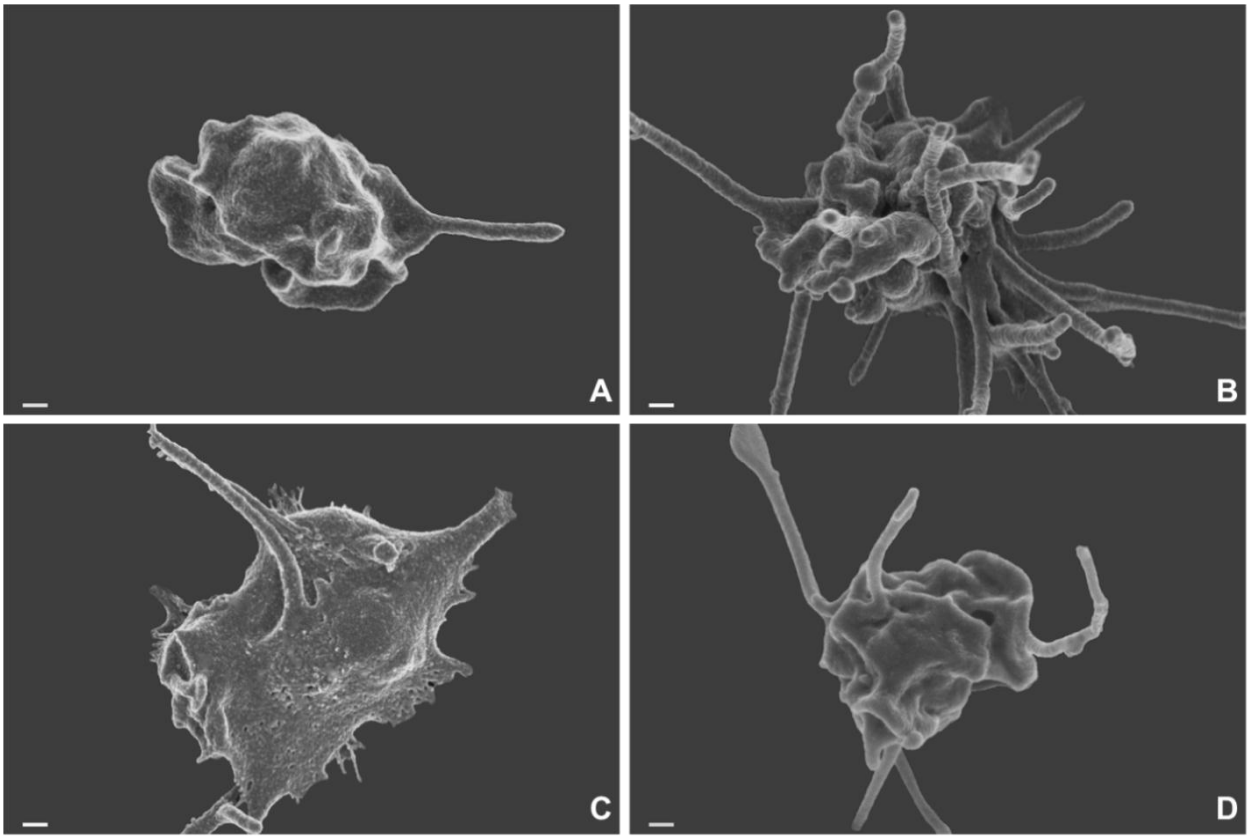


Figure 7.5. Platelet morphology representation of males in group MM. A: Platelet from representative male 0h pre-ischaemia (MM-0), showing typical pseudopodia formation and smooth membrane surfaces. B: Platelet from representative male 2h post-reperfusion (MM-2), showing extensive pseudopodia formation – typically seen in activated platelet states and characteristic of inflammation. C: Platelet from representative male 24h post-reperfusion (MM-24), here platelet swelling is visible with breaks in the membrane and reduced pseudopodia formation compared to 2h after insult. This is typical of necrosis. D: Representative platelet 48h post-reperfusion (MM-48). Recovery begins, and platelets no longer have a necrotic appearance, although pseudopodia formation suggests an activated state. Scale = 200 nm

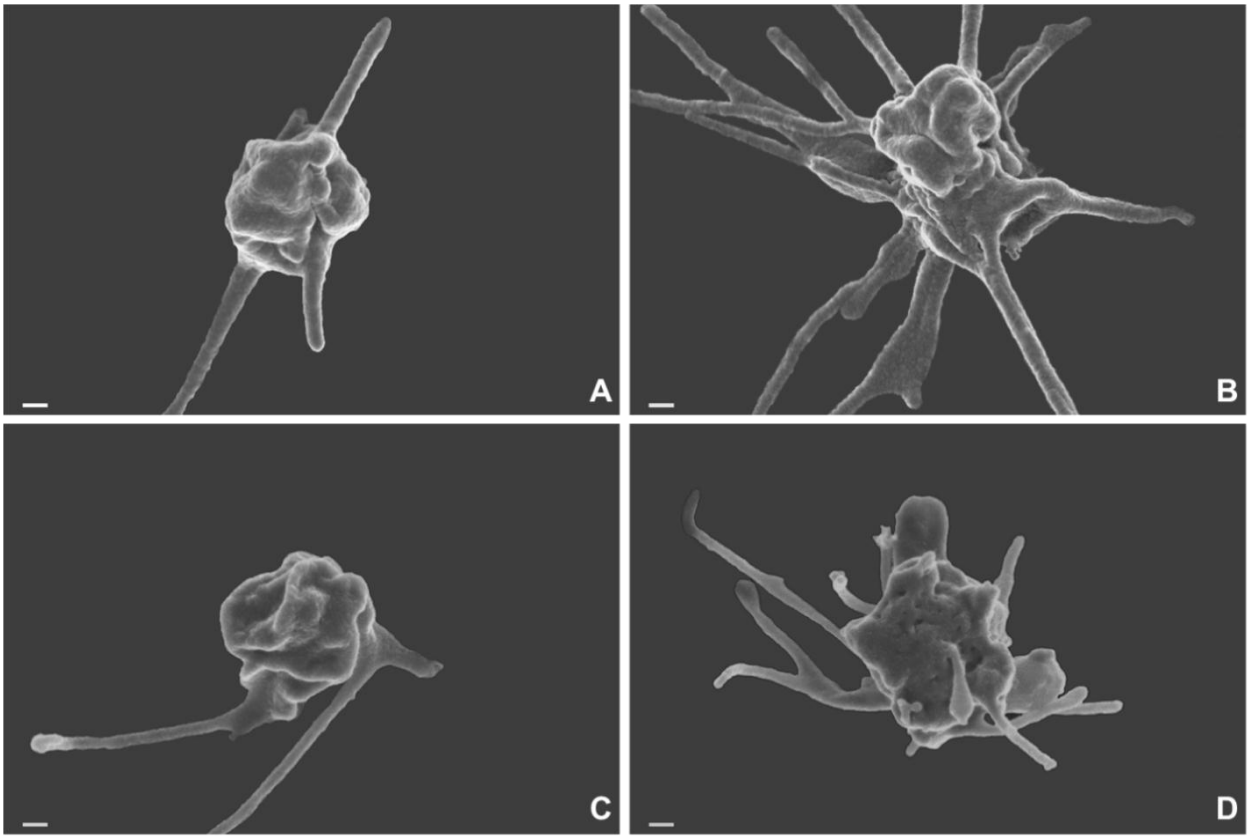


Figure 7.6. Platelet morphology representation of cyclic females in group MF. A: Representative platelet 0h pre-ischaemia (MF-0), showing typical pseudopodia formation and smooth membrane surfaces. B: Representative platelet 2h post-reperfusion (MF-2), showing extensive pseudopodia formation – typically seen in activated platelet states and in inflammation. C: Representative platelet 24h post-reperfusion (MF-24), here platelets are returning to a pre-inflammatory state with little pseudopodia formation. D: Representative platelet 48h post-reperfusion (MF-48), pseudopodia formation typical of activation is visible. Scale = 200 nm

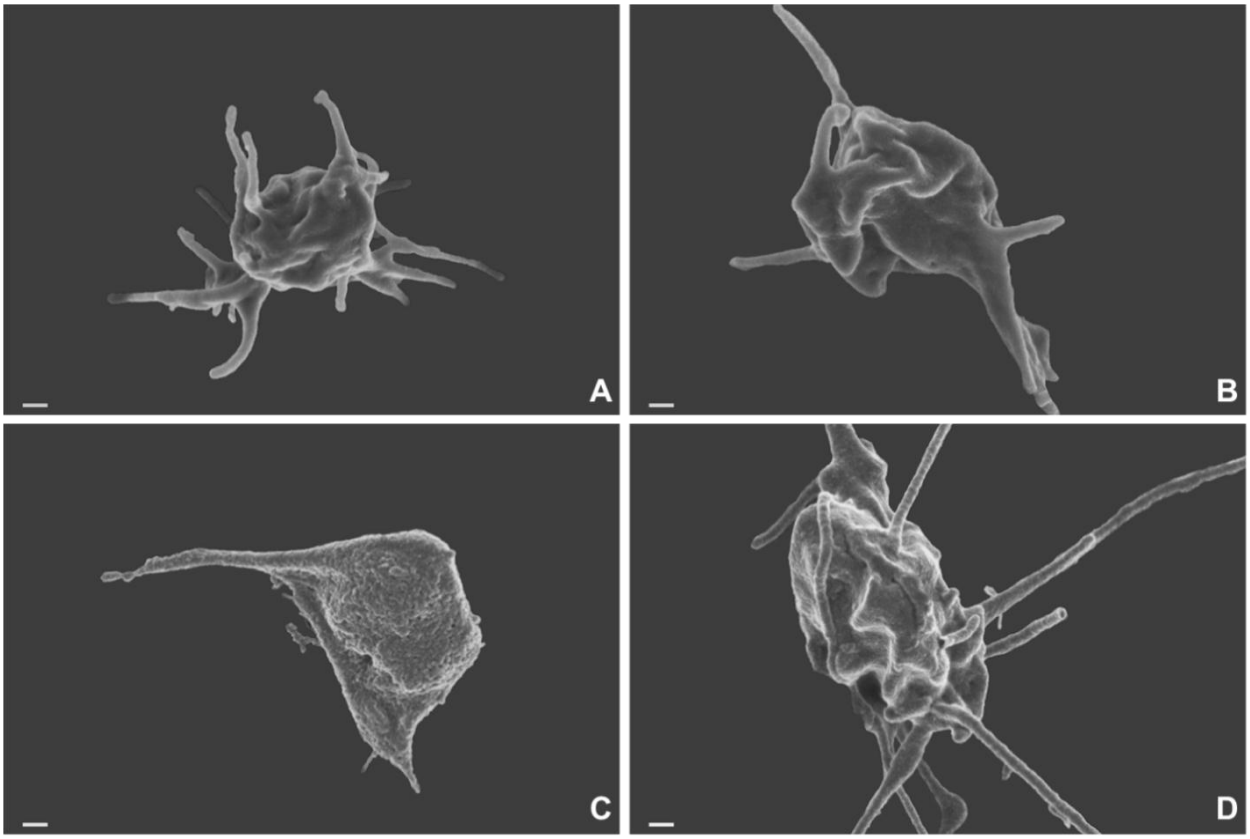


Figure 7.7. Platelet morphology representation of acyclic females in group OVX. A: Representative platelet from acyclic female, 0h pre-ischaemia (OVX-0). Pseudopodia formation is visible, characteristic of thrombotic preparedness. B: Representative platelet from acyclic female, 2h post-reperfusion (OVX-2). Pseudopodia formation is characteristic of mild inflammatory states. C: Representative platelet from acyclic female, 24h post-reperfusion (OVX-24). Here platelet swelling is visible with breaks in the membrane and reduced pseudopodia formation compared to 2h after insult. This is typical of necrosis. D: Representative platelet from acyclic female, 48h after ischaemic (OVX-48) insult. Recovery begins, and platelets no longer have a necrotic appearance, although pseudopodia formation suggests an activated state. Scale = 200 nm

In correlation with events described in the ischaemic cascade in Chapter 2, inflammation is indeed shown to peak early in the morphological evidence presented here and progressively lessen. Apoptosis and necrosis of cerebral tissue takes place in stages, with intermittent recovery of certain areas surrounding lesions. Platelet morphology seems to evidence that the largest shock due to apoptotic and necrotic mechanisms does indeed take place within the first 24h after insult. This corresponds to the knowledge that both treatment and outcome are most effective and favourable respectively, within the first 24h following cerebral ischaemia. Depending on the presence of neuroprotective hormones, the system begins to recover or is at least stable for a period until the second of the biphasic peaks of BBB disruption, after which secondary cerebral injury becomes apparent.

Consistent with literature, 0h pre-ischaemia or control platelets in males (Figure 7.5.A) and cyclic females (Figure 7.6.A) display smooth membranes with minimal pseudopodia present. Acyclic females (Figure 7.7.A), who are expected to be in a state of thrombotic preparedness (with inherent inflammation), are indeed shown to have hyper-reactive platelets in their control state. At 2h post-ischaemia, where initial neural tissue injury and thus inflammation are expected, males (Figure 7.5.B) and cyclic females (Figure 7.6.B) reflect a highly activated state with much pseudopodia present, thus confirming inflammation. Acyclic female (Figure 7.7.B) platelets seem less activated than at 0h, possibly indicating that inflammation induced by ischaemic injury is somehow initially soothed by thrombotic preparedness. By 24h post-ischaemia, the platelets of males (Figure 7.5.C) and acyclic females (Figure 7.7.C) take on similar characteristics, as necrosis is evident in both groups at the period when maximal stress due to cerebral ischaemic consequences is expected. Cyclic females (Figure 7.6.C) at 24h display a return of platelet morphology to normal control morphology, with less pseudopodia present and no evidence of necrosis. This seems to be the point where oestrogen best exerts its anti-inflammatory and anti-apoptotic effects in coagulation. Thus in males and acyclic females, where lower levels of endogenous oestradiol are present, necrotic platelets are found; but cyclic females are protected from the progression of inflammation which would result in more serious cerebral ischaemic consequences. At 48h post-reperfusion, there is no longer a presence of necrotic platelets. This is likely the point where the second phase of BBB disruption is evident subsequent to ischaemic injury, thus affecting the balance in haemostasis again. Therefore, though platelets across all groups are returning to normal morphology (compared to 2h post-reperfusion where much systemic inflammation is evident), a state of inflammation is still apparent. It is important to reiterate here that though early inflammation responses (as at 2h) may potentiate ischaemic

injury, late inflammatory responses (as at 48h) may be beneficial to recovery and the repair of ischaemic lesions. Male (Figure 7.5.D) and cyclic female (Figure 7.6.D) platelet morphology should soon return to normal such as controls at 0h and acyclic female (Figure 7.7.D) platelet morphology should revert to a state of thrombotic preparedness as at 0h.

When forming a correlation between platelet morphology – thus evidence of activation – and coagulation data from statistical analyses, more sense can be made of these. It is apparent that where platelet counts are lowest (MM-2, MF-2, and OVX-2), systemic inflammation was at its highest as visualised in platelet activation. This indicates that less free platelets are available in circulation at times of marked inflammation due to platelet aggregation.

Plasma fibrinogen levels are lowest (MM-2, MF-2, and OVX-2) when inflammation due to cerebral ischaemia is at its highest. In MM-2 and MF-2, this shows that fibrinogen is lysed to form fibrin at the point when platelets are most reactive. In OVX-2, where platelets are less hyper-reactive compared to their usual state of thrombotic preparedness, fibrinogen levels are also lowest in circulation. Fibrinogen levels in plasma are shown to be highest when platelets are necrotic in both MM-24 and OVX-24, as compromised platelets would be to some extent incapable of binding fibrinogen. Levels of fibrinogen in both the aforementioned groups, decrease in circulation upon recovery and clearing of necrotic platelets, as by the point of the second BBB disruption peak, platelets are again more reactive and bind more fibrinogen. In MF-48 the second point of BBB dysfunction is when fibrinogen is at its highest in circulation, though not significantly higher than in MF-24. This may show that ischaemic stress is lessened in cyclic females when compared to males and acyclic females. Fibrinogen levels in circulation are higher in MF-24 and MF-48 because platelets at these times in cyclic females are less reactive than platelets in MF-2 when most inflammation is visible. Less reactive platelets will bind less fibrinogen and thus levels thereof will be higher in circulation.

Chapter 8: Analysis of Red Blood Cell Interaction

8.1. Introduction

A number of pathological conditions exist in which elevated red blood cell (RBC) counts are associated with a predisposition to thrombosis, e.g. haemochromatosis, polycythaemia vera, beta-thalassaemia and even frequent blood transfusions (Brill 2011, Chakraborty *et al.* 2010, Dávalos *et al.* 2000, Day *et al.* 2003, Hod & Spitalnik 2011, Lipinski *et al.* 2012, Nagy *et al.* 2010, Spinella *et al.* 2009, Varma *et al.* 2008). The interaction of blood platelets in a coagulum have been investigated to a large extent, but little is known about the role of RBCs and their interaction in a thrombus. Research groups (Gerch *et al.* 2009) have shown, through *in vitro* experiments, that the addition of RBCs to plasma has a significant effect on the structure and mechanical properties of fibrin clots. Furthermore, the interaction of RBCs with fibrin clots was revealed to be associated with lytic resistance of thrombi (Wohner *et al.* 2011).

In a recent collaboration (Lipinski *et al.* 2012), it was found that the addition of ferric chloride to the whole blood of normal patients revealed comparable ultrastructure to that of thromboembolic cerebral ischaemia patients. It must be noted here that ferrous chloride (dietary iron) is converted to ferric chloride by ferroxidase present in human blood. These trivalent iron ions generate hydroxyl radicals, and these radicals cause nonenzymatic polymerization of fibrinogen. These findings led to the deduction that iron-induced modification of fibrinogen may be responsible for heightened interaction of fibrin with RBCs and may very well explain thrombolytic resistance in patients presenting with thromboembolic cerebral ischaemia (Lipinski 2011).

A whole blood smear from a healthy individual typically shows rounded RBCs with smooth membranes. Once thrombin is added to whole blood, the ultrastructure changes – a clear formation of pseudopodia-like projections is visualised and are in some instances fused to fibrin fibres. At higher magnifications, small indentations have been seen at the sites of connection of the RBC cellular extensions with the fibrin fibres. RBCs have not only been shown to fuse to fibrin fibres, but also to each other through the extension of these projections. Addition of ferric chloride, with and without thrombin, to whole blood smears revealed the formation of unilateral extensions from RBC surfaces. The whole blood coagulum morphology of thromboembolic cerebral ischaemia patients presented with similar morphology to those observed in normal

blood after the addition of ferric chloride, with clearly altered RBC morphology. These results suggest that lytic resistance in patients of thromboembolic cerebral ischaemia may be related to an excessive concentration of divalent iron ions in the blood of these patients, thus affecting RBC interaction with fibrin (Lipinski 2010, Lipinski *et al.* 2012).

This chapter will thus serve to analyse the ultrastructural interaction of RBCs with coagula within and between experimental groups using scanning electron microscopy (SEM), a technique whereby the external morphology of cells and structures can be studied. When studying the interaction of RBCs with fibrin networks in a coagulum, it is necessary to form a whole blood thrombus (Lipinski *et al.* 2012).

8.2. Materials & Methods

Recent collaboration (Lipinski *et al.* 2012) has revealed that whole blood thrombi must be studied, as a modification of RBC morphology and the interaction of RBCs with fibrin networks (generated by the addition of thrombin) is evident in stroke patients. This human thrombin (SANBS, Pretoria, South Africa) mixture is typically prepared from a single regular donor by calcium chloride activation of a euglobulin fraction of plasma obtained by apheresis. Each individual unit is tested and has to be non-reactive for hepatitis B surface antigen (HBsAg), HIV-1 antibody, HIV-2 antibody and HIV p-24 antigen, Hepatitis C virus (HCV) antibody and antibodies to *Treponema pallidum*. These tests are performed by licensed assay methods. This thrombin is 20U/ml and is made up in biological sodium phosphate buffer containing 0.2% human serum albumin. When thrombin is added to whole blood, fibrinogen is converted to fibrin and intracellular platelet components; such as transforming growth factor, platelet derived growth factor and fibroblastic growth factor are released into the coagulum and RBCs are trapped within the networks and interact with fibrin.

8.2.1. Preparation of Whole Blood Coagula

Whole blood was collected from each rat upon termination, via cardiac puncture, and 0.25ml from individual animals of each subgroup pooled for ultrastructural analysis. For every 100µl of blood drawn from an animal, 11µl of sodium citrate was added to prevent the blood from clotting naturally. For investigation of RBC interaction with fibrin, 10µl of whole blood was mixed with an

equal volume of human thrombin on a 0.2µm pore size Microsep polycarb membrane disc of 13mm diameter (Microsep, Johannesburg, South Africa), to form a whole blood thrombus. A repeatable mixing method was once again employed to ensure consistency throughout preparation of the membranes, mixing taking place on the edge of the membrane and spreading of the mixture being performed with a small spatula drawn across the membrane thrice (middle and both sides). The membranes were then placed into a Petri dish on filter paper dampened with 0.075M sodium phosphate (Na_3PO_4) buffer to create a humid environment and subsequently placed in an incubator at 37°C for 2min. This was followed by a washing process, in which the membranes with their coagula were bathed in 0.075M Na_3PO_4 buffer and shaken for 20min. The washing process is performed to remove any blood proteins trapped within the aggregates formed on the membranes (Lipinski *et al.* 2012, Pretorius *et al.* 2006, Pretorius *et al.* 2007).

Washed whole blood coagula were fixed in 2.5% formaldehyde/glutaraldehyde (FA/GA) for 20min. Each membrane was then rinsed three times in 0.075M Na_3PO_4 buffer for 2min each, secondarily fixed with 1% osmium tetroxide (OsO_4) for 20min, and again rinsed three times with 0.075M Na_3PO_4 buffer for 2min each. This was then followed by series dehydration in 30%, 50%, 70%, 90% and three times absolute ethanol (EtOH) for 2min each. Procedures for SEM were completed by critical point drying of the membranes, mounting the membranes on aluminium stubs with double-sided carbon tape, conductive coating by carbon evaporation and examination with a ZEISS ULTRA plus FEG Scanning Electron Microscope (Carl Zeiss Pty Ltd., Randburg, South Africa). Accelerating voltage was set at 1kV for high quality surface analysis and RBCs were imaged with the In-lens secondary electron detector.

8.3. Results & Discussion

To accurately identify differences in ultrastructure between groups, it is necessary to assess RBCs which are outliers of a thrombus rather than those directly associated with a thrombus as this will provide the best indication of "movement" towards a state of change which leads to cellular interaction. Separately, one must also note the interactions of RBCs within a clot to emphasise direct cellular interaction mechanisms. These interactions are likely to take place to some degree across all groups as thrombin was added to each experimental group, thus generating fibrin networks with which RBCs make contact in a thrombus. Control RBCs were expected to be rounded and have smooth membranes, whereas experimental groups were expected to display pseudopodia-like projections. It was also expected that experimental groups, but not control groups, in areas of direct RBC-thrombus interaction would display interfacing morphological features.

Multiple micrographs for each subgroup were studied and a representative micrograph selected for each subgroup within the groups under investigation to signify each in the figures to follow. Groups are again as follows: males (MM), cyclic females (MF) and acyclic females (OVX); with each group subdivided into termination times: 0h pre-ischaemia, 2h, 24h and 48h post-reperfusion.

Figure 8.1.A-D shows representative RBCs of MM in termination subgroups. Pre-ischaemia, MM-0 RBCs show the expected, typical discoid shape and smooth membrane surfaces (Figure 8.1.A). At 2h post-reperfusion, MM-2 RBCs deviate from discoid to a more disrupted shape, but membranes are still smooth. This is the point where the insult would have triggered the inflammatory process and the coagulation system are exposed to the initial stages of equilibrium disturbance (Figure 8.1.B). At 24h post-reperfusion, in MM-24 the impact of the insult influences the RBCs to extend unilateral projections. Membrane surfaces are still smooth (Figure 8.1.C). RBCs undergo these morphological changes to interact with and stabilise fibrin clots. Figure 8.1.D shows a RBC at 48h post-reperfusion for MM-48, where it seemed that the membrane is slowly returning to normal morphology, though still displaying a unilateral projection and a surface membrane pore is visible for the first time.

Figure 8.2.A-D shows representative RBCs for MF. The typical control RBC in MF-0 is similar to that of the control males (Figure 8.2.A). RBCs at 2h post-reperfusion, for MF-2 show more premature unilateral projection formation than visualised in males (Figure 8.2.B). Figure 8.2.C shows a RBC from MF-24 at 24h post-reperfusion, here the surface membrane is no longer smooth and the overall shape has deviated far from the normal discoid form. In Chapter 7 it was suggested that oestrogen might at this time display its protective effect as previously suggested in the literature, delaying apoptosis and exerting anti-inflammatory effects; but it is not possible to confirm this through the assessment of RBC morphology. At 48h post-reperfusion, RBCs of MF-48 display broad unilateral projections with overall shape returning to normal (Figure 8.2.D). Membrane pores or disruptions are however visible as in the male subgroup at this time.

Representative RBCs for OVX are shown in Figure 8.3.A-D. Here, pre-ischaemic OVX-0 RBCs show normal discoid shape, though the membrane is less smooth than noted in the typical morphology of control males and cyclic females (Figure 8.3.A). RBCs at 2h post-reperfusion in OVX-2 showed similar morphology to that of MF-2, with the extension of projections and an inherent discoid shape (Figure 8.3.B). RBCs from OVX-24, at 24h post-reperfusion, showed a loss of discoid shape, as in MF-24, though the membrane surface is smoother (Figure 8.3.C) in acyclic than in cyclic females. At 48h after the insult, RBCs from OVX-48 show a reversion to normal shape, though still with the presence of unilateral projections and a membrane pore visible as in both other groups at this time (Figure 8.3.D).

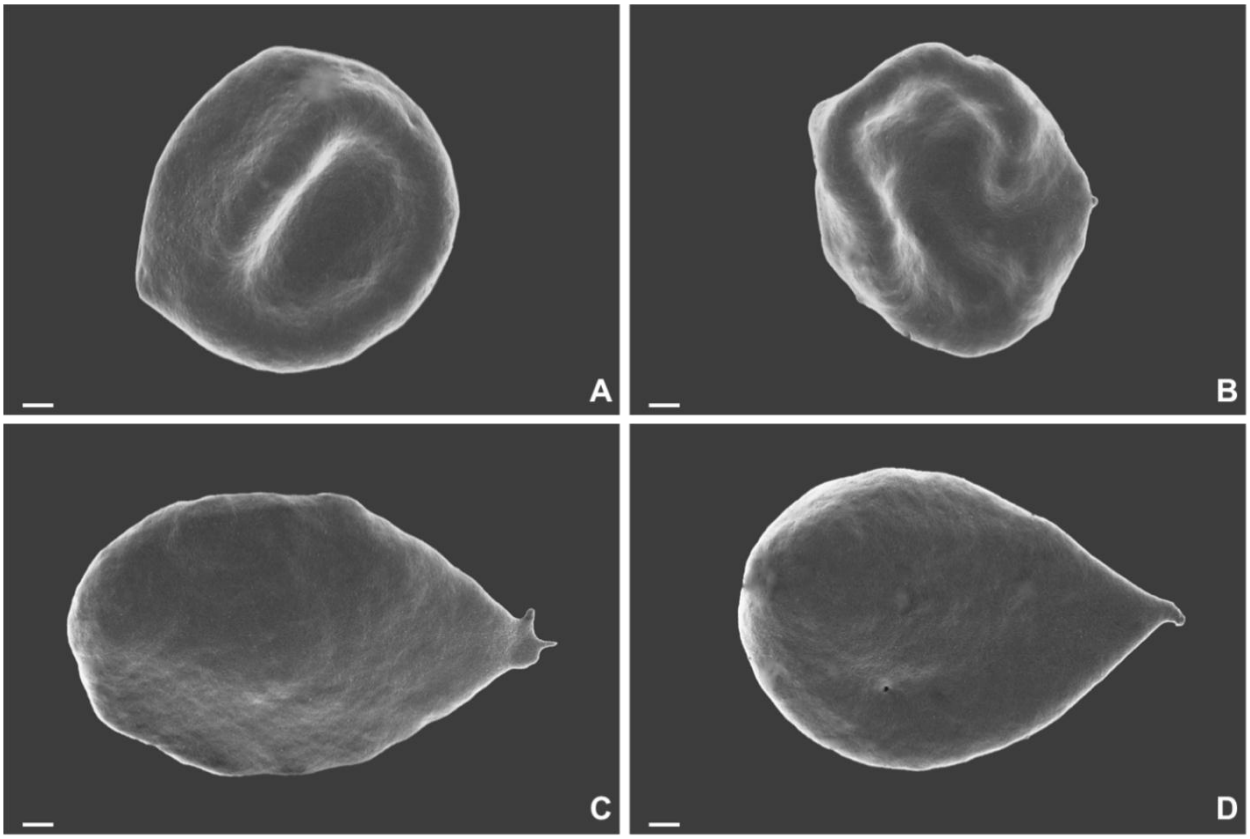


Figure 8.1. Red blood cell morphology representation of males in group MM. A: RBC from representative male 0h pre-ischaemia (MM-0), showing typical discoid shape and smooth membrane surface. B: RBC from representative male 2h post-reperfusion (MM-2), showing slight disruption from discoid shape. C: RBC from representative male 24h post-reperfusion (MM-24), here a unilateral projection is evident with a few processes extending from it. D: Representative RBC from male 48h post-reperfusion (MM-48). A unilateral projection is extended and a membrane pore is visible. Scale = 500 nm

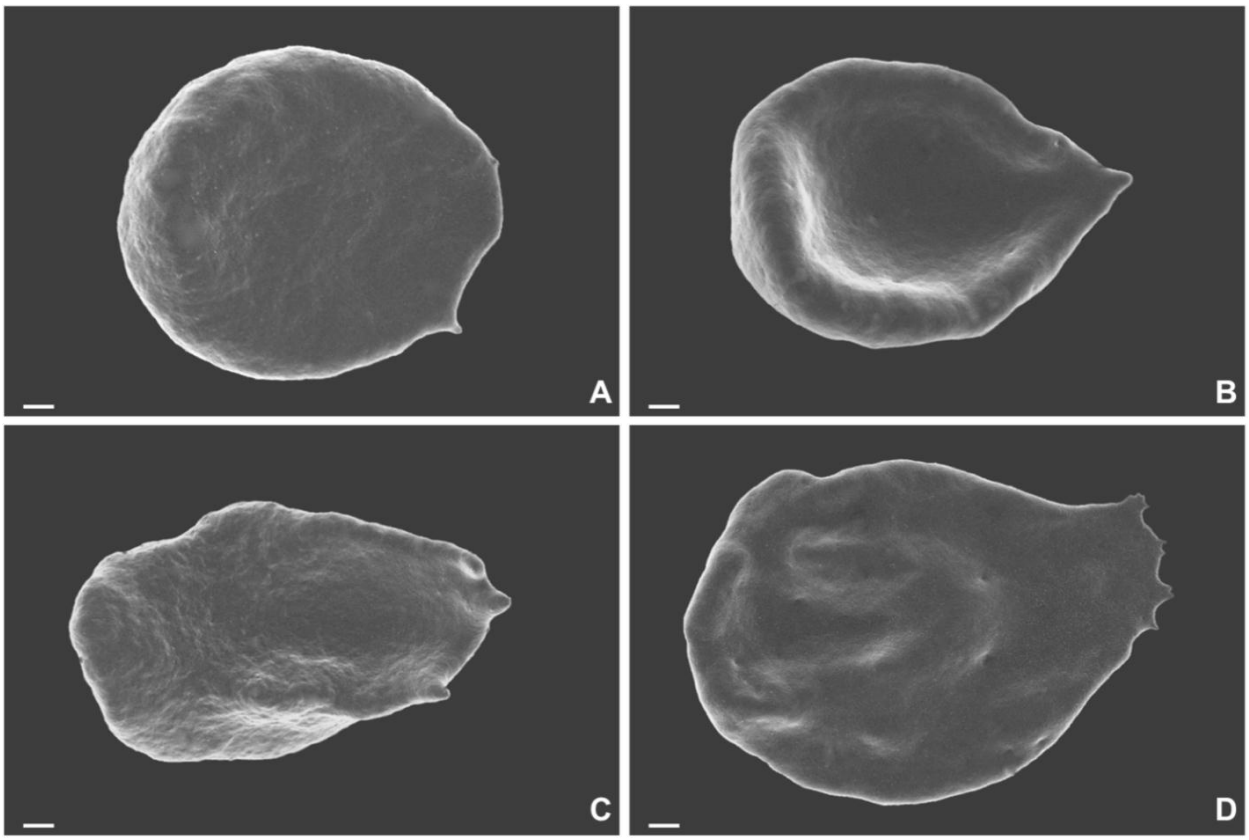


Figure 8.2. Red blood cell morphology representation of cyclic females in group MF. A: Representative RBC 0h pre-ischaemia (MF-0), showing typical discoid shape and smooth membrane surface. B: Representative RBC 2h post-reperfusion (MF-2), showing earlier unilateral projection formation than visualised in males. C: Representative RBC 24h post-reperfusion (MF-24), here the surface membrane is no longer smooth, and shape has deviated far from discoid. D: Representative RBC 48h post-reperfusion (MF-48), unilateral projection is broad but overall shape is returning to normal. Membrane pores are however visible, as in males of the same subgroup. Scale = 500 nm

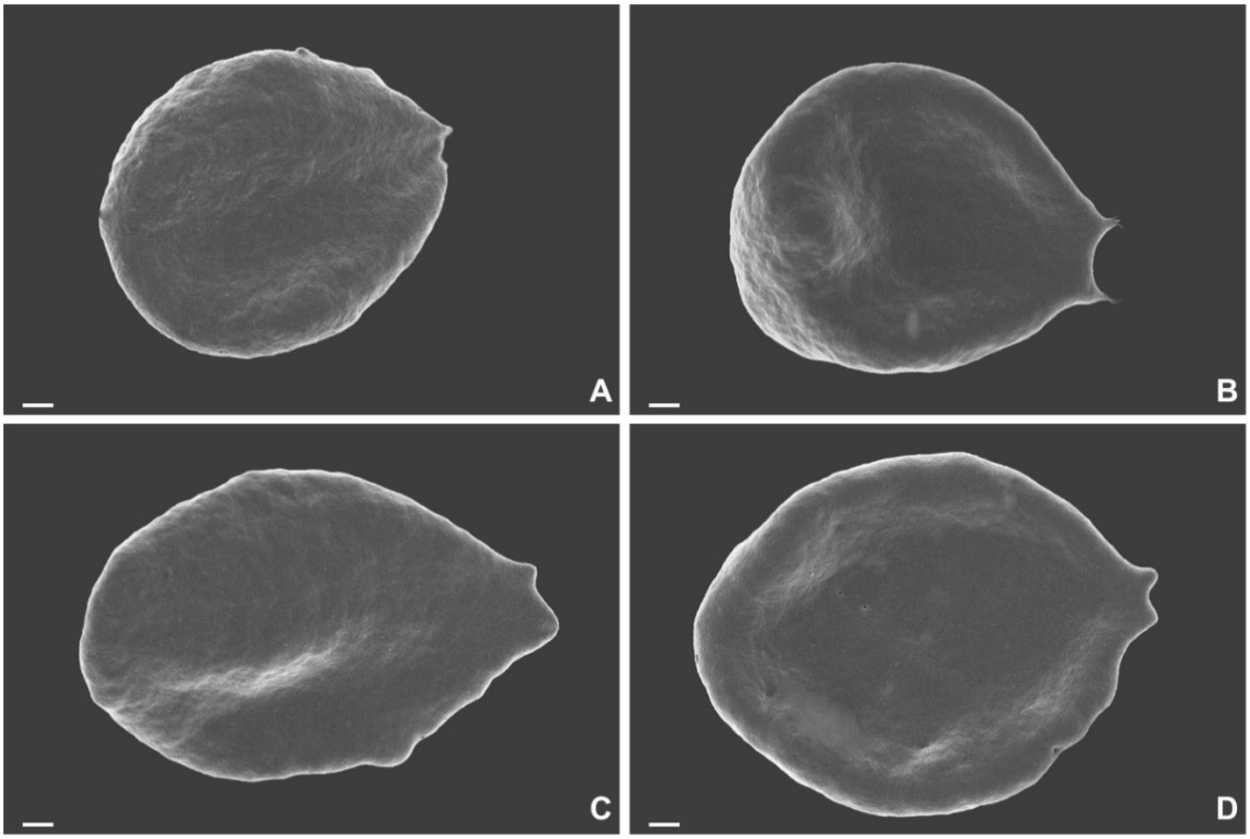


Figure 8.3. Red blood cell morphology representation of acyclic females in group OVX. A: Representative RBC from ovariectomised female, 0h pre-ischaemia (OVX-0). Normal discoid shape is visible, but membrane is not smooth. B: Representative RBC 2h post-reperfusion (OVX-2). Unilateral projections are visible with minor processes at their ends. Shape is still discoid in totality. C: Representative RBC 24h post-reperfusion (OVX-24). Here RBC has also lost its discoid shape like cyclic females, but membrane surface is smoother. D: Representative RBC 48h post-reperfusion (OVX-48). Recovery begins, and RBC becomes more discoid, though still with unilateral projection present and membrane pore present as in both other groups at this time. Scale = 500 nm

Platelet ultrastructure followed the same trends as neural tissue damage results, but RBCs could not be assessed in the same manner as their change in morphology is less specific of time. Overall, there is an indication that RBCs are not as sensitive to changes in haemostasis as platelets are. Their role is however one of stabilising thrombi, and thus they extend unilateral projections to interact with fibrin fibres when the haemostatic system is activated. This was noted by researchers who published data of a clear formation of pseudopodia-like extensions which in some instances were fused to fibrin fibres. They also noted that at higher magnifications, indentations were visible at sites of connection of these cellular extensions with fibrin fibres. They showed that RBCs also fuse to one another through the extension of these projections (Lipinski 2010, Lipinski *et al.* 2012). It is suggested that this morphology and interaction is what leads to lytic resistance in thromboembolic cerebral ischaemia patients.

The morphological interactions of RBCs with fibrin fibres and each other noted by the researchers above are indeed confirmed in this study's ischaemic groups when assessing direct RBC interactions in a thrombus itself rather than the outliers of a thrombus. The most illustrative examples from post-reperfusion subgroups (2h, 24h and 48h) are displayed in Figure 8.4.A-F to demonstrate the different types of interaction that are observed when the haemostatic system is under stress from cerebral insult. RBCs interact with fibrin fibres in various ways – RBCs are often found trapped within a thrombus by the fibrin fibers which strengthen the coagulum (Figure 8.4.A), they are capable of extending projections (not only unilaterally) to connect with fibrin fibres themselves (Figure 8.4.B) and fibrin fibres can also connect directly to the flat surfaces of RBCs (Figure 8.4.C). In terms of RBC interaction with one another – RBCs are capable of connecting directly to each other through the extension of their projections (Figure 8.4.D) and through interdigitation (Figure 8.4.E). Figure 8.4.F displays indentations at a site of previous connection with another RBC.

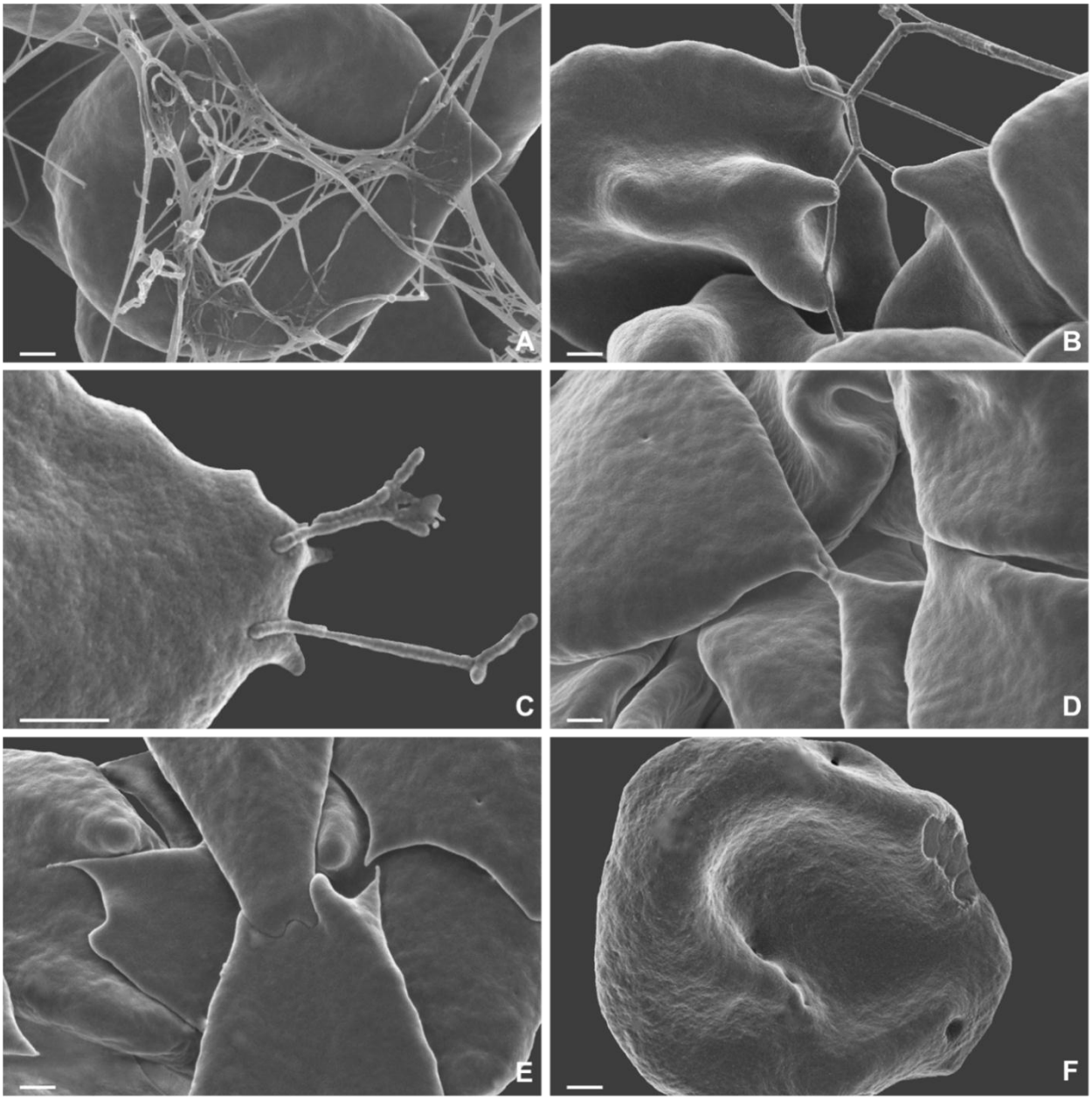


Figure 8.4. Red Blood Cell interactions with fibrin fibres and each other. A: RBC trapped by fibrin network, B: RBCs extending projections to connect with fibrin fibres, C: High magnification of fibrin fibres connecting to RBC with indentations visible at sites of connection, D: RBCs extending projections to connect with each other, E: RBCs with projections interdigitating, F: RBC with indentations from another RBC that was interdigitated with it at the time of fixation. Scale = 500nm

Overall it would appear that when assessing RBCs adjacent to a coagulum, there is a trend in their change of form as RBCs move towards interacting within a thrombus. At 0h or pre-ischaemia, RBCs display the expected discoid morphology. Subsequent to ischaemic insult, RBCs morph to form projections which allow them to interact both with each other and with fibrin fibres in the stabilization of a thrombus. It seems that by 48h post-reperfusion, as RBCs begin to return to normal morphology upon the second BBB dysfunction peak which causes another haemostatic disturbance (evident in platelet morphology in Chapter 7), membrane pores form on the surfaces of RBCs, which were not evident before the 48h point. Transmembrane proteins in RBCs are known to open pores to enable molecules such as glucose to diffuse into the cell for metabolic requirements. These requirements may be accentuated upon recovery from ischaemic injury.

When assessing the coagulum itself, it was found that RBCs do not only extend unilateral projections, but once they are localised and involved in physical interaction, they extend projections multilaterally to connect and interact with fibrin fibres and other RBCs. RBCs thus not only become trapped within a thrombus to stabilise it, but also alter their morphology to strengthen a clot by extending projections, forming connections and interdigitating with cellular constituents in their surroundings. The tighter these bonds, the more resistant these thrombi would conceivably be to lysis and, this is indeed an established characteristic of thrombi in cases of cerebral ischaemia in practice.

Chapter 9: Concluding Discussion

9.1. Introduction

Cerebral ischaemia, particularly by thromboembolism, is one of the forerunning causes of disability and/or death globally (Braeuninger & Kleinschnitz 2009, Elkins & Johnston 2003). After cerebral hypoperfusion, oxidative stress peaks rapidly and then progressively lowers after a few hours. Inflammation should be evident very early as haemostatic activation takes place within minutes of insult, with minimal inflammation still present after a few days. Dysfunction of the blood-brain barrier (BBB) is biphasic, the second peak being substantially higher than the first. The initial peak is evident within hours of ischaemia and the second peak, which is apparent within days (approximately at 48h), is most likely the period at which secondary cerebral injury becomes apparent (Brouns & De Deyn 2009, Gibson *et al.* 2005, Herd & Page 1994, Saenger & Christenson 2010, Wang *et al.* 2007).

It is well known that cyclic females present with a lower incidence of cerebral ischaemia than males and that the risk of cerebral ischaemia increases in both sexes with age (McCullough & Hurn 2003, Mitka 2006, Suzuki *et al.* 2009). Consequently, it is ostensible that sex hormones have a role in neuroprotection and the decline thereof as individuals' age (Braeuninger & Kleinschnitz 2009, Gibson *et al.* 2005, Wolf 1990). Cerebral ischaemia triggers a series of events including inflammation, which is deeply interconnected with thrombosis; as thrombosis amplifies inflammation and inflammation can produce local thrombosis (Myers & Wakefield 2005, Stewart *et al.* 1974). Inflammation is known to potentiate neural injury when triggered by the cascade of cerebral ischaemia (Wang *et al.* 2007). This reveals that the cerebral ischaemic cascade, its association to inflammation and thrombosis, as well as their connection to each other are intricately intertwined.

Oestrogen, especially the 17β-oestradiol isoform, is known to be neuroprotective in a myriad of mechanisms; a few of which include anti-inflammatory actions and the regulation of coagulation factors affecting thrombosis ability, as well as a role in delaying apoptosis (Drača 2009, Manthey & Behl 2006). Central to both thrombosis and inflammation are platelets. Not only is the localisation of platelets essential to thrombus initiation, formation and stabilisation; but in areas of ischaemia/reperfusion injury in cerebral ischaemia, platelets co-localise with leukocytes,

closely linking haemostatic thrombotic and inflammatory responses (Libby & Simon 2001, Ostrovsky *et al.* 1998). It is also understood that sex hormones alter the function and morphology of platelets (Bailey *et al.* 2009, Kadir *et al.* 1999, Leng *et al.* 2004, Mendelsohn & Karas 1999, Peters *et al.* 2002, Wong *et al.* 2008) and changes in their morphology may confirm inflammation before it is chemically apparent. Thus sex hormone levels, inflammatory processes and thrombotic mechanisms are profoundly interconnected in predicting the outcome and consequences of cerebral ischaemia.

In seeking to lessen the consequences of neural injury subsequent to cerebral ischaemia, focus must be placed on the mechanisms by which thrombosis and inflammation can be reduced as soon as possible after insult, through identifying how processes progress in the natural states of haemostasis and neuroprotection. Therefore three groups of animals were studied: males, cyclic and acyclic females. Each of these groups were further divided into four subgroups – designed to study the progression of cerebral ischaemia – consisting of 0h or pre-ischaemic controls, 2h post-reperfusion for immediate injury determination, 24h post-reperfusion for determination of maximal tissue injury and 48h post-reperfusion for analysis around the onset of possible regeneration. Analysis of multiple factors allowed us to elucidate that oestrogen not only directly affects neural tissue but also has a role in haemostasis, mechanisms of which would appease neural injury. Understanding where oestrogen as well as other hormones act to soothe the consequences of ischaemic injury may assist greatly in the improvement of treatment strategies.

9.2. Findings

From neural tissue injury results it was deduced that subsequent to induction of cerebral ischaemia, neural tissue in males progressively deteriorated from 2h to 48h post-reperfusion due to the absence of high levels of oestrogen and therefore the inability to curb the inflammatory response and suppress apoptosis. Cyclic female neural tissue presented with initial injury at 2h though less severe consequences were apparent than in males. By 24h regeneration variables became apparent, with oestrogen possibly compensating to curb the peak in inflammation and initiating recovery by stalling apoptosis and necrosis. At 48h, where the second and highest peak of BBB dysfunction manifested, secondary neural loss became evident. Acyclic females seemed to display a smaller degree of initial injury than cyclic females, which was proposed to be a result of the inherent state of inflammation found in menopausal females – termed

thrombotic preparedness – and thus the inflammatory shock to the haemostatic system was lessened initially. By 24h, acyclic females presented with greatest neural tissue injury, due to the absence of high levels of oestrogen and therefore the inability to suppress necrosis and initiate recovery. At 48h however, the neural tissue of acyclic females seemed to have recovered to a great extent, almost similar to control levels. Return to the state of thrombotic preparedness in these females may potentially have curbed the injury initiated by the second peak of BBB dysfunction. Overall, across all time intervals, cyclic females presented significantly less neural tissue injury than males and acyclic females.

Males and cyclic females were found to have similar neural tissue protein levels, whereas acyclic females were shown to have significantly less soluble neural tissue protein. This served to confirm that proteins, including oestrogen receptors, must be present in similar concentrations in both males and females for hormone treatment to protect males from ischaemic injury in other research as endogenous oestrogen protects cyclic females. Furthermore, it was confirmed that certain neural proteins, particularly oestrogen receptors were indeed downregulated subsequent to ovariectomy, thus rendering hormone treatment in older acyclic females ineffective. Analysis of the correlation between neural tissue injury and neural tissue protein revealed a significant negative correlation between the two. This served to confirm that there is indeed an upregulation of protein synthesis upon neural injury for the purpose of both neural repair and the clearing of irreparably damaged cells.

In the analysis of 17β-oestradiol levels, it was found that cyclic females had significantly higher levels of female reproductive hormone than both males and acyclic females, confirming that ovariectomy had been performed successfully. Subsequent to cerebral ischaemia/reperfusion, it was found that circulating 17β-oestradiol was upregulated across all groups at 2h post-reperfusion where initial ischaemic injury was apparent. The expression of oestrogen receptors is known to be upregulated subsequent to cerebral ischaemia; therefore oestrogen too must be upregulated for increased uptake in the neural tissue. In both female groups, data enabled confirmation that where 17β-oestradiol levels were lowest in circulation, the least neural tissue injury was evident, strengthening the idea that as the expression of oestrogen receptors is upregulated, more oestrogen is taken up in neural tissue to act in neuroprotection. Analysis of the correlation between neural tissue injury and 17β-oestradiol levels revealed a significant negative correlation between the two. This confirmed that oestrogen is neuroprotective as higher levels of oestrogen gave rise to neural injury of lesser degree.

Platelet ultrastructure followed the same trends realised in the neural tissue injury results. Cyclic females were perceived to be the gold-standard in assessing the progression of events subsequent to cerebral ischaemia. At 0h pre-ischaemia, platelets displayed perfect control morphology, with smooth membranes and the extension of few pseudopodia. Upon initial injury assessment at 2h post-ischaemia, platelets demonstrated typical inflammatory morphology, with the extension of a multitude of pseudopodia. At 24h post-ischaemia, where maximal damage was expected, oestrogen was shown to play its most significant role in that platelets returned to pre-inflammatory morphology due to the noteworthy anti-inflammatory mechanisms exerted by the presence of high levels of oestrogen. In these females, 24h was indeed the point when the smallest neural tissue injury percentage was apparent. At 48h post-reperfusion, platelets appeared more activated once again, though less so than platelets at 2h post-reperfusion. It was proposed that this reactivation was reflective of the second phase of BBB disruption which again changed the balance in haemostasis to a more inflammatory state. In males and acyclic females, insignificant concentrations of oestrogen were present rendering them incapable of protecting or masking the coagulation system from haemostatic disturbance subsequent to cerebral ischaemia. Therefore, at 24h post-reperfusion, both these groups expressed a prominent necrotic platelet ultrastructure which did not exist in cyclic females who had full access to the protective effects of oestrogen. In males as well as acyclic females, platelet recovery or return to the direction of normalised morphology was noted at 48h post-reperfusion. This corresponded to what was seen in the neural tissue assessment of acyclic females and correlated therefore perfectly with the ultrastructure of the platelets. In the males however, 48h was the point when maximal neural tissue damage was evident. Thus commencement of recovery in the males, though not immediately present in neural tissue integrity seemed evident in platelet morphology.

To the evidence of high levels of inflammation visualised by platelet activation, correlation was made to reduced free platelets in counts. This provided evidence that in inflamed states, more platelets aggregate and thus are not freely available in circulation for platelet counts. Platelet counts were additionally shown to be significantly highest in acyclic females and higher in males than in cyclic females. Plasma fibrinogen levels were also found to be at their lowest when inflammation was at its highest, showing that fibrinogen is lysed to form fibrin and thus less was available in circulation when platelets were most reactive. Where platelets were found to be necrotic at 24h post-reperfusion in males and acyclic females, fibrinogen levels were at their highest in circulation, and these levels lowered again upon recovery from platelet necrosis. The

finding that less fibrinogen was present in circulation when inflammation was pronounced confirmed that more reactive platelets bind more fibrinogen. Fibrinogen levels were also found to be higher in males and acyclic females than in cyclic females, confirming that coagulation factors and platelet counts are lowered by oestrogen. Analysis of the correlation between both platelet counts and fibrinogen levels to 17β-oestradiol levels also revealed significant negative correlations. This confirmed that oestrogen lowers platelet counts and fibrinogen levels in addition to lessening their reactivity.

The final morphological assessment of red blood cell interaction in a coagulum revealed an interactive trend within a thrombus visible through a change in cellular shape. At 0h pre-ischaemic red blood cells displayed typical discoid morphology, but post-reperfusion they extended projections which allowed them to connect and interact with adjacent fibrin fibres and other red blood cells. In so doing, red blood cells become intertwined within a thrombus to stabilise and strengthen its structure. It is known that the tighter these bonds, the more resistant thrombi are to lysis, which is an established characteristic of thrombi in cases of cerebral ischaemia.

In essence, neural tissue injury was shown to be lessened in cyclic females compared to males and acyclic females who presented with higher levels of tissue injury. Oestrogen receptors are known to be upregulated in neural tissue upon ischaemic insult. Confirming this, it was found that circulating oestrogen too is upregulated subsequent to cerebral ischaemic to allow for increased uptake by oestrogen receptors in neural tissue upon insult. It is known that oestrogen supplementation prior to induction of cerebral ischaemia is capable of protecting neural tissue in males and acyclic females (only directly after ovariectomy but not after prolonged absence of the hormone). In validation of this, it was shown that soluble neural proteins which include oestrogen receptors are present at similar levels in both males and cyclic females, but were downregulated in acyclic females after ovariectomy. Oestrogen was shown to have a definite role in regulating thrombosis, as coagulation factor levels were lower in cyclic females than in males and acyclic females and cellular reactivity was also better controlled in the presence of high levels of oestrogen. Platelet ultrastructure undeniably reflected the presence of inflammation subsequent to cerebral ischaemia and to a large extent mimicked the levels of neural tissue injury as well as the phases of the cerebral ischaemic cascade. Finally, red blood cells were shown to stabilise thrombi through cellular interactions which could contribute to the lysis resistance of clots characteristic in patients of cerebral ischaemia.

9.3. Implications

It was hypothesised that oestrogen would display neuroprotective and anti-inflammatory properties in an exclusively hyperglycaemic two-vessel occlusion model for induction of experimental cerebral ischaemia. The hypothesis would be supported by findings of lesser neural tissue injury in cyclic females than in males and acyclic females. In addition, it was expected that the ultrastructure of coagula and thrombotic interactions would be altered from normal in animals subjected to experimental cerebral ischaemia, due to alteration of their inflammatory profiles by ischaemic cascade phases.

Collectively the results of this study strongly suggest that oestrogen is indeed neuroprotective through various actions including roles in the regulation of thrombosis and inflammation, targeting neural cells, inhibiting apoptosis and exerting anti-inflammatory and antioxidant effects. It is evident that under the influence of oestrogen in cyclic females, there is reduced neural tissue injury as well as a smaller degree of inflammation evident in platelet activation morphology when compared to males and acyclic females. Furthermore it is unmistakeable that neural injury is closely shadowed, if not preceded, by inflammatory changes in the coagulation system, particularly manifested in platelet ultrastructure. It is therefore suggested that platelets may indeed be used successfully to follow the progression of events of cerebral ischaemia and possibly assist in the assessment of treatment strategies and their effects on haemostasis.

This research leads to an understanding that inflammation is evident very early after ischaemic insult and if such inflammation is not curbed, necrosis of platelets and more severe injury to neural tissue follows. Thus the development of agents which not only target thrombosis, i.e. lysing clots which may have caused ischaemia through obstruction of blood flow, but also which control inflammation must be entered into for advancement in treatment. In fact, even before it has been determined whether a stroke has been caused by thromboembolism or haemorrhage, it may be beneficial to immediately target inflammation to prevent most severe consequences in human patients.

9.4. Direction

Because it is not possible to obtain more than 4ml of blood from female animals of breeding age, it was not possible to allocate blood for further analysis of coagulation factors and stroke markers. It would thus be beneficial to undertake a secondary study, employing the same methodology, again quantifying cerebral ischaemic injury in addition to quantifying thrombin levels (to facilitate data of coagulation activation) and stroke markers (to support data of neural ischaemic injury severity). Furthermore, it will be of interest to include two groups – males and ovariectomised (acyclic) females with slow-release oestradiol implants for hormone supplementation – to further assess the effects of oestrogen on cerebral ischaemic consequences. Of particular interest would be the assessment of platelets in these groups to determine if hormone supplementation curbs or worsens thrombotic preparedness and also determine whether supplementation of oestrogen prevents necrosis of platelets at 24h post-ischaemia. Moreover, because platelets are shown to be highly reactive at 2h post-reperfusion due to high levels of inflammation, necrotic at 24h post-reperfusion and recovered but still inflamed at 48h post-reperfusion; it would be of great interest to assess the progression of events between these two time intervals, especially to determine if at any point apoptosis precedes the platelet necrosis seen at 24h post-reperfusion and how platelets recover after the period where they are shown to be necrotic.

In terms of treatment strategies, mediators of inflammatory processes are of interest. It is known that neutrophils contribute to neural injury amongst others. Administration of antioxidants and/or anti-inflammatory agents to animals before and/or soon after cerebral ischaemia will be useful to determine whether cyclic females are protected to a greater degree and whether males and acyclic females are protected to a degree similar to that inherently afforded by the presence of oestrogen in cyclic females. In essence, expanding on this research can serve to uncover a multitude of mechanisms by which persons can be protected from cerebral ischaemia and by which patients may be afforded a more optimistic outcome subsequent to suffering cerebral ischaemia.

Chapter 10: References

Ace Animals, Inc. Sprague Dawley. 2006. Available at: http://www.aceanimals.com/SpragueDawley.htm Accessed: 20/06/2010

Ajjan R, Lim BC, Standeven KF, Harrand R, Dolling S, Phoenix F, Greaves R, Abou-Saleh RH, Connell S, Smith DA, Weisel JW, Grant PJ, Ariens RA. Common variation in the C-terminal region of the fibrinogen beta-chain: effects on fibrin structure, fibrinolysis and clot rigidity. *Blood* 2008; 111:643-50

Alkayed NJ, Harukuni I, Kimes AS, London ED, Traystman RJ, Hurn PD. Gender-linked brain injury in experimental stroke. *Stroke* 1998; 29:159-65

Alkayed NJ, Murphy SJ, Traystman RJ, Hurn PD, Miller VM. Neuroprotective effects of female gonadal steroids in reproductively senescent female rats. *Stroke* 2000; 31:161-8

Altman R. Risk factors in coronary atherosclerosis athero-inflammation: the meeting point. *Thrombosis Journal* 2003; 1:4 (11 pages)

Alzahrani SH & Ajjan RA. Coagulation and fibrinolysis in diabetes. *Diabetes and Vascular Disease Research* 2010; 7:260-73

André P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD. CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. *Nature Medicine* 2002; 8:247-52

Bailey AL, Scantlebury DC, Smyth SS. Thrombosis and antithrombotic therapy in women. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2009; 29:284-8

Bake S & Sohrabji F. 17beta-estradiol differently regulates blood-brain barrier permeability in young and aging female rats. *Endocrinology* 2004; 145:5471-5

Barone FC, Hillegass LM, Tzimas MN, Schmidt DB, Foley JJ, White RF, Price WJ, Feuerstein GZ, Clark RK, Griswold DE, Sarau HM. Time-related changes in myeloperoxidase activity and leukotriene B4 receptor binding reflect leukocyte influx in cerebral focal stroke. *Molecular and Chemical Neuropathology* 1995, 24:13-30

Bar-Shavit R, Kahn AJ, Mann KG, Wilner GD. Identification of a thrombin sequence with growth factor activity on macrophages. *Proceedings of the National Academy of Sciences USA* 1986; 83:976-80

Bastyr EJ 3rd, Kadrofske MM, Vinik AI. Platelet activity and phosphoinositide turnover increase with advancing age. *American Journal of Medicine* 1990; 88:601-6

Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartowski HM. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke* 1986; 17:1304-8

Bio-Rad Laboratories. Bio-Rad Protein Assay. 2012. Available at: http://www.bio-rad.com Accessed: 27/06/2012

Boer GJ, Dozy MH, Uylings HBM. Cerebellar DNA and tissue water changes in the brain of diabetes insipidus brattleboro rats are already present at birth. *International Journal of Developmental Neuroscience* 1984; 2:301-4

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976; 72:248-54

Braeuninger S & Kleinschnitz C. Rodent models of focal cerebral ischemia: procedural pitfalls and translational problems. *Experimental & Translational Stroke Medicine* 2009; 1:8 (11 pages)

Brand K, Fowler BJ, Edgington TS, Mackman N. Tissue factor mRNA in THP-1 monocytic cells is regulated at both transcriptional and posttranscriptional levels in response to lipopolysaccharide. *Molecular and Cellular Biology* 1991; 11:4732-8

Brill A. A ride with ferric chloride. *Journal of Thrombosis and Haemostasis* 2011; 9:776-8

Brouns R & De Deyn PP. The complexity of neurobiological processes in acute ischemic stroke. *Clinical Neurology and Neurosurgery* 2009; 111:483-95

Burstein SA. Cytokines, platelet production and hemostasis. *Platelets* 1997; 8:93-104

Butcher EC. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* 1991; 67:1033-6

Butenas S & Mann KG. Blood coagulation. *Biochemistry (Moscow)* 2002; 67:3-12

Camera M, Giesen PL, Fallon J, Aufiero BM, Taubman M, Tremoli E, Nemerson Y. Cooperation between VEGF and TNF-alpha is necessary for exposure of active tissue factor on the surface of human endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* 1999; 19:531-7

Carswell HV, Dominiczak AF, Macrae IM. Estrogen status affects sensitivity to focal cerebral ischemia in stroke-prone spontaneously hypertensive rats. *American Journal of Physiology. Heart and Circulatory Physiology* 2000; 278:H290-4

Chakraborty I, Mitra S, Gachhui R, Kar M. Non-haem iron-mediated oxidative stress in haemoglobin E beta-thalassaemia. *Annals Academy of Medicine Singapore* 2010; 39:13-6

Chen CJ, Cheng FC, Liao SL, Chen WY, Lin NN, Kuo JS. Effects of naloxone on lactate, pyruvate metabolism and antioxidant enzyme activity in rat cerebral ischemia/reperfusion. *Neuroscience Letters* 2000; 287:113-6

Chu X, Qi C, Zou L, Fu X. Intraluminal suture occlusion and ligation of the distal branch of internal carotid artery: an improved rat model of focal cerebral ischemia-reperfusion. *Journal of Neuroscience Methods* 2008; 168:1-7

Compton SJ & Jones CG. Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry* 1985; 151:369-74

Connolly ES Jr, Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, Stern DM, Solomon RA, Gutierrez-Ramos JC, Pinsky DJ. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *The Journal of Clinical Investigation* 1996; 97:209-16

Danesh J, Lewington S, Thompson SG, Lowe GD, Collins R. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *JAMA: Journal of the American Medical Association* 2005; 294:1799-809

Danton GH & Dietrich WD. Inflammatory mechanisms after ischemia and stroke. *Journal of Neuropathology and Experimental Neurology* 2003; 62:127-36

Dávalos A, Castillo J, Marrugat J, Fernandez-Real JM, Armengou A, Cacabelos P, Rama R. Body iron stores and early neurologic deterioration in acute cerebral infarction. *Neurology* 2000; 54:1568-74

Davis M, Mendelow AD, Perry RH, Chambers IR, James OF. Experimental stroke and neuroprotection in the aging rat brain. *Stroke* 1995; 26: 1072-8

Day SM, Duquaine D, Mundada LV, Menon RG, Khan BV, Raiagopalan S, Fay WP. Chronic iron administration increases vascular oxidative stress and accelerates arterial thrombosis. *Circulation* 2003; 107:2601-6

Day SM, Reeve JL, Pedersen B, Farris DM, Myers DD, Im M, Wakefield TW, Mackman N, Fay WP. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. *Blood* 2005; 105:192-8

D'Erasmo E, Acca M, Celi FS, Medici F, Palmerini T, Pisani D. Plasma fibrinogen and platelet count in stroke. *Journal of Medicine* 1993; 24:185-91

Demopoulos HB, Milvy P, Kakari S, Ransohoff J. Molecular aspects of membrane structure in cerebral edema. In: Reulen HJ & Shurmann K (Eds), Steroids and Brain Edema. Springer-Verlag, New York. 1972; 29-39

Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood* 1996; 88:146-57

Dietrich WD, Alonso O, Busto R. Moderate hyperglycaemia worsens acute blood-brain barrier injury after forebrain ischemia in rats. *Stroke* 1993; 24:111-6

Drača S. Are female sex steroids neuroprotective in experimental stroke. *Medical Hypotheses* 2009; 73:1051-2

Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *The American Journal of Pathology* 1989; 134:1087-97

Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finkelstein SP, Rau SW, Wise PM. Estradiol protects against ischemic injury. *Journal of Cerebral Blood Flow and Metabolism* 1998; 18:1253-8

Dubal DB, Zhu H, Yu J, Rau SW, Shughrue PJ, Merchenthaler I, Kindy MS, Wild PM. Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. *Proceedings of the National Academy of Sciences USA* 2001; 98:1952-7

Duhamel RC, Meezan E, Brendel K. The addition of SDS to the Bradford dye-binding protein assay, a modification with increased sensitivity to collagen. *Journal of Biochemical and Biophysical Methods* 1981; 5:67-74

Eklöf B & Siesjö BK. Cerebral blood flow in ischemia caused by carotid artery ligation in the rat. *Acta Physiologica Scandinavica* 1973; 87:69-77

Eklöf B & Siesjö BK. The effect of bilateral carotid artery ligation upon the blood flow and energy state of the rat brain. *Acta Physiologica Scandinavica* 1972; 86:155-65

Elkins JS & Johnston SC. Thirty-year projections for deaths from ischemic stroke in the United States. *Stroke* 2003; 34:2109-12

Emms H & Lewis GP. Sex and hormonal influences on platelet sensitivity and coagulation in the rat. *British Journal of Pharmacology* 1985; 86:557-63

Esmon CT. Inflammation and thrombosis. *Journal of Thrombosis and Haemostasis* 2003; 1:1343-8

Fatah K, Hamsten A, Blombäck B, Blombäck M. Fibrin gel network characteristics and coronary heart disease: relations to plasma fibrinogen concentration, acute phase protein, serum lipoproteins and coronary atherosclerosis. *Thrombosis and Haemostasis* 1992; 68:130-5

Fatah K, Silveira A, Tornvall P, Karpe F, Blombäck M, Hamsten A. Proneness to formation of tight and rigid fibrin gel structures in men with myocardial infarction at a young age. *Thrombosis and Haemostasis* 1996; 76:535-40

Ferreiro J, Gómez-Hospital JA, Angiolillo DJ. Platelet abnormalities in diabetes mellitus. *Diabetes and Vascular Disease Research* 2010; 7:251-9

Franchini M. Hemostasis and aging. *Critical Reviews in Oncolology/Hematology* 2006; 60:144-51

Frenette PS, Denis CV, Weiss L, Jurk K, Subbarao S, Kehrel B, Hartwig JH, Vestweber D, Wagner DD. P-selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions *in vivo*. *The Journal of Experimental Medicine* 2000; 191:1413-22

Friedenauer S & Berlet HH. Sensitivity and variability of the Bradford protein assay in the presence of detergents. *Analytical Biochemistry*1989; 178:263-8

Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB, Michelson AD. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *Journal of the American College of Cardiology* 1998; 31:352-8

Gailani D & Renné T. Intrinsic pathway of coagulation and arterial thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2007; 27:2507-13

Gaston LW, Brooks JE, Blumenthal HJ, Miller CE. A study of blood coagulation following an acute stroke. *Stroke* 1971; 2:81-7

Gerch KC, Nagaswami C, Weisel JW. Fibrin network structure and clot mechanical properties are altered by incorporation of erythrocytes. *Thrombosis and Haemostasis* 2009; 102:1169-75

Gharacholou SM & Becker RC. Hemostasis and thrombosis in older adults. *Journal of Thrombosis and Thrombolysis* 2009; 27:249-51

Gibson CL, Constantin D, Prior MJ, Bath PM, Murphy SP. Progesterone suppresses the inflammatory response and nitric oxide synthase-2 expression following cerebral ischemia. *Experimental Neurology* 2005; 193:522-30

Gimbrone MA Jr. Vascular endothelium: an integrator of pathophysiologic stimuli in atherosclerosis. *The American Journal of Cardiology* 1995; 75:67B-70B

Ginsberg MD & Busto R. Rodent models of cerebral ischemia. *Stroke* 1989; 20:1627-42

Goel MS & Diamond SL. Neutrophil enhancement of fibrin deposition under flow through platelet-dependent and -independent mechanisms. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2001; 21:2093-8

Graham DI, McIntosh TK, Maxwell WL, Nicoll JA. Recent advances in neurotrauma. *Journal of Neuropathology and Experimental Neurology* 2000; 59:641-51

Grant PJ. Inflammatory, atherothrombotic aspects of type 2 diabetes. *Current Medical Research Opinion* 2005; 21:S5-12

Guha M & Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal* 2001; 13:85-94

Hallenbeck JM. Significance of the inflammatory response in brain ischemia. *Acta Neurochirurgica Supplement* 1996; 66:27-31

Harlan Laboratories, Inc. Sprague Dawley Rat, Neuroscience. 2008. Available at: www.harlan.com/.../588b64edc48f460f9635de28b9ef60a1.pdf Accessed: 29/06/2010

Hawiger J. Mechanisms involved in platelet vessel wall interaction. *Thrombosis and Haemostasis* 1995; 74:369-72

Hawk T, Zhang YQ, Rajakumar G, Day AL, Simpkins JW. Testosterone increases and estradiol decreases middle cerebral artery occlusion lesion size in male rats. *Brain Research* 1998; 796:296-8

Henn V, Slupsky JR, Gräfe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; 391:591-4

Herd CM & Page CP. Pulmonary immune cells in health and disease: platelets. *The European Respiratory Journal* 1994; 7:1145-60

Hod EA & Spitalnik SL. Harmful effects of transfusion of older red blood cells: iron and inflammation. *Transfusion* 2011; 51:881-5

Hoyte LC & Buchan AM. Animal models of stroke. In: Hof PR & Mobbs CV (Eds), Handbook of the Neuroscience of Aging. Elsevier Inc., Italy. 2009; 575-82

Hume R. The relationship to age and cerebral vascular accidents of fibrin and fibrinolytic activity. *Journal of Clinical Pathology* 1961; 14:167-71

Hynes RO. Fibronectins. Springer Verlag, New York. 1990

Ivanova MM, Mazhawidza W, Dougherty SM, Klinge CM. Sex differences in estrogen receptor subcellular location and activity in lung adenocarcinoma cells. *American Journal of Respiratory Cell and Molecular Biology* 2010; 42:320-30

Jayachandran M & Miller VM. Human platelets contain estrogen receptor alpha, caveolin-1 and estrogen receptor associated proteins. *Platelets* 2003; 14:75-81

Jeziersky MK & Sohrabji F. Neurotrophin expression in the reproductively senescent forebrain is refractory to estrogen stimulation. *Neurobiology of Aging* 2001; 22:309-19

Jezierski MK & Sohrabji F. Region- and peptide-specific regulation of the neurotrophins by estrogen. *Brain Research: Molecular Brain Research* 2000; 85:77-84

Jia J, Guan D, Zhu W, Alkayed NJ, Wang MM, Hua Z, Xu Y. Estrogen inhibits Fas-mediated apoptosis in experimental stroke. *Experimental Neurology* 2009; 215:48-52

Joshi CN, Jani SK, Murthy PS. An optimised triphenyltetrazolium chloride method for identification of cerebral infarcts. *Brain Research Protocols* 2004; 13:11-7

Kadir RA, Economides DL, Sabin CA, Owens D, Lee CA. Variations in coagulation factors in women: effects of age, ethnicity, menstrual cycle and combined oral contraceptive. *Thrombosis and Haemostasis* 1999; 82:1456-61

Kiyota K. Soluble protein fraction of the brain tissue in relation to maturation of the brain. *Psychiatry and Clinical Neurosciences* 1959; 13:15-22

Kleinschnitz C, Pozgajova M, Pham M, Bendszus M, Nieswandt B, Stoll G. Targeting platelets in acute experimental stroke: impact of glycoprotein Ib, VI, and IIb/IIIa blockade on infarct size, functional outcome, and intracranial bleeding. *Circulation* 2007; 115:2323-30

Kohn DF & Clifford CB. Biology and diseases of rats. In: Fox JG, Anderson LC, Loew FM, Quimby FW (Eds), Laboratory animal medicine. Academic Press, New York. 2002; 121-65

Konstantopoulos K, Neelamegham S, Burns AR, Hentzen E, Kansas GS, Snapp KR, Berg EL, Hellums JD, Smith CW, McIntire LV, Simon SI. Venous levels of shear support neutrophil-platelet adhesion and neutrophil aggregation in blood via P-selectin and beta2-integrin. *Circulation* 1998; 98:873-82

Kranzhöfer R, Clinton SK, Ishii K, Coughlin SR, Fenton JW 2nd, Libby P. Thrombin potently induces cytokine production by human vascular smooth muscle cells but not in mononuclear phagocytes. *Circulation Research* 1996; 79:286-94

Kristofiková Z, Benesová O, Tejkalová H. Changes in water solubility of proteins in aging rat brain. *Archives of Gerontology and Geriatrics 1991;* 12:41-8

Kuijper PH, Gallardo Torres HI, van der Linden JA, Lammers JW, Sixma JJ, Koenderman L, Zwaginga JJ. Platelet-dependent primary hemostasis promotes selectin- and integrin-mediated neutrophil adhesion to damaged endothelium under flow conditions. *Blood* 1996; 87:3271-81

Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpley M, Fukai T, Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* 2001; 103:1282-8

Lee AJ, Fowkes GR, Lowe GD, Rumley A. Determinants of fibrin D-dimer in the Edinburgh Artery Study. *Arteriosclerosis, Thrombosis, and Vascular Biology* 1995; 15:1094-7

Leng XH, Hong SY, Larrucea S, Zhang W, Li TT, López JA, Bray PF. Platelets of female mice are intrinsically more sensitive to agonists than are platelets of males. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2004; 24:376-81

Liao S, Chen W, Kuo J, Chen C. Association of serum estrogen level and ischemic neuroprotection in female rats. *Neuroscience Letters* 2001; 297:159-62

Libby P & Simon DI. Inflammation and thrombosis: the clot thickens. *Circulation* 2001; 130:1718-20

Lipinski B. Hydroxyl radical and its scavengers in health and disease. *Oxidative Medicine and Cellular Longevity* 2011; 2011:809696 (9 pages)

Lipinksi B. Modification of fibrin structure as a possible cause of thrombolytic resistance. *Journal of Thrombosis and Thrombolysis* 2010; 29:296-8

Lipinski B, Pretorius E, Oberholzer HM, van der Spuy WJ. Interaction of fibrin with red blood cells: the role of iron. *Ultrastructural Pathology* 2012; 36:79-84

Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *The Journal of Cell Biology* 1991; 115:223-34

Lowe GD, Rumley A, Mackie IJ. Plasma fibrinogen. *Annals of Clinical Biochemistry* 2004; 41:430-40

Lowe GD, Rumley A, Woodward M, Morrison CE, Philippou H, Lane DA, Tunstall-Pedoe H. Epidemiology of coagulation factors, inhibitors and activation markers: the Third Glasgow MONICA Survey. I. Illustrative reference ranges by age, sex and hormone use. *British Journal of Haematology* 1997; 97:775-84

Mackman N. Role of tissue factor in hemostasis, thrombosis, and vascular development. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2004; 24:1015-22

Macrae IM & Carswell HV. Oestrogen and stroke: the potential for harm as well as benefit. *Biochemical Society Transactions* 2006; 34:1362-5

Malý J. Diabetes mellitus and prothrombotic activity. *Vnitrni Lekarstvi* 2010; 56:284-8

Mann KG, van't Veer C, Cawthern K, Butenas S. The role of tissue factor pathway in initiation of coagulation. *Blood Coagulation and Fibrinolysis* 1998; 9:S3-7

Manthey D & Behl C. From structural biochemistry to expression profiling: neuroprotective activities of estrogen. *Neuroscience* 2006; 138:845-50

Mari D, Coppola R, Provenzano R. Hemostasis factors and aging. *Experimental Gerontology* 2008; 43:66-73

McBean DE & Kelly PAT. Rodent models of global cerebral ischemia: a comparison of two-vessel occlusion and four-vessel occlusion. *General Pharmacology* 1998; 30:431-4

McCann UD & Ricaurte GA. Caveat emptor: editors beware. *Neuropsychopharmacology* 2001; 24:333-6

McCullough LD & Hurn PD. Estrogen and ischemic neuroprotection: an integrated view. *Trends in Endocrinology and Metabolism* 2003; 14:228-35

McIlwain H & Bachelard HS. Biochemistry and the Central Nervous System. Churchill Livingstone, Edinburgh. 1985

Mendelsohn ME & Karas RH. The protective effects of estrogen on the cardiovascular system. *The New England Journal of Medicine* 1999; 340:1801-11

Miller DL, Yaron R, Yellin MJ. CD40L-CD40 interactions regulate endothelial cell surface tissue factor and thrombomodulin expression. *Journal of Leukocyte Biology* 1998; 63:373-9

Mitka M. Studies explore stroke's gender gap. *JAMA: The Journal of the American Medical Association* 2006, 295:1755-6

Morganti-Kossmann MC, Rancan M, Otto VI, Stahel PF, Kossmann T. Role of cerebral inflammation after traumatic brain injury: a revisited concept. *Shock* 2001; 16:165-77

Muszbek L, Bagoly Z, Bereczky Z, Katona E. The involvement of blood coagulation factor XIII in fibrinolysis and thrombosis. *Cardiovascular and Hematological Agents in Medicinal Chemistry* 2008; 6:190-205

Myers DD & Wakefield TW. Inflammation-dependent thrombosis. *Frontiers in Bioscience* 2005; 10:2750-7

Nagy E, Eaton JW, Jeney V, Soares MP, Varga Z, Galajda Z, Szentmiklósi J, Méhes G, Csonka T, Smith A, Vercellotti GM, Balla G, Balla J. Red cells, hemoglobin, heme, iron, and atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2010; 30:1347-53

Nagy I. Ageing of the cellular membrane: basic principles and pharmacological interventions. *Geriatrika* 1985; 1:102-11

Nagy I, Nagy K, Nagy V, Kalmár A, Nagy E. Alterations in total content and solubility characteristics of proteins in rat brain and liver during ageing and centrophenoxine treatment. *Experimental Gerontology* 1981; 16:229-40

Nathan C. Points of control in inflammation. *Nature* 2002; 420:846-52

Nemerson Y. Tissue factor and hemostasis. *Blood* 1988; 71:1-8

Ni H, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *The Journal of Clinical Investigation* 2000; 106:385-92

Ostrovsky L, King AJ, Bond S, Mitchell D, Lorant DE, Zimmerman GA, Larsen R, Niu XF, Kubes P. A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process. *Blood* 1998; 91:3028-36

Ott I, Neumann FJ, Gawaz M, Schmitt M, Schömig A. Increased neutrophil-platelet adhesion in patients with unstable angina. *Circulation* 1996; 94:1239-46

Park EM, Cho S, Frys KA, Glickstein SB, Zhou P, Anrather J, Ross ME, Iadecola C. Inducible nitric oxide synthase contributes to gender differences in ischemic brain injury. *Journal of Cerebral Blood Flow and Metabolism* 2006; 26:392-401

Pendurthi UR, Alok D, Rao LV. Binding of factor VIIa to tissue factor induces alterations in gene expression in human fibroblast cells: up-regulation of poly(A) polymerase. *Proceedings of the National Academy of Sciences USA* 1997; 94:12598-603

Peters LL, Cheever EM, Ellis HR, Magnani PA, Svenson KL, Von Smith R, Bogue MA. Large-scale, high-throughput screening for coagulation and hematologic phenotypes in mice. *Physiological Genomics* 2002; 11:185-93

Preston E & Webster J. Spectrophotometric measurement of experimental brain injury. *Journal of Neuroscience Methods* 2000; 94:187-92

Pretorius E & Oberholzer HM. Ultrastructural changes of platelets and fibrin networks in human asthma: a qualitative case study. *Blood Coagulation and Fibrinolysis* 2009c; 20:146-9

Pretorius E, Bornman MS, Reif S, Oberholzer HM, Franz RC. Ultrastructural changes of platelet aggregates and fibrin networks in a patient with renal clear cell adenocarcinoma: a scanning electron microscopy study. *Microscopy Research and Technique* 2009a; 72:679-83

Pretorius E, Briedenhann S, Marx J, Franz RC. Structural changes in the fibrin network of a Pretoria family with dysfibrinogenemia: a scanning electron microscopical study. *Ultrastructural Pathology* 2006; 30:167-76

Pretorius E, Briedenhann S, Marx J, Smit E, van der Merwe C, Pieters M, Franz RC. Ultra-structural comparison of the morphology of three different platelet and fibrin fibre preparations. *Anatomical Record (Hoboken)* 2007; 290:188-98

Pretorius E, Engelbrecht MJ, Duim W. Thromboembolic ischemic stroke and the presence of necrotic platelets: a scanning electron microscopy investigation. *Ultrastructural Pathology* 2012; 36:19-22

Pretorius E, Smit E, Oberholzer HM, Steyn E, Briedenhann S, Franz RC. Investigating the ultrastructure of platelets of HIV patients treated with the immuno-regulator, Canova: a qualitative scanning electron microscopy study. *Histology and Histopathology* 2009b; 24:399-405

Pretorius E, Oberholzer HM, van der Spuy WJ, Meiring JH. Age-related changes in fibrin networks and platelets of individuals over 75: a scanning electron microscopy study showing ‘‘thrombotic preparedness’’. *Journal of Thrombosis and Thrombolysis* 2010; 29:271-5

Pretorius E, Oberholzer HM, van der Spuy WJ, Swanepoel AC, Soma P. Qualitative scanning electron microscopy analysis of fibrin networks and platelet abnormalities in diabetes. *Blood Coagulation and Fibrinolysis* 2011b, 22:463-7

Pretorius E, Swanepoel AC, Oberholzer HM, van der Spuy WJ, Duim W, Wessels PF. A descriptive investigation of the ultrastructure of fibrin networks in thrombo-embolic ischemic stroke. *Journal of Thrombosis and Thrombolysis* 2011a; 31:507-13

Pretorius E. The role of platelet and fibrin ultrastructure in identifying disease patterns. *Pathophysiology of Haemostasis and Thrombosis* 2008; 36:251-8

Prewitt AK & Wilson ME. Changes in estrogen receptor-alpha mRNA in the mouse cortex during development. *Brain Research* 2007; 1134:62-9

Probst RJ, Lim JM, Bird DN, Pole GL, Sato AK, Claybaugh JR. Gender differences in the blood volume of conscious Sprague-Dawley rats. *Journal of the American Association for Laboratory Animal Science* 2006; 45:49-52

Pulsinelli WA, Waldman S, Rawlinson D, Plum F. Moderate hyperglycaemia augments ischemic brain damage: a neuropathological study in the rat. *Neurology* 1982; 32:1239-46

Que M, Schiene K, Witte OW, Zilles K. Widespread up-regulation of N-methyl-D-aspartate receptors after focal photothrombotic lesion in rat brain. *Neuroscience Letters* 1999; 273:77-80

Rauch U & Nemerson Y. Tissue factor, the blood, and the arterial wall. *Trends in Cardiovascular Medicine* 2000; 10:139-43

Ringler DH & Dabich L. Hematology and clinical biochemistry. In: Baker HJ, Lindsey JR, Weisbroth SH (Eds), The laboratory rat. Academic Press, Orlando (FL). 1979; 105-21

Ross R. Platelets, platelet-derived growth factor, growth control, and their interactions with the vascular wall. *Journal of Cardiovascular Pharmacology* 1985; 7:S186-90

Roth GJ. Platelets and blood vessels: the adhesion event. *Immunology Today* 1992; 13:100-5

Saenger AK & Christenson RH. Stroke biomarkers: progress and challenges for diagnosis, prognosis, differentiation, and treatment. *Clinical Chemistry* 2010; 56:21-33

Saha JK, Xia J, Grondin JM, Engle SK, Jakubowski JA. Acute hyperglycaemia induced by ketamine/xylazine anaesthesia in rats: mechanisms and implications for preclinical models. *Experimental Biology and Medicine (Maywood)* 2005; 230:777-84

Sakai T, Johnson KJ, Murozono M, Sakai K, Magnuson MA, Wieloch T, Cronberg T, Isshiki A, Erickson HP, Fässler R. Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing hemostasis. *Nature Medicine* 2001; 3:324-30

Schecter AD, Giesen PL, Taby O, Rosenfield CL, Rossikhina M, Fyfe BS, Kohtz DS, Fallon JT, Nemerson Y, Taubman MB. Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation. *The Journal of Clinical Investigation* 1997; 100:2276-85

Schmid-Elsaesser R, Zausinger S, Hungerhuber E, Baethmann A, Reulen HJ. A critical reevaluation of the intraluminal thread model of focal cerebral ischemia: evidence of inadvertent premature reperfusion and subarachnoid hemorrhage in rats by laser-doppler flowmetry. *Stroke* 1998, 29:2162-70

Selvamani A & Sohrabji F. Reproductive age modulates the impact of focal ischemia on the forebrain as well as the effects of estrogen treatment in female rats. *Neurobiology of Aging* 2010; 31:1618-28

Shebuski RJ & Kilgore KS. Role of inflammatory mediators in thrombogenesis. *The Journal of Pharmacology and Experimental Therapeutics* 2002; 300:729-35

Siesjö BK. Acidosis and ischemic brain damage. *Neurochemical Pathology* 1988; 9:31-88

Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, Bodor N, Day AL. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *Journal of Neurosurgery* 1997; 87:724-30

Singer CA, Rogers KL, Strickland TM, Dorsa DM. Estrogen protects primary cortical neurons from glutamate toxicity. *Neuroscience Letters* 1996; 212:13-6

Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. *Journal of Immunological Methods* 1993; 157:203-7

Spinella PC, Carroll CL, Staff I, Gross R, Mc Quay J, Keibel L, Wade CE, Holcomb JB. Duration of red blood cell storage is associated with increased incidence of deep vein thrombosis and in hospital mortality in patients with traumatic injuries. *Critical Care* 2009; 13:R151 (11 pages)

Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994; 76:301-14

Steele EC Jr, Guo Q, Namura S. Filamentous middle cerebral artery occlusion causes ischemic damage to the retina in mice. *Stroke* 2008; 39:2099-104

Stewart GJ, Ritchie WG, Lynch PR. Venous endothelial damage produced by massive sticking and emigration of leukocytes. *American Journal of Pathology* 1974; 74:507-32

Suzuki S, Brown CM, Dela Cruz CD, Yang E, Bridwell DA, Wise PM. Timing of estrogen therapy after ovariectomy dictates the efficacy of its neuroprotective and antiinflammatory actions. *Proceedings of the National Academy of Sciences USA* 2007; 104:6013-8

Suzuki S, Brown CM, Wise PM. Neuroprotective effects of estrogens following ischemic stroke. *Frontiers in Neuroendocrinology* 2009; 30:201-11

Taylor FB Jr, Chang A, Esmon CT, D'Angelo A, Vigano-D'Angelo S, Blick KE. Protein C prevents the coagulopathic and lethal effects of Escherichia coli infusion in the baboon. *The Journal of Clinical Investigation* 1987; 79:918-25

ten Cate JW, van der Poll T, Levi M, ten Cate H, van Deventer SJ. Cytokines: triggers of clinical thrombotic disease. *Journal of Thrombosis and Haemostasis* 1997; 78:415-9

Todd M, McDevitt E, McDowell F. Stroke and blood coagulation. *Stroke* 1973; 4:400-5

Toung TJ, Traystman RJ, Hurn PD. Estrogen-mediated neuroprotection after experimental stroke in male rats. *Stroke* 1998; 29:1666-70

Traystman RJ. Animal models of focal and global cerebral ischemia. *ILAR Journal* 2003; 44:85-95

Tsuchiya D, Hong S, Kayama T, Panter SS, Weinstein PR. Effect of suture size and carotid clip application upon blood flow and infarct volume after permanent and temporary middle cerebral artery occlusion in mice. *Brain Research* 2003; 970:131-9

Undas A, Podolec P, Zawilska K, Pieculewicz M, Jedliński I, Stepień E, Konarska-Kuszeweska E, Weglarz P, Duszyńska M, Hanschke E, Przewlocki T, Tracz W. Altered fibrin clot structure/function in patients with cryptogenic ischemic stroke. *Stroke* 2009; 40:1499-1501

Varma S, Sharma A, Malhotra P, Kumari S, Jain S, Varma N. Thrombotic complications of polycythemia vera. *Hematology* 2008; 13:319-23

Varma MR, Varga AJ, Knipp BS, Sukheepod P, Upchurch GR, Kunkel SL, Wakefield TW, Henke PK. Neutropenia impairs venous thrombosis resolution in the rat. *Journal of Vascular Surgery* 2003; 38:1090-8

Vegeto E, Benedusi V, Maggi A. Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. *Frontiers in Neuroendocrinology* 2008; 29:507-19

Vergouwen MD, Anderson RE, Meyer FB. Gender differences and the effects of synthetic exogenous and non-synthetic estrogens in focal cerebral ischemia. *Brain Research* 2000; 878:88-97

Wagner DD & Burger PC. Platelets in inflammation and thrombosis. *Aterioscleroses, Thrombosis, and Vascular Biology* 2003; 23:2131-7

Wakefield TW, Strieter RM, Prince MR, Downing LJ, Greenfield LJ. Pathogenesis of venous thrombosis: a new insight. *Cardiovascular Surgery* 1997; 5:6-15

Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. *Journal of Neuroimmunology* 2007; 184:53-68

Weber C & Springer TA. Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to alphaIIbbeta3 and stimulated by platelet-activating factor. *The Journal of Clinical Investigation* 1997; 100:2085-93

Weisel JW & Litvinov RI. The biochemical and physical process of fibrinolysis and effects of clot structure and stability on the lysis rate. *Cardiovascular and Hematological Agents in Medicinal Chemistry* 2008; 6:161-80

Weisel JW. Structure of fibrin: impact on clot stability. *Journal of Thrombosis and Haemostasis* 2007; 5:S116-24

White JG. Views of the platelet cytoskeleton at rest and at work. *Annals of the New York Academy of Sciences* 1987; 509:156-76

Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proceedings of the National Academy of Sciences USA* 1989; 86:2839-43

Wohner N. Role of cellular elements in thrombus formation and dissolution. *Cardiovascular & Hematological Agents in Medicinal Chemistry* 2008; 6:224-8

Wohner N, Sótonyi P, Machovich R, Szabó L, Tenekedjiev K, Silva MM, Longstaff C, Koley K. Lytic resistance of fibrin containing red blood cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2011; 31:2306-13

Wolberg AS & Campbell RA. Thrombin generation, fibrin clot formation and hemostasis. *Transfusion and Apheresis Science* 2008; 38:15-23

Wolberg AS. Thrombin generation and fibrin clot structure. *Blood Reviews* 2007; 21:131-42

Wolf PA. An overview of the epidemiology of stroke. *Stroke* 1990; 21:S114-6

Wong JH, Dukes J, Levy RE, Sos B, Mason SE, Fong TS, Weiss EJ. Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. *The Journal of Clinical Investigation* 2008; 118:2969-78

Xue QS, Yu BW, Wang ZJ, Chen HZ. Effects of ketamine, midazolam, thiopental, and propofol on brain ischemia injury in rat cerebral cortical slices. *Acta Pharmacologica Sinica* 2004; 25:115-20

Zhang X, Selli ML, Baglioni S, Hauri A, Chiari R, Dottorini M, Todisco T, Nenci GG, Gresele P. Platelets from asthmatic patients migrate *in vitro* in response to allergen stimulation. *Thrombosis and Haemostasis* 1993; 69:1356

Zhang Z, Chopp M, Zhang RL, Goussev A. A mouse model of embolic focal cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism* 1997; 17:1081-8

Appendices

Appendix A

SAS Analysis Data

Data processing and analysis was performed using SAS® (Version 9.3 running on a desktop computer on Windows XP service pack 3). In a multiple ANOVA group effect, time effect and interaction between group and time was assessed. If differences between the levels of group and/or time were detected using the General Linear Model (GLM) procedure, specific differences were tested for using Fisher's Least Squares Means (LSM) to assess pair-wise differences. Data summary for the outcomes report LSmeans and standard error at 95% confidence intervals.

Groups are MM – mature males, indicated as Group 1, MF – mature intact females, indicated as Group 2, and OVX – mature acyclic females, indicated as Group 3. Times are 0h pre-ischaemia, 2h, 24h and 48h post-reperfusion.

A.1. Animal Mass

The GLM Procedure
Class Level Information

Class	Levels	Values
GROUP	3	1 2 3
TIME	4	0 2 24 48

Number of Observations Read 72
Number of Observations Used 72

The GLM Procedure
Dependent Variable: ANIMAS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	731896.4444	66536.0404	145.10	<.0001
Error	60	27514.0000	458.5667		
Corrected Total	71	759410.4444			

R-Square	Coeff Var	Root MSE	ANIMAS Mean
0.963769	6.584473	21.41417	325.2222

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	2	719352.6944	359676.3472	784.35	<.0001
TIME	3	7372.3333	2457.4444	5.36	0.0025
GROUP*TIME	6	5171.4167	861.9028	1.88	0.0991

The GLM Procedure
Least Squares Means

GROUP	ANIMAS LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	462.041667	4.371149	<.0001	1
2	226.041667	4.371149	<.0001	2
3	287.583333	4.371149	<.0001	3

Least Squares Means for effect GROUP
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: ANIMAS

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		<.0001
3	<.0001	<.0001	

TIME	ANIMAS LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	339.944444	5.047368	<.0001	1
2	319.111111	5.047368	<.0001	2
24	313.222222	5.047368	<.0001	3
48	328.611111	5.047368	<.0001	4

Least Squares Means for effect TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: ANIMAS

i/j	1	2	3	4
1		0.0049	0.0004	0.1176
2	0.0049		0.4126	0.1883
3	0.0004	0.4126		0.0351
4	0.1176	0.1883	0.0351	

GROUP	TIME	ANIMAS LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	490.833333	8.742298	<.0001	1
1	2	458.666667	8.742298	<.0001	2

The GLM Procedure
Least Squares Means

GROUP	TIME	ANIMAS LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	24	449.500000	8.742298	<.0001	3
1	48	449.166667	8.742298	<.0001	4
2	0	238.000000	8.742298	<.0001	5
2	2	222.000000	8.742298	<.0001	6
2	24	213.500000	8.742298	<.0001	7
2	48	230.666667	8.742298	<.0001	8
3	0	291.000000	8.742298	<.0001	9
3	2	276.666667	8.742298	<.0001	10
3	24	276.666667	8.742298	<.0001	11
3	48	306.000000	8.742298	<.0001	12

Least Squares Means for effect GROUP*TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: ANIMAS

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.0117	0.0014	0.0013	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	0.0117		0.4613	0.4453	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	0.0014	0.4613		0.9786	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	0.0013	0.4453	0.9786		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001		0.2006	0.0521	0.5553	<.0001	0.0027	0.0027	<.0001
6	<.0001	<.0001	<.0001	<.0001	0.2006		0.4944	0.4860	<.0001	<.0001	<.0001	<.0001
7	<.0001	<.0001	<.0001	<.0001	0.0521	0.4944		0.1701	<.0001	<.0001	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	0.5553	0.4860	0.1701		<.0001	0.0004	0.0004	<.0001
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.2509	0.2509	0.2298
10	<.0001	<.0001	<.0001	<.0001	0.0027	<.0001	<.0001	0.0004	0.2509		1.0000	0.0209
11	<.0001	<.0001	<.0001	<.0001	0.0027	<.0001	<.0001	0.0004	0.2509	1.0000		0.0209
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.2298	0.0209	0.0209	

BYGROUP=1 Effect=GROUP

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
1	ANIMAS	1	462.041667	4.371149		A
2	ANIMAS	2	226.041667	4.371149		C
3	ANIMAS	3	287.583333	4.371149		B

BYGROUP=2 Effect=GROUP_TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
4	ANIMAS	1	490.833333	8.742298	0	A
5	ANIMAS	1	458.666667	8.742298	2	B
6	ANIMAS	1	449.500000	8.742298	24	B
7	ANIMAS	1	449.166667	8.742298	48	B
8	ANIMAS	2	238.000000	8.742298	0	E
9	ANIMAS	2	222.000000	8.742298	2	E
10	ANIMAS	2	213.500000	8.742298	24	E
11	ANIMAS	2	230.666667	8.742298	48	E
12	ANIMAS	3	291.000000	8.742298	0	CD
13	ANIMAS	3	276.666667	8.742298	2	D
14	ANIMAS	3	276.666667	8.742298	24	D
15	ANIMAS	3	306.000000	8.742298	48	C

BYGROUP=3 Effect=TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
16	ANIMAS		339.944444	5.047368	0	A
17	ANIMAS		319.111111	5.047368	2	BC
18	ANIMAS		313.222222	5.047368	24	C
19	ANIMAS		328.611111	5.047368	48	AB

A.2. Neural Tissue Injury

The GLM Procedure
Class Level Information

Class	Levels	Values
GROUP	3	1 2 3
TIME	3	2 24 48

Number of Observations Read 324
Number of Observations Used 324

The GLM Procedure
Dependent Variable: NTI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	21899.47757	2737.43470	66.09	<.0001
Error	315	13047.32018	41.42006		
Corrected Total	323	34946.79775			

R-Square	Coeff Var	Root MSE	NTI Mean
0.626652	41.45065	6.435842	15.52652

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	2	12326.26934	6163.13467	148.80	<.0001
TIME	2	31.65526	15.82763	0.38	0.6827
GROUP*TIME	4	9541.55297	2385.38824	57.59	<.0001

The GLM Procedure
Least Squares Means

GROUP	NTI LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	23.8972962	0.6192892	<.0001	1
2	9.2166047	0.6192892	<.0001	2
3	13.4656467	0.6192892	<.0001	3

Least Squares Means for effect GROUP
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: NTI

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		<.0001
3	<.0001	<.0001	

TIME	NTI LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
2	15.8212886	0.6192892	<.0001	1
24	15.6644081	0.6192892	<.0001	2
48	15.0938508	0.6192892	<.0001	3

Least Squares Means for effect TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: NTI

i/j	1	2	3
1		0.8580	0.4068
2	0.8580		0.5152
3	0.4068	0.5152	

GROUP	TIME	NTI LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	2	21.0446487	1.0726404	<.0001	1
1	24	21.3484514	1.0726404	<.0001	2
1	48	29.2987884	1.0726404	<.0001	3
2	2	14.7979059	1.0726404	<.0001	4

The GLM Procedure
Least Squares Means

GROUP	TIME	NTI LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
2	24	2.7804173	1.0726404	0.0100	5
2	48	10.0714909	1.0726404	<.0001	6
3	2	11.6213112	1.0726404	<.0001	7
3	24	22.8643557	1.0726404	<.0001	8
3	48	5.9112732	1.0726404	<.0001	9

Least Squares Means for effect GROUP*TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: NTI

i/j	1	2	3	4	5	6	7	8	9
1		0.8414	<.0001	<.0001	<.0001	<.0001	<.0001	0.2312	<.0001
2	0.8414		<.0001	<.0001	<.0001	<.0001	<.0001	0.3184	<.0001
3	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001		<.0001	0.0020	0.0371	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	0.0398
6	<.0001	<.0001	<.0001	0.0020	<.0001		0.3077	<.0001	0.0064
7	<.0001	<.0001	<.0001	0.0371	<.0001	0.3077		<.0001	0.0002
8	0.2312	0.3184	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
9	<.0001	<.0001	<.0001	<.0001	0.0398	0.0064	0.0002	<.0001	

BYGROUP=1 Effect=GROUP

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
1	NTI	1	23.8972962	0.6192892		A
2	NTI	2	9.2166047	0.6192892		C
3	NTI	3	13.4656467	0.6192892		B

BYGROUP=2 Effect=GROUP_TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
4	NTI	1	21.0446487	1.0726404	2	B
5	NTI	1	21.3484514	1.0726404	24	B
6	NTI	1	29.2987884	1.0726404	48	A
7	NTI	2	14.7979059	1.0726404	2	C
8	NTI	2	2.7804173	1.0726404	24	F
9	NTI	2	10.0714909	1.0726404	48	D
10	NTI	3	11.6213112	1.0726404	2	D
11	NTI	3	22.8643557	1.0726404	24	B
12	NTI	3	5.9112732	1.0726404	48	E

BYGROUP=3 Effect=TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
13	NTI		15.8212886	0.6192892	2	A
14	NTI		15.6644081	0.6192892	24	A
15	NTI		15.0938508	0.6192892	48	A

A.3. Brain Density

The GLM Procedure
Class Level Information

Class	Levels	Values
GROUP	3	1 2 3
TIME	4	0 2 24 48

Number of Observations Read 72
Number of Observations Used 72

The GLM Procedure
Dependent Variable: BDENS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	1.66630787	0.15148253	5.21	<.0001
Error	60	1.74407407	0.02906790		
Corrected Total	71	3.41038194			

R-Square	Coeff Var	Root MSE	BDENS Mean
0.488599	20.14029	0.170493	0.846528

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	2	0.31674190	0.15837095	5.45	0.0067
TIME	3	0.40222608	0.13407536	4.61	0.0057
GROUP*TIME	6	0.94733989	0.15788998	5.43	0.0002

The GLM Procedure
Least Squares Means

GROUP	BDENS LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0.82465278	0.03480176	<.0001	1
2	0.93645833	0.03480176	<.0001	2
3	0.77847222	0.03480176	<.0001	3

Least Squares Means for effect GROUP
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: BDENS

i/j	1	2	3
1		0.0267	0.3519
2	0.0267		0.0021
3	0.3519	0.0021	

TIME	BDENS LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.92870370	0.04018561	<.0001	1
2	0.72546296	0.04018561	<.0001	2
24	0.85648148	0.04018561	<.0001	3
48	0.87546296	0.04018561	<.0001	4

Least Squares Means for effect TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: BDENS

i/j	1	2	3	4
1		0.0007	0.2087	0.3526
2	0.0007		0.0246	0.0106
3	0.2087	0.0246		0.7395
4	0.3526	0.0106	0.7395	

GROUP	TIME	BDENS LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	0.76250000	0.06960352	<.0001	1
1	2	0.78055556	0.06960352	<.0001	2

The GLM Procedure
Least Squares Means

GROUP	TIME	BDENS LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	24	0.99722222	0.06960352	<.0001	3
1	48	0.75833333	0.06960352	<.0001	4
2	0	1.23611111	0.06960352	<.0001	5
2	2	0.68472222	0.06960352	<.0001	6
2	24	0.81388889	0.06960352	<.0001	7
2	48	1.01111111	0.06960352	<.0001	8
3	0	0.78750000	0.06960352	<.0001	9
3	2	0.71111111	0.06960352	<.0001	10
3	24	0.75833333	0.06960352	<.0001	11
3	48	0.85694444	0.06960352	<.0001	12

Least Squares Means for effect GROUP*TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: BDENS

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.8551	0.0203	0.9664	<.0001	0.4326	0.6035	0.0142	0.8004	0.6035	0.9664	0.3412
2	0.8551		0.0316	0.8222	<.0001	0.3342	0.7361	0.0225	0.9440	0.4832	0.8222	0.4408
3	0.0203	0.0316		0.0182	0.0182	0.0024	0.0674	0.8883	0.0372	0.0051	0.0182	0.1593
4	0.9664	0.8222	0.0182		<.0001	0.4575	0.5746	0.0127	0.7680	0.6332	1.0000	0.3205
5	<.0001	<.0001	0.0182	<.0001		<.0001	<.0001	0.0258	<.0001	<.0001	<.0001	0.0003
6	0.4326	0.3342	0.0024	0.4575	<.0001		0.1944	0.0016	0.3006	0.7896	0.4575	0.0853
7	0.6035	0.7361	0.0674	0.5746	<.0001	0.1944		0.0496	0.7896	0.3006	0.5746	0.6634
8	0.0142	0.0225	0.8883	0.0127	0.0258	0.0016	0.0496		0.0267	0.0034	0.0127	0.1226
9	0.8004	0.9440	0.0372	0.7680	<.0001	0.3006	0.7896	0.0267		0.4408	0.7680	0.4832
10	0.6035	0.4832	0.0051	0.6332	<.0001	0.7896	0.3006	0.0034	0.4408		0.6332	0.1437
11	0.9664	0.8222	0.0182	1.0000	<.0001	0.4575	0.5746	0.0127	0.7680	0.6332		0.3205
12	0.3412	0.4408	0.1593	0.3205	0.0003	0.0853	0.6634	0.1226	0.4832	0.1437	0.3205	

BYGROUP=1 Effect=GROUP

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
1	BDENS	1	0.82465278	0.03480176		B
2	BDENS	2	0.93645833	0.03480176		A
3	BDENS	3	0.77847222	0.03480176		B

BYGROUP=2 Effect=GROUP_TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
4	BDENS	1	0.76250000	0.06960352	0	D
5	BDENS	1	0.78055556	0.06960352	2	D
6	BDENS	1	0.99722222	0.06960352	24	BC
7	BDENS	1	0.75833333	0.06960352	48	D
8	BDENS	2	1.23611111	0.06960352	0	A
9	BDENS	2	0.68472222	0.06960352	2	D
10	BDENS	2	0.81388889	0.06960352	24	CD
11	BDENS	2	1.01111111	0.06960352	48	B
12	BDENS	3	0.78750000	0.06960352	0	D
13	BDENS	3	0.71111111	0.06960352	2	D
14	BDENS	3	0.75833333	0.06960352	24	D
15	BDENS	3	0.85694444	0.06960352	48	BCD

BYGROUP=3 Effect=TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
16	BDENS		0.92870370	0.04018561	0	A
17	BDENS		0.72546296	0.04018561	2	B
18	BDENS		0.85648148	0.04018561	24	A
19	BDENS		0.87546296	0.04018561	48	A

A.4. Neural Tissue Protein

The GLM Procedure
Class Level Information

Class	Levels	Values
GROUP	3	1 2 3
TIME	4	0 2 24 48

Number of Observations Read 72
Number of Observations Used 72

The GLM Procedure
Dependent Variable: NTPRO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	0.00004871	0.00000443	1.37	0.2125
Error	60	0.00019445	0.00000324		
Corrected Total	71	0.00024316			

R-Square	Coeff Var	Root MSE	NTPRO Mean
0.200337	11.26597	0.001800	0.015979

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	2	0.00002505	0.00001253	3.87	0.0264
TIME	3	0.00000487	0.00000162	0.50	0.6828
GROUP*TIME	6	0.00001879	0.00000313	0.97	0.4559

The GLM Procedure
Least Squares Means

GROUP	NTPRO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0.01636250	0.00036747	<.0001	1
2	0.01642917	0.00036747	<.0001	2
3	0.01514583	0.00036747	<.0001	3

Least Squares Means for effect GROUP
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: NTPRO

i/j	1	2	3
1		0.8984	0.0226
2	0.8984		0.0164
3	0.0226	0.0164	

TIME	NTPRO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.01615000	0.00042431	<.0001	1
2	0.01577778	0.00042431	<.0001	2
24	0.01631111	0.00042431	<.0001	3
48	0.01567778	0.00042431	<.0001	4

Least Squares Means for effect TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: NTPRO

i/j	1	2	3	4
1		0.5374	0.7892	0.4344
2	0.5374		0.3777	0.8682
3	0.7892	0.3777		0.2955
4	0.4344	0.8682	0.2955	

GROUP	TIME	NTPRO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	0.01635000	0.00073493	<.0001	1
1	2	0.01595000	0.00073493	<.0001	2

The GLM Procedure
Least Squares Means

GROUP	TIME	NTPRO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	24	0.01766667	0.00073493	<.0001	3
1	48	0.01548333	0.00073493	<.0001	4
2	0	0.01668333	0.00073493	<.0001	5
2	2	0.01693333	0.00073493	<.0001	6
2	24	0.01590000	0.00073493	<.0001	7
2	48	0.01620000	0.00073493	<.0001	8
3	0	0.01541667	0.00073493	<.0001	9
3	2	0.01445000	0.00073493	<.0001	10
3	24	0.01536667	0.00073493	<.0001	11
3	48	0.01535000	0.00073493	<.0001	12

Least Squares Means for effect GROUP*TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: NTPRO

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.7017	0.2101	0.4077	0.7495	0.5767	0.6666	0.8857	0.3728	0.0725	0.3479	0.3398
2	0.7017		0.1038	0.6551	0.4832	0.3479	0.9618	0.8107	0.6097	0.1542	0.5767	0.5659
3	0.2101	0.1038		0.0399	0.3479	0.4832	0.0944	0.1634	0.0344	0.0030	0.0307	0.0296
4	0.4077	0.6551	0.0399		0.2528	0.1681	0.6899	0.4931	0.9491	0.3241	0.9110	0.8984
5	0.7495	0.4832	0.3479	0.2528		0.8107	0.4540	0.6436	0.2277	0.0357	0.2101	0.2045
6	0.5767	0.3479	0.4832	0.1681	0.8107		0.3241	0.4832	0.1497	0.0200	0.1370	0.1329
7	0.6666	0.9618	0.0944	0.6899	0.4540	0.3241		0.7739	0.6436	0.1681	0.6097	0.5986
8	0.8857	0.8107	0.1634	0.4931	0.6436	0.4832	0.7739		0.4540	0.0974	0.4258	0.4167
9	0.3728	0.6097	0.0344	0.9491	0.2277	0.1497	0.6436	0.4540		0.3561	0.9618	0.9491
10	0.0725	0.1542	0.0030	0.3241	0.0357	0.0200	0.1681	0.0974	0.3561		0.3813	0.3900
11	0.3479	0.5767	0.0307	0.9110	0.2101	0.1370	0.6097	0.4258	0.9618	0.3813		0.9873
12	0.3398	0.5659	0.0296	0.8984	0.2045	0.1329	0.5986	0.4167	0.9491	0.3900	0.9873	

BYGROUP=1 Effect=GROUP

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
1	NTPRO	1	0.01636250	0.00036747		A
2	NTPRO	2	0.01642917	0.00036747		A
3	NTPRO	3	0.01514583	0.00036747		B

BYGROUP=2 Effect=GROUP_TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
4	NTPRO	1	0.01635000	0.00073493	0	ABC
5	NTPRO	1	0.01595000	0.00073493	2	ABC
6	NTPRO	1	0.01766667	0.00073493	24	A
7	NTPRO	1	0.01548333	0.00073493	48	BC
8	NTPRO	2	0.01668333	0.00073493	0	AB
9	NTPRO	2	0.01693333	0.00073493	2	AB
10	NTPRO	2	0.01590000	0.00073493	24	ABC
11	NTPRO	2	0.01620000	0.00073493	48	ABC
12	NTPRO	3	0.01541667	0.00073493	0	BC
13	NTPRO	3	0.01445000	0.00073493	2	C
14	NTPRO	3	0.01536667	0.00073493	24	BC
15	NTPRO	3	0.01535000	0.00073493	48	BC

BYGROUP=3 Effect=TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
16	NTPRO		0.01615000	0.00042431	0	A
17	NTPRO		0.01577778	0.00042431	2	A
18	NTPRO		0.01631111	0.00042431	24	A
19	NTPRO		0.01567778	0.00042431	48	A

A.5. Oestradiol Level

The GLM Procedure
Class Level Information

Class	Levels	Values
TIME	4	0 2 24 48

Number of Observations Read 24
Number of Observations Used 24

The GLM Procedure
Dependent Variable: OESTRA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1807.000000	602.333333	7.19	0.0018
Error	20	1676.333333	83.816667		
Corrected Total	23	3483.333333			

R-Square	Coeff Var	Root MSE	OESTRA Mean
0.518756	14.72677	9.155144	62.16667

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME	3	1807.000000	602.333333	7.19	0.0018

The GLM Procedure
Least Squares Means

TIME	OESTRA LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	56.3333333	3.7375720	<.0001	1
2	68.1666667	3.7375720	<.0001	2
24	72.8333333	3.7375720	<.0001	3
48	51.3333333	3.7375720	<.0001	4

Least Squares Means for effect TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: OESTRA

i/j	1	2	3	4
1		0.0367	0.0054	0.3555
2	0.0367		0.3878	0.0047
3	0.0054	0.3878		0.0006
4	0.3555	0.0047	0.0006	

---------- **BYGROUP=1 Effect=TIME** ----------

Obs	Dependent	TIME	LSMean	Standard Error	Letter Group
1	OESTRA	0	56.3333333	3.7375720	B
2	OESTRA	2	68.1666667	3.7375720	A
3	OESTRA	24	72.8333333	3.7375720	A
4	OESTRA	48	51.3333333	3.7375720	B

The GLM Procedure
Class Level Information

Class	Levels	Values
GROUP	3	1 2 3

Number of Observations Read 18
Number of Observations Used 18

The GLM Procedure
Dependent Variable: OESTRA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	46538.11111	23269.05556	10.52	0.0014
Error	15	33191.50000	2212.76667		
Corrected Total	17	79729.61111			

R-Square	Coeff Var	Root MSE	OESTRA Mean
0.583699	47.86438	47.04005	98.27778

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	2	46538.11111	23269.05556	10.52	0.0014

The GLM Procedure
Least Squares Means

GROUP	OESTRA LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	68.666667	19.204022	0.0028	1
2	169.833333	19.204022	<.0001	2
3	56.333333	19.204022	0.0103	3

Least Squares Means for effect GROUP
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: OESTRA

i/j	1	2	3
1		0.0020	0.6562
2	0.0020		0.0008
3	0.6562	0.0008	

---------- **BYGROUP=1 Effect=GROUP** ----------

Obs	Dependent	GROUP	LSMean	Standard Error	Letter Group
1	OESTRA	1	68.666667	19.204022	B
2	OESTRA	2	169.833333	19.204022	A
3	OESTRA	3	56.333333	19.204022	B

A.6. Platelet Count

The GLM Procedure
Class Level Information

Class	Levels	Values
GROUP	3	1 2 3
TIME	4	0 2 24 48

Number of Observations Read	72
Number of Observations Used	61

The GLM Procedure
Dependent Variable: PLATES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	1567967.417	142542.492	8.26	<.0001
Error	49	845235.533	17249.705		
Corrected Total	60	2413202.951			

R-Square	Coeff Var	Root MSE	PLATES Mean
0.649745	34.55074	131.3381	380.1311

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	2	612099.8033	306049.9016	17.74	<.0001
TIME	3	258903.3747	86301.1249	5.00	0.0042
GROUP*TIME	6	591135.9911	98522.6652	5.71	0.0001

The GLM Procedure
Least Squares Means

GROUP	PLATES LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	380.316667	33.377289	<.0001	1
2	246.625000	32.834533	<.0001	2
3	503.641667	28.117814	<.0001	3

Least Squares Means for effect GROUP
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: PLATES

i/j	1	2	3
1		0.0063	0.0068
2	0.0063		<.0001
3	0.0068	<.0001	

TIME	PLATES LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	465.422222	31.971937	<.0001	1
2	290.655556	31.971937	<.0001	2
24	368.200000	47.957905	<.0001	3
48	383.166667	30.956694	<.0001	4

Least Squares Means for effect TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: PLATES

i/j	1	2	3	4
1		0.0003	0.0980	0.0706
2	0.0003		0.1847	0.0429
3	0.0980	0.1847		0.7943
4	0.0706	0.0429	0.7943	

GROUP	TIME	PLATES LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	624.600000	58.736198	<.0001	1
1	2	253.833333	53.618568	<.0001	2

The GLM Procedure
Least Squares Means

GROUP	TIME	PLATES LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	24	375.500000	92.870083	0.0002	3
1	48	267.333333	53.618568	<.0001	4
2	0	202.833333	53.618568	0.0004	5
2	2	294.333333	53.618568	<.0001	6
2	24	255.500000	92.870083	0.0083	7
2	48	233.833333	53.618568	<.0001	8
3	0	568.833333	53.618568	<.0001	9
3	2	323.800000	58.736198	<.0001	10
3	24	473.600000	58.736198	<.0001	11
3	48	648.333333	53.618568	<.0001	12

Least Squares Means for effect GROUP*TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: PLATES

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		<.0001	0.0278	<.0001	<.0001	0.0001	0.0015	<.0001	0.4865	0.0007	0.0752	0.7666
2	<.0001		0.2621	0.8594	0.5044	0.5957	0.9877	0.7931	0.0001	0.3833	0.0080	<.0001
3	0.0278	0.2621		0.3181	0.1138	0.4527	0.3654	0.1926	0.0776	0.6401	0.3764	0.0142
4	<.0001	0.8594	0.3181		0.3991	0.7233	0.9126	0.6606	0.0002	0.4811	0.0125	<.0001
5	<.0001	0.5044	0.1138	0.3991		0.2334	0.6255	0.6845	<.0001	0.1347	0.0013	<.0001
6	0.0001	0.5957	0.4527	0.7233	0.2334		0.7188	0.4288	0.0007	0.7126	0.0287	<.0001
7	0.0015	0.9877	0.3654	0.9126	0.6255	0.7188		0.8407	0.0052	0.5371	0.0528	0.0006
8	<.0001	0.7931	0.1926	0.6606	0.6845	0.4288	0.8407		<.0001	0.2635	0.0041	<.0001
9	0.4865	0.0001	0.0776	0.0002	<.0001	0.0007	0.0052	<.0001		0.0034	0.2369	0.2996
10	0.0007	0.3833	0.6401	0.4811	0.1347	0.7126	0.5371	0.2635	0.0034		0.0775	0.0002
11	0.0752	0.0080	0.3764	0.0125	0.0013	0.0287	0.0528	0.0041	0.2369	0.0775		0.0328
12	0.7666	<.0001	0.0142	<.0001	<.0001	<.0001	0.0006	<.0001	0.2996	0.0002	0.0328	

---------- BYGROUP=1 Effect=GROUP ----------

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
1	PLATES	1	380.316667	33.377289		B
2	PLATES	2	246.625000	32.834533		C
3	PLATES	3	503.641667	28.117814		A

---------- BYGROUP=2 Effect=GROUP_TIME ----------

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
4	PLATES	1	624.600000	58.736198	0	AB
5	PLATES	1	253.833333	53.618568	2	E
6	PLATES	1	375.500000	92.870083	24	CDE
7	PLATES	1	267.333333	53.618568	48	E
8	PLATES	2	202.833333	53.618568	0	E
9	PLATES	2	294.333333	53.618568	2	E
10	PLATES	2	255.500000	92.870083	24	DE
11	PLATES	2	233.833333	53.618568	48	E
12	PLATES	3	568.833333	53.618568	0	ABC
13	PLATES	3	323.800000	58.736198	2	DE
14	PLATES	3	473.600000	58.736198	24	BCD
15	PLATES	3	648.333333	53.618568	48	A

---------- BYGROUP=3 Effect=TIME ----------

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
16	PLATES		465.422222	31.971937	0	A
17	PLATES		290.655556	31.971937	2	B
18	PLATES		368.200000	47.957905	24	AB
19	PLATES		383.166667	30.956694	48	A

A.7. Fibrinogen Level

The GLM Procedure
Class Level Information

Class	Levels	Values
GROUP	3	1 2 3
TIME	4	0 2 24 48

Number of Observations Read	72
Number of Observations Used	63

The GLM Procedure
Dependent Variable: FIBRINO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	154.7000220	14.0636384	14.08	<.0001
Error	51	50.9305050	0.9986374		
Corrected Total	62	205.6305270			

R-Square	Coeff Var	Root MSE	FIBRINO Mean
0.752320	26.40816	0.999318	3.784127

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	2	41.90881937	20.95440969	20.98	<.0001
TIME	3	98.19139997	32.73046666	32.78	<.0001
GROUP*TIME	6	17.34745413	2.89124235	2.90	0.0166

The GLM Procedure
Least Squares Means

GROUP	FIBRINO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	4.72500000	0.21394130	<.0001	1
2	2.68041667	0.23257871	<.0001	2
3	3.69962500	0.22111450	<.0001	3

Least Squares Means for effect GROUP
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: FIBRINO

i/j	1	2	3
1		<.0001	0.0016
2	<.0001		0.0025
3	0.0016	0.0025	

TIME	FIBRINO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	2.72444444	0.23554162	<.0001	1
2	2.19261111	0.29481933	<.0001	2
24	5.43133333	0.25802291	<.0001	3
48	4.45833333	0.23554162	<.0001	4

Least Squares Means for effect TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: FIBRINO

i/j	1	2	3	4
1		0.1648	<.0001	<.0001
2	0.1648		<.0001	<.0001
3	<.0001	<.0001		0.0075
4	<.0001	<.0001	0.0075	

GROUP	TIME	FIBRINO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	3.23000000	0.40797005	<.0001	1
1	2	2.76200000	0.44690879	<.0001	2

The GLM Procedure
Least Squares Means

GROUP	TIME	FIBRINO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	24	7.49800000	0.44690879	<.0001	3
1	48	5.41000000	0.40797005	<.0001	4
2	0	2.17333333	0.40797005	<.0001	5
2	2	1.43333333	0.57695677	0.0163	6
2	24	3.30000000	0.44690879	<.0001	7
2	48	3.81500000	0.40797005	<.0001	8
3	0	2.77000000	0.40797005	<.0001	9
3	2	2.38250000	0.49965922	<.0001	10
3	24	5.49600000	0.44690879	<.0001	11
3	48	4.15000000	0.40797005	<.0001	12

Least Squares Means for effect GROUP*TIME
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: FIBRINO

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.4429	<.0001	0.0004	0.0729	0.0141	0.9084	0.3154	0.4290	0.1948	0.0005	0.1170
2	0.4429		<.0001	<.0001	0.3352	0.0745	0.3986	0.0879	0.9895	0.5738	<.0001	0.0260
3	<.0001	<.0001		0.0011	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0026	<.0001
4	0.0004	<.0001	0.0011		<.0001	<.0001	0.0010	0.0079	<.0001	<.0001	0.8875	0.0336
5	0.0729	0.3352	<.0001	<.0001		0.2999	0.0684	0.0064	0.3059	0.7471	<.0001	0.0012
6	0.0141	0.0745	<.0001	<.0001	0.2999		0.0135	0.0014	0.0642	0.2193	<.0001	0.0003
7	0.9084	0.3986	<.0001	0.0010	0.0684	0.0135		0.3987	0.3852	0.1771	0.0011	0.1662
8	0.3154	0.0879	<.0001	0.0079	0.0064	0.0014	0.3987		0.0760	0.0308	0.0076	0.5640
9	0.4290	0.9895	<.0001	<.0001	0.3059	0.0642	0.3852	0.0760		0.5507	<.0001	0.0205
10	0.1948	0.5738	<.0001	<.0001	0.7471	0.2193	0.1771	0.0308	0.5507		<.0001	0.0084
11	0.0005	<.0001	0.0026	0.8875	<.0001	<.0001	0.0011	0.0076	<.0001	<.0001		0.0306
12	0.1170	0.0260	<.0001	0.0336	0.0012	0.0003	0.1662	0.5640	0.0205	0.0084	0.0306	

BYGROUP=1 Effect=GROUP

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
1	FIBRINO	1	4.72500000	0.21394130		A
2	FIBRINO	2	2.68041667	0.23257871		C
3	FIBRINO	3	3.69962500	0.22111450		B

BYGROUP=2 Effect=GROUP_TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
4	FIBRINO	1	3.23000000	0.40797005	0	CDE
5	FIBRINO	1	2.76200000	0.44690879	2	DEF
6	FIBRINO	1	7.49800000	0.44690879	24	A
7	FIBRINO	1	5.41000000	0.40797005	48	B
8	FIBRINO	2	2.17333333	0.40797005	0	EF
9	FIBRINO	2	1.43333333	0.57695677	2	F
10	FIBRINO	2	3.30000000	0.44690879	24	CDE
11	FIBRINO	2	3.81500000	0.40797005	48	CD
12	FIBRINO	3	2.77000000	0.40797005	0	DEF
13	FIBRINO	3	2.38250000	0.49965922	2	EF
14	FIBRINO	3	5.49600000	0.44690879	24	B
15	FIBRINO	3	4.15000000	0.40797005	48	C

BYGROUP=3 Effect=TIME

Obs	Dependent	GROUP	LSMean	Standard Error	TIME	Letter Group
16	FIBRINO		2.72444444	0.23554162	0	C
17	FIBRINO		2.19261111	0.29481933	2	C
18	FIBRINO		5.43133333	0.25802291	24	A
19	FIBRINO		4.45833333	0.23554162	48	B

Appendix B

Scientific Manuscript Published in Peer-Reviewed ISI Journal

van der Spuy WJ & Pretorius E. Interrelation between inflammation, thrombosis, and neuroprotection in cerebral ischaemia. *Reviews in the Neurosciences* 2012; 23:269-78. DOI 10.1515/revneuro-2012-0028

Published as Commissioned Review

Rev. Neurosci., Vol. xx(x): xxx–xxx, 2012 • Copyright © by Walter de Gruyter • Berlin • Boston. DOI 10.1515/revneuro-2012-0028

Interrelation between inflammation, thrombosis, and neuroprotection in cerebral ischemia

Wendy J. van der Spuy[1] and Etheresia Pretorius[2,*]

[1] Department of Anatomy, School of Medicine, Faculty of Health Sciences, University of Pretoria, South Africa
[2] Department of Physiology, School of Medicine, Faculty of Health Sciences, University of Pretoria, Private Bag x323, Arcadia 0007, South Africa

*Corresponding author
e-mail: resia.pretorius@up.ac.za

Abstract

Stroke by mechanism of thrombotic cerebral ischemia is one of the leading causes of death and/or disability worldwide. Current research is under consensus that there are sex-based differences in both the prevalence and presentation of stroke and thrombosis. Here we discuss the interrelation between thrombosis and inflammation and call attention to points in the cerebral ischemic cascade where estrogen may be involved in neuroprotection. Cerebral ischemia triggers a series of events including inflammation, which is deeply interrelated with thrombosis; inflammation not only produces local thrombosis, but thrombosis can also amplify inflammation especially through the synergism of leukocyte and platelet activity. Research involving experimental animal models of cerebral ischemia has shown that sex hormones, especially estrogen, offer a degree of neuroprotection. Mechanisms of this neuroprotection may be linked to certain anti-inflammatory properties of estrogen, as well as estrogen's regulation of thrombosis through the lowering of coagulation factors, among others. It is also understood that sex hormones alter the function and morphology of platelets and fibrin networks, and changes in platelet and fibrin network morphology offer one of the earliest confirmations of inflammation. Sex hormone levels, inflammatory processes, and thrombotic mechanisms are profoundly interconnected in predicting the outcome of cerebral ischemia.

Keywords: anti-inflammatory; coagulation; estrogen; leukocytes; platelets.

Introduction

Stroke is currently the second leading cause of death and/or disability worldwide, and the existence of a larger aging population suggests that stroke research will become more important in the future (Elkins and Johnston, 2003; Braeuninger and Kleinschnitz, 2009). Thrombotic cerebral ischemia accounts for more than half of all cases of stroke worldwide. It results from an occlusion of cerebral vasculature, reducing or preventing the supply of oxygen to the cerebrum, thus hampering metabolic demand, and ultimately leading to death of brain tissue by ischemic stroke.

Research suggests that there are sex-based differences in the prevalence and presentation of both stroke and thrombosis (Bailey et al., 2009). Sex hormones not only alter procoagulant protein expression (Lowe et al., 2004) and the function of blood and vascular cells (Kadir et al., 1999; Butenas and Mann, 2002; Leng et al., 2004), but differences in platelet function (Liao et al., 2001; Suzuki et al., 2009) and in thrombosis activity (Bailey et al., 2009) have also been noted. Estrogen is to a degree neuroprotective (Liao et al., 2001; Suzuki et al., 2009; Selvamani and Sohrabji, 2010) in certain cases of induced cerebral ischemia, as females appear to suffer less severe consequences of stroke, including lesser neural tissue loss, than their male counterparts (McCullough and Hurn, 2003; Suzuki et al., 2009). In the absence of ovarian hormone production at menopause, females are again at higher risk to strokes than their male counterparts, and this risk continues to increase with age, as women have a longer life expectancy than men (Mitka, 2006; Suzuki et al., 2009). Thus, both sex and age play an important role in the occurrence of thrombotic events and the severity of neural damage subsequent to a stroke.

Platelets and fibrin play an important role in the normal coagulation process where they are involved in the maintenance of hemostasis (Herd and Page, 1994). Their activation may be due to damage of the vessel wall or activation of the endothelium by chemicals, cytokines, and also inflammatory processes (Camera et al., 1999; Butenas and Mann, 2002). Activated platelets synthesize/secrete inducers of platelet aggregation and adhere to the injured vessel wall, as well as aggregate to each other in order to form a platelet-rich plug or thrombus, which secures hemostasis; this plug is then stabilized by fibrin formation as fibrinogen is activated by binding to activated platelets. Fibrinogen is the major plasma protein coagulation factor (Lowe et al., 2004), and though plasma levels thereof are decreased by estrogen during the menstrual cycle, these levels are known to be higher in females than in males (Bailey et al., 2009). Although fibrin forms the core matrix of a thrombus, its structure depends also on the cellular elements embedded in its meshwork and the overall rate of coagulation reactions initiated by platelet aggregation (Wohner, 2008). Morphological changes of fibrin networks may therefore occur due to several kinetic and modulating factors present in plasma.

A thrombotic event is associated with a change in hemostasis and cellular components that play a fundamental role

in blood platelets and fibrin network formation. It is well known that thrombotic events are the most common cause of stroke and resultant cerebral ischemia (Braeuninger and Kleinschnitz, 2009). Furthermore, it is known that cerebral ischemia triggers a cascade of inflammatory processes, among others (Herd and Page, 1994; Gibson et al., 2005; Wang et al., 2007). Inflammation again causes an alteration in platelet activation (Camera et al., 1999; Butenas and Mann, 2002) and possibly further thrombotic events. It is suggested that estrogen provides neuroprotection through certain anti-inflammatory mechanisms, among others (Vegeto et al., 2008; Suzuki et al., 2009).

Cerebral ischemia

With two-thirds of deaths from stroke complications occurring in developing regions of the world, such as sub-Saharan Africa, stroke is the second leading cause of death worldwide. The existence of a larger aging population suggests that stroke research will become more important in the future (Elkins and Johnston, 2003; Wang et al., 2007; Braeuninger and Kleinschnitz, 2009).

Stroke incidence and resultant cerebral ischemia can be linked to coagulation processes. The cascade of blood coagulation is initiated when subendothelial tissue factor is exposed to the flow of blood subsequent to damage or activation of the vessel endothelium by chemicals, cytokines, or inflammatory processes (Butenas and Mann, 2002). The formation of a thrombus at a site of vessel injury is thus a hemostatic process. A thrombotic event, however, is associated with a change in hemostasis and cellular components that play a fundamental role in blood platelet and fibrin network formation. Overactivity of any one component of the coagulation cascade can result in the formation of tight and rigid fibrin networks (Fatah et al., 1992), which can cause blockage of one or multiple cerebral blood vessels, resulting in cerebral ischemia.

Cerebral ischemia is known to trigger a series of complex events initiating with cerebral hypoperfusion and comprising bioenergetic failure of cellular components, excitotoxicity, oxidative stress, biphasic dysfunction of the blood-brain barrier, microvascular injury, hemostatic activation, inflammation, and formation of edema, as well as apoptosis and ultimate necrosis of neuronal, glial, and endothelial cells. This cascade of events is dependent on variables, such as onset and duration of ischemia, effectiveness of reperfusion, and resultant infarct size or tissue loss (Danton and Dietrich, 2003; Gibson et al., 2005; Brouns and De Deyn, 2009; Saenger and Christenson, 2010).

Blood-brain barrier disruption in ischemic stroke appears to be dependent on the response to and aggressiveness of reperfusion. Increased permeability of the blood-brain barrier takes place within the first 24 hours of an ischemic event, with further damage occurring 48–72 hours after ischemia in the absence of sufficient reperfusion (Saenger and Christenson, 2010). Inflammation itself is recognized as a key element of the pathological progression of ischemic stroke. The destructive or beneficial nature of inflammation seems to be dependent on the severity of ischemia. It is thus likely that early inflammatory responses may potentiate ischemic injury, whereas late responses may be beneficial to recovery and repair of ischemic lesions (Wang et al., 2007). Inflammation and thrombosis are deeply interrelated, as not only can inflammation produce local thrombosis, but thrombosis can also amplify inflammation (Libby and Simon, 2001).

Thrombosis

Thrombosis, the most common cause of stroke, is influenced by factors including endothelial injury, blood stasis or turbulent flow, and hypercoagulability of blood (Myers and Wakefield, 2005). Endothelial injury is the most common cause of hemostatic coagulation processes, and it is vital that these processes are understood. Endothelial damage to vasculature initiates a local inflammatory response, promoting a state of prothrombosis, which is driven by tissue factor, adhesion molecules, and proinflammatory cytokines and prothrombotic microparticles (Libby and Simon, 2001). Various disease states are also found to promote tissue factor exposure within vascular walls to blood flow, leading to the initiation of non-hemostatic coagulation processes (Mackman, 2004; Myers and Wakefield, 2005). Thrombosis and inflammation are interrelated (Stewart et al., 1974; Myers and Wakefield, 2005), as inflammation produces local thrombosis, and thrombosis can amplify inflammation (Libby and Simon, 2001). A thrombus, whether formed through hemostatic or non-hemostatic mechanisms, consists of platelets and fibrin as well as trapped red and white blood cells, which stabilize its structure.

Platelets and fibrin play an important role in the coagulation process, where they are involved in the maintenance of hemostasis. Platelets have a life span of 8–12 days, and though devoid of a nucleus, possess many features of classical inflammatory cells. Like neutrophils, they can undergo chemotaxis (Zhang et al., 1993), phagocytose foreign particles, contain and release adhesive proteins, activate complement, interact with foreign bodies, alter vascular tone, enhance vascular permeability, as well as store and metabolize various vasoactive substances, and release inflammatory mediators (Herd and Page, 1994). Structurally, platelet surfaces consist of a typical bilayer membrane composed of lipids, proteins, and carbohydrates. Surface glycoproteins are essential to their function and play a primary role in their adhesion to exposed subendothelial matrix proteins, interaction with thrombin, and exposure of fibrinogen receptors to facilitate aggregation (Roth, 1992; Herd and Page, 1994). Internally, platelets are only capable of limited protein synthesis, and few mitochondria are present, which contribute to energy metabolism of the platelet. In addition, a random cytoplasmic distribution of lysosomes, glycogen granules, and peroxisomes are present (Herd and Page, 1994). Following activation, platelets change shape from a discoid to a spherical form. This process is mediated by a contractile microtubular system, morphologically characterized by an

extension of dendritic pseudopodia (White, 1987; Herd and Page, 1994).

Platelets are activated by a number of stimuli resulting in the expression and/or activation of surface receptors, secretion of vasoactive substances, adhesion, aggregation, and finally, thrombus formation. The activation may be due to damage of the vascular wall or activation of the endothelium by chemicals, cytokines, and also inflammatory processes (Camera et al., 1999; Butenas and Mann, 2002). Upon activation, platelets cover the exposed subendothelial matrix and mediate additional platelet and leukocyte recruitment through the release of microparticles that mediate local leukocyte-leukocyte and leukocyte-endothelial cell interactions – mechanisms which play a role in both thrombosis and inflammation (Wagner and Burger, 2003). Platelets are essential in the initial stages of thrombus formation because they adhere and aggregate at sites of vascular wall injury and then serve as a surface for coagulation reactions; the overall rate of which determines the final structure of fibrin (Wohner, 2008). Thrombi thus form locally in a vessel when injury occurs or endothelial activation takes place – as a hemostatic mechanism to repair the insult. Platelets, during adhesion to endothelium, are activated and release proinflammatory cytokines that further stimulate the endothelium (Weber and Springer, 1997; Wagner and Burger, 2003), promote hemoattraction of leukocytes, stimulate smooth muscle cell and fibroblast proliferation, promote collagen synthesis, and thus contribute directly to lesion progression and maturation (Ross, 1985; Wagner and Burger, 2003).

Fibrinogen is the major plasma protein coagulation factor and the best known precursor of fibrin, playing an important role in platelet aggregation by linking activated platelets, and therefore playing a key role in hemostasis and thrombosis. Activated platelets synthesize/secrete inducers of platelet aggregation, adhere to the injured vessel wall, and aggregate to each other in order to form a platelet-rich plug that secures hemostasis; this plug is then stabilized by fibrin formation as fibrinogen is activated by binding to activated platelets. Thus, not all circulating fibrinogen is functional or clottable. As the tissue repair process takes place, the fibrin plug is digested by fibrinolytic enzymes (Lowe et al., 2004). On the one hand, low levels of plasma fibrinogen are associated with an increased risk for bleeding, as platelet aggregation as well as fibrin plug formation is impaired. Elevated fibrinogen levels, on the other hand, may well be associated with the risk of stroke (Danesh et al., 2005), as elevated fibrinogen synthesis is inclined to shift the hemostatic balance in favor of coagulation/thrombosis.

Interestingly, thrombi are found to form readily and rapidly in the complete absence of fibrinogen in animal models. However, these thrombi are unstable and fail to resist shear stress, resulting in frequent thromboembolization, with downstream vessel occlusion (Ni et al., 2000; Wagner and Burger, 2003). Fibrinogen/fibrin complexes are thus required to secure thrombus stability for anchorage to the site of injury. In addition, this stability is dependent on fibrinogen/fibrin interaction with platelet integrin (a surface protein), which also slows down the growth of the thrombus (Hawiger, 1995; Ni et al., 2000; Wagner and Burger, 2003). Fibronectin is known to support platelet adhesion and distribution (Hynes, 1990; Wagner and Burger, 2003). Deficiency of plasma fibronectin though does not affect initial platelet adhesion (Sakai et al., 2001; Wagner and Burger, 2003), but delays thrombus formation quite substantially as platelets are continuously shed. Therefore, fibronectin is an important mediator of platelet-platelet interactions within thrombi as they form and grow. This mediation takes place through fibronectin's rapid binding to activated integrins, thus cross-linking platelets. Fibrin is then generated, which anchors the growing thrombus to the site of vascular injury (Ni et al., 2000; Wagner and Burger, 2003).

Platelet adhesion and activation are thus regulated by specific proteins on the platelet surface, and fibrinogen as well as fibronectin plays a fundamental role in the coagulation process (Ni et al., 2000; Lowe et al., 2004). Fibrin assembly (through the coagulation pathway and involvement of the platelets) from fibrinogen proceeds in a highly ordered fashion. Fibrin forms a network, which functions to stabilize the primary platelet plug. Although fibrin forms the core matrix of a thrombus, its structure depends also on the cellular elements embedded in its meshwork. Morphological changes of fibrin networks may therefore occur due to several kinetic and modulating factors present in plasma. Fibrinolysis plays an important role in hemostasis too. Leukocyte-derived enzymes, such as elastase, influence fibrinolysis by direct digestion of fibrin or indirectly modulating it by partial degradation of zymogens and inhibitors of coagulation and fibrinolytic proteases (Wohner, 2008).

Thrombosis research has shown that sex hormones have complex effects on vascular walls, coagulation proteins, and platelets – all which may alter thrombosis. In line with this, females have shown cyclic patterns in their coagulation proteins, which correspond to menstrual cycle patterns (Kadir et al., 1999; Bailey et al., 2009); in addition, they possess slightly higher fibrinogen levels than their male counterparts, though estrogen is known to decrease fibrinogen plasma levels (Mendelsohn and Karas, 1999; Bailey et al., 2009). There also seem to be sex-related differences in platelet function. It is known that isolated female platelets bind more fibrinogen and have a greater maximal aggregation extent than male platelet isolates, and this platelet reactivity is altered in ovariectomized females (Leng et al., 2004; Bailey et al., 2009). Males have been shown to have higher platelet counts and faster clotting times than their female counterparts, thus making them more susceptible to thrombosis – possibly due to the differences in growth hormone secretion between the sexes (pulsatile in males, sustained in females), which in turn influence protein production of coagulation and thrombosis regulators (Wong et al., 2008; Bailey et al., 2009). Isolates of male rat platelets have been shown to display greater maximal aggregation *in vitro* than platelets isolated from female rats; this aggregation is reduced in male rats that have undergone castration (Emms and Lewis, 1985; Bailey et al., 2009). It has been noted that both megakaryocytes and platelets express the estrogen receptor beta (ERβ) as well as the androgen receptor (Jayachandran and Miller, 2003; Bailey et al., 2009); thus,

it is almost certain that the sex hormones have an effect on thrombosis. Estrogen is hypothesized to have a direct effect on platelet function, whereas androgen seems to regulate megakaryocyte biology and platelet production (Peters et al., 2002; Bailey et al., 2009).

The occurrence of thrombosis is thus hemostasis in the wrong place, which results from local activation of platelets and coagulation, and also from increased concentrations of plasma fibrinogen (Lowe et al., 2004). As mentioned, females have elevated fibrinogen levels compared to their male counterparts, though estrogen does lower fibrinogen levels (Mendelsohn and Karas, 1999; Bailey et al., 2009), and in menopause, these plasma-lowering effects of estrogen are ruled out, rendering fibrinogen levels even higher than in cyclic females (Lowe et al., 1997, 2004). Many diseases like cancer, thrombotic disease, bleeding disorders, asthma, and even conditions like HIV/AIDS are associated with changes in platelet and fibrin structure. Fibrin structure itself has been shown to play a role in the development of vascular complications (Pretorius et al. 2006, 2007).

Cerebral ischemia may be a consequence of thrombosis when a change in hemostasis occurs, for example, in the instance of a blood disorder or diminished blood flow (due to age or even normal thrombus formation), resulting in a local accumulation of coagulation factors and thus increasing platelet aggregation. These changes in hemostasis thus alter the coagulation cascade and result in the formation of rigid fibrin networks, which do not digest as programed. These rigid thrombi may cause ischemia locally if they diminish or inhibit the blood flow in a vessel for an extended period, or pieces thereof may break free, termed a thromboembolus, and become lodged in another vessel where they can cause ischemia by inhibition of blood flow.

Inflammation

Inflammation is characterized by interactions among endothelial cells, platelets, and leukocytes, and causes endothelial activation regardless of the mechanisms by which inflammation itself was activated. Endothelial activation sets off the cell adhesion cascade, which results in the adherence and aggregation of platelets, chemokine deposition by platelets on the activated endothelial surface, the expression of cell adhesion molecules by endothelial cells and platelets, and ultimately, activation of leukocytes. Chemokines activate leukocytes, and further binding to adhesion molecules mediate the process of leukocyte rolling (on the activated 'sticky' endothelium), adhesion (through binding to fibrinogen), and transmigration into the subendothelial tissue (Butcher, 1991; Springer, 1994; Diacovo et al., 1996; Kuijper et al., 1996; Weber and Springer, 1997; Konstantopoulos et al., 1998; Wagner and Burger, 2003). Platelets are thus central to both thrombosis and inflammation.

The objective of inflammation is therefore to recruit leukocytes rapidly to a site of vascular injury. Endothelial dysfunction or injury promotes activation of the coagulation cascade by exposure of tissue factor (Gimbrone, 1995; Day et al., 2005; Myers and Wakefield, 2005), as well as the activation of inflammatory process (Laursen et al., 2001; Altman, 2003; Myers and Wakefield, 2005). Inflammatory mediators then promote coagulation through further elevation of tissue factor (Drake et al., 1989; Esmon, 2003), which elevates fibrinogen synthesis, and fibrinogen levels will continue to rise under inflammatory conditions (Taylor et al., 1987; Esmon, 2003) unless hemostatic factors counteract this. Tissue factor is a membrane-bound protein that functions as a procoagulant (Libby and Simon, 2001), triggering thrombin generation, which then prompts activation of the coagulation cascade (Nemerson, 1988; Mann et al., 1998; Myers and Wakefield, 2005). Thrombin in turn amplifies the inflammatory response by activating the endothelium, resulting in the formation of more tissue factor (Pendurthi et al., 1997; Miller et al., 1998; Esmon, 2003) and high levels of platelet-activating factor (Bar-Shavit et al., 1986; Esmon, 2003), which is a neutrophil agonist (Lorant et al., 1991; Esmon, 2003) enhancing leukocyte activation and adhesion, as well as increasing inflammatory cytokines (Henn et al., 1998; André et al., 2002, Esmon, 2003). Inflammatory cytokines have been shown to increase platelet reactivity, which increases thrombogenic potential (Burstein, 1997; Esmon, 2003), further linking inflammation and thrombosis.

Monocytes do not express tissue factor unless they are stimulated by inflammatory mediators to transcribe the gene for tissue factor (Wilcox et al., 1989; Brand et al., 1991; Libby and Simon, 2001); thus, their recruitment and activation can lead to thrombogenesis. Activated monocytes that express tissue factor on their surfaces (Rauch and Nemerson, 2000) facilitate monocyte-platelet and monocyte-endothelial interactions through binding mechanisms of cell adhesion molecules (Wakefield et al., 1997; Shebuski and Kilgore, 2002; Myers and Wakefield, 2005). These interactions, driven by inflammatory mediators and tissue factor, lead to accelerated fibrin formation and deposition into a developing thrombus (Shebuski and Kilgore, 2002; Myers and Wakefield, 2005). Thus, the specific interaction between cell adhesion molecules and their leukocyte receptors is what stimulates fibrin formation (Goel and Diamond, 2001; Myers and Wakefield, 2005), and procoagulant microparticles derived both from activated leukocytes and platelets amplify the coagulation process (Frenette et al., 2000; Myers and Wakefield, 2005).

Like endothelial cells and activated monocytes, smooth muscle of blood vessels can express tissue factor when exposed through endothelial breakage, thus also contributing to thrombogenesis (Schecter et al., 1997; Libby and Simon, 2001). Besides production of procoagulant tissue factor, the smooth muscle can also undergo inflammatory activation when exposed to thrombin and products of thrombosis (Kranzhöfer et al., 1996; Libby and Simon, 2001), thus amplifying the inflammatory response and promoting systemic procoagulant effects due to increased fibrinogen levels in circulation (Libby and Simon, 2001).

The progression of the inflammatory response subsequent to a stimulus hence reflects a balance between prothrombotic and anticoagulant activities. The ability of proinflammatory cytokines to downregulate antithrombotic proteins and

upregulate prothrombotic proteins shifts this balance toward a procoagulant state (ten Cate et al., 1997; Myers and Wakefield, 2005); in addition, these cytokines induce the immune defense mechanism and mediate leukocyte recruitment. Inflammatory cells are important to the process of thrombus recanalization and organization. Although it may seem intuitive that a decrease in inflammation will decrease thrombogenesis, once a clot forms, the presence of neutrophils is important for recanalization (Varma et al., 2003; Myers and Wakefield, 2005). Accordingly, inflammation leads to an imbalance between the pro- and anticoagulant properties of endothelium that can lead to local stimulation of the coagulation cascade (Nathan, 2002; Wagner and Burger, 2003). Early inflammatory responses may consequently contribute to damage, whereas late/delayed inflammatory responses are necessary to facilitate repair.

As mentioned previously, cerebral ischemia triggers inflammatory processes (Herd and Page, 1994; Gibson et al., 2005; Wang et al., 2007). The first inflammatory cells that enter the brain subsequent to trauma are neutrophils, followed by monocytes, and later on, resident microglia, astrocytes, and neurons are also activated (Morganti-Kossmann et al., 2001). Leukocytes and, after a few hours post-injury, microglia secrete proinflammatory cytokines and chemokines, the severity of which play detrimental roles in the pathophysiology of stroke (Morganti-Kossmann et al., 2001; Wang et al., 2007; Suzuki et al., 2009). Significant leukocyte influx into cerebral parenchyma and tissue remodeling are characteristics of cerebral ischemia/reperfusion (Barone et al., 1995; Wang et al., 2007). Interestingly, infarct volume has been shown to be reduced significantly through the inhibition of neutrophil infiltration, as it is evident that neutrophils wield the most damage to ischemic lesions once reperfusion is undertaken (Connolly et al., 1996; Guha and Mackman, 2001; Wang et al., 2007).

In areas of ischemia/reperfusion injury, platelets colocalize with leukocytes – an interaction linking hemostatic thrombotic and inflammatory responses (Ostrovsky et al., 1998; Libby and Simon, 2001). The inflammatory reaction subsequent to cerebral ischemia is characterized by neutrophil adherence to blood vessels 4–6 hours after onset of ischemia and their infiltration into the neural tissue with subsequent release of proinflammatory mediators, potentiating injury (Hallenbeck, 1996; Wang et al., 2007), by resultant accumulation and activation of monocytes in the area of lesion. Platelets promote accumulation of both neutrophils and monocytes at sites of injury, and neutrophil-platelet aggregates specifically influence cellular responses by inducing further leukocyte activation, enhancing cell-adhesion molecule expression, and generating signals that promote platelet integrin (surface protein) activation and chemokine synthesis (Ott et al., 1996; Furman et al., 1998; Libby and Simon, 2001). Chemokines stimulate cytoskeletal reorganization of neutrophils and monocytes to facilitate their motility, proliferation of fibroblasts and astrocytes for glial scar formation, apoptosis and necrosis of neurons, and the phagocytic ability of macrophages and microglia to remove the debris of damaged tissue (Morganti-Kossmann et al., 2001).

Neuroprotection

Sex hormones target the central and peripheral nervous systems, affecting brain development and differentiation and influencing neuronal functions (Manthey and Behl, 2006; Drača, 2009). In humans, it is accepted that premenopausal or cyclic women present with a lower incidence of ischemic stroke than men; this distinction is, however, no longer present when postmenopausal or acyclic women are compared to men. Furthermore, the ischemic stroke risk increases in both sexes with age (Wolf, 1990; Gibson et al., 2005; Braeuninger and Kleinschnitz, 2009). Thus, sex hormones must have a role in neuroprotection and the decline thereof with age.

Experimental animal studies have not only reported that young females present with smaller cerebral infarcts and thus less neural tissue injury than their male counterparts (Alkayed et al., 1998; Braeuninger and Kleinschnitz, 2009), but also that high endogenous estradiol levels during the estrus cycle seem to correlate with smaller infarct size in females (Carswell et al., 2000; Braeuninger and Kleinschnitz, 2009). This advantage is abolished in ovariectomized animals, due to the loss of endogenous female sex hormones (Simpkins et al., 1997; Alkayed et al., 1998, 2000; Hawk et al., 1998; Liao et al., 2001; Gibson et al., 2005; Park et al., 2006; Drača, 2009; Selvamani and Sohrabji, 2010), and the consequences of cerebral ischemia in aged animals are more severe than in young animals (Davis et al., 1995; Alkayed et al., 1998).

The female sex hormone 17β-estradiol has been shown to be the principal circulating estrogen protecting the brain from damage, by reducing infarct size after experimental cerebral ischemia, through attenuation of markers of apoptosis by activation of mediators of cell survival signaling pathways. It seems that when administered several days before inducing cerebral ischemia, physiological levels of estradiol attenuate brain injury through the suppression of neuronal apoptosis and genomic actions by acting through mechanisms of the classical nuclear estrogen receptors (Liao et al., 2001; Prewitt and Wilson, 2007; Jia et al., 2009; Suzuki et al., 2009); this is, however, not the case with acute administration of 17β-estradiol at the time of injury, as this does not reduce the extent of infarction (Dubal et al., 1998; Suzuki et al., 2009).

In studies of the neuroprotective extent of estradiol, researchers have shown that a single high-dose injection (1 mg/kg) of 17β-estradiol administered to male rats immediately before experimental cerebral ischemia was capable of reducing cortical tissue loss, and that castration of male rats, resulting in the loss of testosterone, did not alter these results (Toung et al., 1998). Moreover, injection of exogenous 17β-estradiol was only neuroprotective in the male brain, revealing that endogenous estrogen is sufficient to protect the female brain and that exogenous 17β-estradiol had no additional protective effect (Toung et al., 1998). Thus, the hypothesis that estrogen is a major mediator of sex differences displayed in stroke is heavily strengthened (Drača, 2009).

17β-Estradiol salvages the brain from ischemic injury, even enhancing recovery and reducing infarct size in ovariectomized (Simpkins et al., 1997; Selvamani and Sohrabji, 2010) and reproductively senescent or aged females (Alkayed et al.,

2000; Liao et al., 2001), as well as in male animals (Hawk et al., 1998). In the case of acyclic females, this is subject to administration of 17β-estradiol at the onset of senescence or ovariectomy and not in older acyclic females (Bake and Sohrabji, 2004; Suzuki et al., 2009; Selvamani and Sohrabji, 2010). This seems to be due to the upregulation of estrogen receptor alpha (ERα) close to the onset of senescence, in response to declining estrogenic stimuli from the ovaries, inadvertently providing a substrate for exogenous estrogen. However, in older acyclic females, the ERα is already downregulated, and thus, exogenous estrogen becomes deleterious (Jezierski and Sohrabji, 2001; Selvamani and Sohrabji, 2010). Ischemic injury itself has been found to increase the expression of ERα in the cerebral cortex, without influencing ERβ expression. Consequently, it is believed that it is this ERα re-expression after ischemic injury that mediates 17β-estradiol's profound neuroprotection against ischemia (Dubal et al., 2001; Suzuki et al., 2007, 2009).

Cerebral ischemia triggers a complex series of events, including excitotoxicity, inflammation, and formation of edema, as well as apoptosis and necrosis (Danton and Dietrich, 2003; Gibson et al., 2005; Saenger and Christenson, 2010) – all of which are reduced by estradiol through free radical scavenger action, among others (Demopoulos et al., 1972; Singer et al., 1996; Gibson et al., 2005; Drača, 2009). Estradiol seems to target neural cells by indirect transcriptional mechanisms and by direct mechanisms, stabilizing neurotransmission, inhibiting apoptosis, reducing cerebral edema, and exerting anti-inflammatory and antioxidant effects (Manthey and Behl, 2006; Drača, 2009). Figure 1 proposes further points at which estradiol wields an influence in neuroprotection against cerebral ischemia.

Postischemic inflammation strongly contributes to the extent of cerebral injury, and 17β-estradiol may exert protection through anti-inflammatory (Figure 1) actions (Vegeto et al., 2008; Suzuki et al., 2009). In fact, the presence of initial neural inflammation is negatively correlated with serum estradiol levels (Wang et al., 2007). The proposed anti-inflammatory action of estradiol is strengthened by findings that 17β-estradiol is neuroprotective when administered immediately upon ovariectomy but not when administered after 10 weeks of hypoestrogenicity, demonstrating that a prolonged period of hypoestrogenicity disrupts not only the neuroprotective but also the anti-inflammatory actions of estradiol (Suzuki et al., 2007, 2009). The first cellular response in inflammation is the activation and accumulation of neutrophils (Morganti-Kossmann et al., 2001). It is evident in models of transient cerebral ischemia that tissue loss is reduced significantly through the inhibition of neutrophil infiltration, as it is apparent that neutrophils wield the most damage to ischemic lesions once reperfusion is undertaken (Connolly et al., 1996; Guha and Mackman, 2001; Wang et al., 2007). It would seem that neutrophil accumulation is also negatively correlated with serum estradiol levels (Liao et al., 2001), strengthening evidence for the neuroprotective role of the female sex hormone even more. The anti-inflammatory properties of 17β-estradiol in the cerebral circulation thus influence the incidence, outcome, and severity of injury in stroke

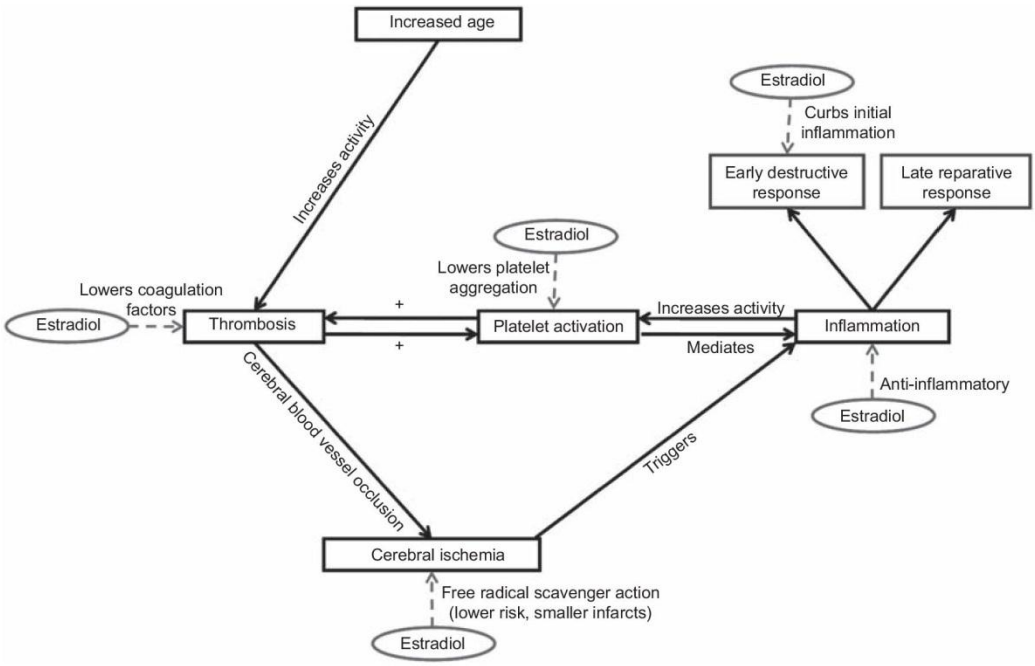


Figure 1 Possible points of estradiol's influence in neuroprotection against cerebral ischemia.
Estradiol affects thrombosis activity through the lowering of coagulation factors and platelet aggregation. It displays anti-inflammatory mechanisms and lowers the negative consequences of cerebral infarction through free radical scavenger action.

by attenuating ischemia-induced inflammatory responses (Suzuki et al., 2009).

Not only does estradiol exert protective anti-inflammatory actions subsequent to cerebral ischemia, but it also has a role in thrombosis (Figure 1) regulation (Wong et al., 2008; Bailey et al., 2009), the alteration of which may result in cerebral ischemia. Coagulation factors and proteins are lowered by the presence of estrogen, though some factors (i.e., fibrinogen) are inherently higher in females than in males (Mendelsohn and Karas, 1999; Bailey et al., 2009); in addition, there are cyclic patterns in these coagulation proteins that correspond to the menstrual cycle in females (Kadir et al., 1999; Bailey et al., 2009). Functionally, female platelet isolates, though capable of binding more fibrinogen and displaying a greater maximal aggregation extent than male platelet isolates (Leng et al., 2004; Bailey et al., 2009), actually do not aggregate as quickly as the larger number of male platelets do, thus to some degree rendering females less susceptible to thrombosis. In the absence of estradiol in acyclicity, female platelets are again more susceptible to thrombosis (Wong et al., 2008; Bailey et al., 2009). Platelets are indeed found to express ERβ, which is hypothesized to have a direct effect on platelet function (Peters et al., 2002; Jayachandran and Miller, 2003; Bailey et al., 2009). Thus, it becomes clear that there are not only sex-based differences in coagulation processes but also age-based differences; as in a hypoestrogenic state, females not only have higher coagulation factors but also higher maximal platelet aggregation capabilities than males, rendering them more prone to thrombosis in an acyclic state.

Finally, it must be noted that estradiol replacement is not universally neuroprotective. It has been suggested that the neuroprotective effects of estrogen are more evident in transient than in permanent models of cerebral ischemia (Macrae and Carswell, 2006; Selvamani and Sohrabji, 2010). This is suggested due to findings that in severe ischemic injury, there are no sex differences in infarct size and also no reduction of the infarct with 17β-estradiol administration (Vergouwen et al., 2000; Selvamani and Sohrabji, 2010). Permanent ischemia leads to severe metabolic impairment in the cerebral cortex, which results in necrosis of many neurons in the region within several hours following injury. Regions surrounding the core of ischemia can be salvaged from apoptosis through the powerful neuroprotective action of 17β-estradiol (Prewitt and Wilson, 2007; Suzuki et al., 2009), but the effects of estradiol at the ischemic core are only visible in transient ischemic models. Conclusively, 17β-estradiol protects the brain through suppression of neuronal apoptosis during the initial 24 hours after injury, in part by suppressing the inflammatory response, and enhances neurogenesis within the first 96 hours after ischemic stroke (Suzuki et al., 2009).

Conclusions

Platelets are central to both thrombosis and inflammation. Not only is platelet localization essential to thrombus initiation, formation, and stabilization, but in areas of ischemia/reperfusion injury in cerebral ischemia, platelets and leukocytes colocalize, linking hemostatic thrombotic and inflammatory responses. Estrogen is neuroprotective in a myriad of mechanisms – a few of which include anti-inflammatory actions and regulation of coagulation factors affecting thrombosis ability. The cascade of cerebral ischemia, its association to inflammation and thrombosis, and their connection to each other are thus intricately intertwined.

References

Alkayed, N.J., Harukuni, I., Kimes, A.S., London, E.D., Traystman, R.J., and Hurn, P.D. (1998). Gender-linked brain injury in experimental stroke. Stroke *29*, 159–165.

Alkayed, N.J., Murphy, S.J., Traystman, R.J., and Hurn, P.D. (2000). Neuroprotective effects of female gonadal steroids in reproductively senescent female rats. Stroke *31*, 161–168.

Altman, R. (2003). Risk factors in coronary atherosclerosis atheroinflammation: the meeting point. Thromb. J. *1*, 1–11.

André, P., Prasad, K.S., Denis, C.V., He, M., Papalia, J.M., Hynes, R.O., Phillips, D.R., and Wagner, D.D. (2002). CD40L stabilizes arterial thrombi by a β3 integrin – dependent mechanism. Nat. Med. *8*, 247–252.

Bailey, A.L., Scantlebury, D.C., and Smyth, S.S. (2009). Thrombosis and antithrombotic therapy in women. Arterioscler. Thromb. Vasc. Biol. *29*, 284–288.

Bake, S. and Sohrabji, F. (2004). 17β-Estradiol differentially regulates blood-brain barrier permeability in young and aging female rats. Endocrinology *145*, 5471–5475.

Barone, F.C., Hillegass, L.M., Tzimas, M.N., Schmidt, D.B., Foley, J.J., White, R.F., Price, W.J., Feuerstein, G.Z., Clark, R.K., Griswold, D.E., et al. (1995). Time-related changes in myeloperoxidase activity and leukotriene B4 receptor binding reflect leukocyte influx in cerebral focal stroke. Mol. Chem. Neuropathol. *24*, 13–30.

Bar-Shavit, R., Kahn, A.J., Mann, K.G., and Wilner, G.D. (1986). Identification of a thrombin sequence with growth factor activity on macrophages. Proc. Natl. Acad. Sci. USA *83*, 976–980.

Braeuninger, S. and Kleinschnitz, C. (2009). Rodent models of focal cerebral ischemia: procedural pitfalls and translational problems. Exp. Transl. Stroke Med. *25*, 1–8.

Brand, K., Fowler, B.J., Edgington, T., and Mackman, N. (1991). Tissue factor mRNA in THP-1 monocytic cells is regulated at both transcriptional and posttranscriptional levels in response to lipopolysaccharide. Mol. Cell Biol. *11*, 4732–4738.

Brouns, R. and De Deyn, P.P. (2009). The complexity of neurobiological processes in acute ischemic stroke. Clin. Neurol. Neurosurg. *111*, 483–495.

Burstein, S.A. (1997). Cytokines, platelet production and hemostasis. Platelets *8*, 93–104.

Butcher, E.C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell *67*, 1033–1036.

Butenas, S. and Mann, K.G. (2002). Blood coagulation. Biochemistry *67*, 3–12.

Camera, M., Giesen, P.L., Fallon, J., Aufiero, B.M., Taubman, M., Tremoli, E., and Nemerson, Y. (1999). Cooperation between VEGF and TNF-α is necessary for exposure of active tissue factor on the surface of human endothelial cells. Arterioscler. Thromb. Vasc. Biol. *19*, 531–537.

Carswell, H.V., Dominiczak, A.F., and Macrae, I.M. (2000). Estrogen status affects sensitivity to focal cerebral ischemia in stroke-

prone spontaneously hypertensive rats. Am. J. Physiol. Heart Circ. Physiol. *278*, H290–H294.

Connolly Jr., E.S., Winfree, C.J., Springer, T.A., Naka, Y., Liao, H., Yan, S.D., Stern, D.M., Solomon, R.A., Gutierrez-Ramos, J.C., and Pinsky, D.J. (1996). Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. J. Clin. Invest. *97*, 209–216.

Danesh, J., Lewington, S., Thompson, S.G., Lowe, G.D.O., Collins, R., Kostis, J.B., Wilson, A.C., Folsom, A.R., Wu, K., Benderly, M., et al. (2005). Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. J. Am. Med. Assoc. *294*, 1799–1809.

Danton, G.H. and Dietrich, W.D. (2003). Inflammatory mechanisms after ischemia and stroke. J. Neuropathol. Exp. Neurol. *62*, 127–136.

Davis, M., Mendelow, A.D., Perrry, R.H., Chambers, I.R., and James, O.F. (1995). Experimental stroke and neuroprotection in the aging rat brain. Stroke *26*, 1072–1078.

Day, S.M., Reeve, J.L., Pedersen, B., Farris, D.M., Myers, D.D., Im, M., Wakefield, T.W., Mackman, N., and Fay, W.P. (2005). Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. Blood *105*, 192–198.

Demopoulos, H.B., Milvy, P., Kakari, S., and Ransohoff, J. (1972). Molecular aspects of membrane structure in cerebral edema. In: Steroids and Brain Edema, H. Reulen and K. Shurmann, eds. (Springer-Verlag, New York), pp. 29–39.

Diacovo, T.G., Roth, S.J., Buccola, J.M., Bainton, D.F., and Springer, T.A. (1996). Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the β2-integrin CD11b/CD18. Blood *88*, 146–157.

Drača, S. (2009). Are female sex steroids neuroprotective in experimental stroke. Med. Hypotheses *73*, 1051–1052.

Drake, T.A., Morrissey, J.H., and Edgington, T.S. (1989). Selective cellular expression of tissue factor in human tissues: implications for disorders of hemostasis and thrombosis. Am. J. Pathol. *134*, 1087–1097.

Dubal, D.B., Kashon, M.L., Pettigrew, L.C., Ren, J.M., Finkelstein, S.P., Rau, S.W., and Wise, P.M. (1998). Estradiol protects against ischemic injury. J. Cereb. Blood Flow Metab. *18*, 1253–1258.

Dubal, D.B., Zhu, H., Yu, J., Rau, S.W., Shughrue, P.J., Merchenthaler, I., Kindy, M.S., and Wild, P.M. (2001). Estrogen receptor α, not β is a critical link in estradiol-mediated protection against brain injury. Proc. Natl. Acad. Sci. USA *98*, 1952–1957.

Elkins, J.S. and Johnston, S.C. (2003). Thirty-year projections for details from ischemic stroke in the United States. Stroke *34*, 2109–2112.

Emms, H. and Lewis, G.P. (1985). Sex and hormonal influences on platelet sensitivity and coagulation in the rat. Br. J. Pharmacol. *86*, 557–563.

Esmon, C.T. (2003). Inflammation and thrombosis. J. Thromb. Haemost. *1*, 1343–1348.

Fatah, K., Hamsten, A., Blombäck, B., and Blombäck, M. (1992). Fibrin gel network characteristics and coronary heart disease: relations to plasma fibrinogen concentration, acute phase protein, serum lipoproteins and coronary atherosclerosis. Thromb. Haemost. *68*, 130–135.

Frenette, P.S., Denis, C.V., Weiss, L., Jurk, K., Subbarao, S., Kehrel, B., Hartwig, J.H., Vestweber, D., and Wagner, D.D. (2000). P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions *in vivo*. J. Exp. Med. *191*, 1413–1422.

Furman, M.I., Benoit, S.E., Barnard, M.R., Valeri, C.R., Borbone, M.L., Becker, R.C., Hechtman, H.B., and Michelson, A.D. (1998). Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. J. Am. Coll. Cardiol. *31*, 352–358.

Gibson, C.L., Constantin, D., Prior, M.J., Bath, P.M., and Murphy, S.P. (2005). Progesterone suppresses the inflammatory response and nitric oxide synthase-2 expression following cerebral ischemia. Exp. Neurol. *193*, 522–530.

Gimbrone Jr., M.A. (1995). Vascular endothelium: an integrator of pathophysiologic stimuli in atherosclerosis. Am. J. Cardiol. *75*, B67–B70.

Goel, M.S. and Diamond, S.L. (2001). Neutrophil enhancement of fibrin deposition under flow through platelet-dependent and -independent mechanisms. Arterioscler. Thromb. Vasc. Biol. *21*, 2093–2098.

Guha, M. and Mackman, N. (2001). LPS induction of gene expression in human monocytes. Cell Signal. *13*, 85–94.

Hallenbeck, J.M. (1996). Significance of the inflammatory response in brain ischemia. Acta Neurochir. *66*, S27–S31.

Hawiger, J. (1995). Mechanisms involved in platelet vessel wall interaction. Thromb. Haemost. *74*, 369–372.

Hawk, T., Zhang, Y.Q., Rajakumar, G., Day, A.L., and Simpkins, J.W. (1998). Testosterone increases and estradiol decreases middle cerebral artery occlusion lesion size in male rats. Brain Res. *796*, 296–298.

Henn, V., Slupsky, J.R., Gräfe, M., Anagnostopoulos, I., Förster, R., Müller-Berghaus, G., and Kroczek, R.A. (1998). CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature *391*, 591–594.

Herd, C.M. and Page, C.P. (1994). Pulmonary immune cells in health and disease: platelets. Eur. Respir. J. *7*, 1145–1160.

Hynes, R.O. (1990). Fibronectins (Springer-Verlag, New York).

Jayachandran, M. and Miller, V.M. (2003). Human platelets contain estrogen receptor α, caveolin-1 and estrogen receptor associated proteins. Platelets *14*, 75–81.

Jezierski, M.K. and Sohrabji, F. (2001). Neurotrophin expression in the reproductively senescent forebrain is refractory to estrogen stimulation. Neurobiol. Aging *22*, 309–319.

Jia, J., Guan, D., Zhu, W., Alkayed, N.J., Wang, M.M., Hua, Z., and Xu, Y. (2009). Estrogen inhibits Fas-mediated apoptosis in experimental stroke. Exp. Neurol. *215*, 48–52.

Kadir, R.A., Economides, D.L., Sabin, C.A., Owens, D., and Lee, C.A. (1999). Variations in coagulation factors in women: effects of age, ethnicity, menstrual cycle and combined oral contraceptive. Thromb. Haemost. *82*, 1456–1461.

Konstantopoulos, K., Neelamegham, S., Burns, A.R., Hentzen, E., Kansas, G.S., Snapp, K.R., Berg, E.L., Hellums, J.D., Smith, C.W., McIntire, L.V., et al. (1998). Venous levels of shear support neutrophil-platelet adhesion and neutrophil aggregation in blood via P-selectin and β2-integrin. Circulation *98*, 873–882.

Kranzhöfer, R., Clinton, S.K., Ishii, K., Coughlin, S.R., Fenton 2nd, J.W., and Libby, P. (1996). Thrombin potently induces cytokine production by human vascular smooth muscle cells but not in mononuclear phagocytes. Circ. Res. *79*, 286–294.

Kuijper, P.H., Gallardo Torres, H.I., van der Linden, J.A., Lammers, J.W., Sixma, J.J., Koenderman, L., and Zwaginga, J.J. (1996). Platelet-dependent primary hemostasis promotes selectin- and integrin-mediated neutrophil adhesion to damaged endothelium under flow conditions. Blood *87*, 3271–3281.

Laursen, J.B., Somers, M., Kurz, S., Warnholtz, A., McCann, L., Freeman, B.A., Tarpley, M., Fukai, T., and Harrison, D.G. (2001). Endothelial regulation of vasomotion in apoE-deficient

mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. Circulation *103*, 1282–1288.

Leng, X.H., Hong, S.Y., Larrucea, S., Zhang, W., Li, T.T., López, J.A., and Bray, P.F. (2004). Platelets of female mice are intrinsically more sensitive to agonists than are platelets of males. Arterioscler. Thromb. Vasc. Biol. *24*, 376–381.

Liao, S.L., Chen, W.Y., Kuo, J.S., and Chen, C.J. (2001). Association of serum estrogen level and ischemic neuroprotection in female rats. Neurosci. Lett. *297*, 159–162.

Libby, P. and Simon, D.I. (2001). Inflammation and thrombosis: the clot thickens. Circulation *130*, 1718–1720.

Lorant, D.E., Patel, K.D., McIntyre, T.M., McEver, R.P., Prescott, S.M., and Zimmerman, G.A. (1991). Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. J. Cell Biol. *115*, 223–224.

Lowe, G.D.O., Rumley, A., Woodward, M., Morrison, C.E., Philippou, H., Lane, D.A., and Tunstall-Pedoe, H. (1997). Epidemiology of coagulation factors, inhibitors and activation markers: the Third Glasgow MONICA Survey. I. Illustrative reference ranges by age, sex and hormone use. Br. J. Haematol. *97*, 775–784.

Lowe, G.D.O., Rumley, A., and Mackie, I.J. (2004). Plasma fibrinogen. Ann. Clin. Biochem. *41*, 430–440.

Mackman, N. (2004). Role of tissue factor in hemostasis, thrombosis, and vascular development. Arterioscler. Thromb. Vasc. Biol. *24*, 1015–1022.

Macrae, I.M. and Carswell, H.V. (2006). Oestrogen and stroke: the potential for harm as well as benefit. Biochem. Soc. Trans. *34*, 1362–1365.

Mann, K.G., van't Veer, C., Cawthern, K., and Butenas, S. (1998). The role of tissue factor pathway in initiation of coagulation. Blood Coagul. Fibrinolysis *9*, S3–S7.

Manthey, D. and Behl, C. (2006). From structured biochemistry to expression profiling: neuroprotective activities of estrogen. Neuroscience *138*, 845–850.

McCullough, L.D. and Hurn, P.D. (2003). Estrogen and ischemic neuroprotection: an integrated view. Trends Endocrinol. Metab. *14*, 228–235.

Mendelsohn, M.E. and Karas, R.H. (1999). The protective effects of estrogen on the cardiovascular system. N. Engl. J. Med. *340*, 1801–1811.

Miller, D.L., Yaron, R., and Yellin, M.J. (1998). CD40L-CD40 interactions regulate endothelial cell surface tissue factor and thrombomodulin expression. J. Leukoc. Biol. *63*, 373–379.

Mitka, M. (2006). Studies explore stroke's gender gap. J. Am. Med. Assoc. *295*, 1755–1756.

Morganti-Kossmann, M.C., Rancan, M., Otto, V.I., Stahel, P.F., and Kossmann, T. (2001). Role of cerebral inflammation after traumatic brain injury: a revisited concept. Shock *16*, 165–177.

Myers, D.D. and Wakefield, T.W. (2005). Inflammation-dependent thrombosis. Front. Biosci. *10*, 2750–2757.

Nathan, C. (2002). Points of control in inflammation. Nature *420*, 846–852.

Nemerson, Y. (1988). Tissue factor and hemostasis. Blood *71*, 1–8.

Ni, H., Denis, C.V., Subbarao, S., Degen, J.L., Sato, T.N., Hynes, R.O., and Wagner, D.D. (2000). Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J. Clin. Invest. *106*, 385–392.

Ostrovsky, L., King, A.J., Bond, S., Mitchell, D., Lorant, D.E., Zimmerman, G.A., Larsen, R., Niu, X.F., and Kubes, P. (1998). A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process. Blood *91*, 3028–3036.

Ott, I., Neumann, F.J., Gawaz, M., Schmitt, M., and Schömig, A. (1996). Increased neutrophil-platelet adhesion in patients with unstable angina. Circulation *94*, 1239–1246.

Park, E.M., Cho, S., Frys, K.A., Glickstein, S.B., Zhou, P., Anrather, J., Ross, M.E., and Iadecola, C. (2006). Inducible nitric oxide synthase contributes to gender differences in ischemic brain injury. J. Cereb. Blood Flow Metab. *26*, 392–401.

Pendurthi, U.R., Alok, D., and Rao, L.V.M. (1997). Binding of factor VIIa to tissue factor induces alterations in gene expression in human fibroblast cells: up-regulation of poly(A) polymerase. Proc. Natl. Acad. Sci. USA *94*, 12598–12603.

Peters, L.L., Cheever, E.M., Ellis, H.R., Magnani, P.A., Svenson, K.L., Von Smith, R., and Bogue, M.A. (2002). Large-scale, high-throughput screening for coagulation and hematologic phenotypes in mice. Physiol. Genomics *11*, 185–193.

Pretorius, E., Briedenhann, S., Marx, J., and Franz, R.C. (2006). Structural changes in the fibrin network of a pretoria family with dysfibrinogenemia: a scanning electron microscopical study. Ultrastruct. Pathol. *30*, 167–176.

Pretorius, E., Briedenhann, S., Marx, J., Smit, E., Van Der Merwe, C.F., Pieters, M., and Franz, R.C. (2007). Ultrastructural comparison of the morphology of three different platelet and fibrin fibre preparations. Anat. Rec. (Hoboken). *290*, 188–198.

Prewitt, A.K. and Wilson, M.E. (2007). Changes in estrogen receptor-α mRNA in the mouse cortex during development. Brain Res. *1134*, 62–69.

Rauch, U. and Nemerson, Y. (2000). Tissue factor, the blood, and the arterial wall. Trends Cardiovasc. Med. *10*, 139–143.

Ross, R. (1985). Platelets, platelet-derived growth factor, growth control, and their interactions with the vascular wall. J. Cardiovasc. Pharmacol. *7*, S186–S190.

Roth, G.J. (1992). Platelets and blood vessels: the adhesion event. Immunol. Today *13*, 100–105.

Saenger, A.K. and Christenson, R.H. (2010). Stroke biomarkers: progress and challenges for diagnosis, prognosis, differentiation, and treatment. Clin. Chem. *56*, 21–33.

Sakai, T., Johnson, K.J., Murozono, M., Sakai, K., Magnuson, M.A., Wieloch, T., Cronberg, T., Isshiki, A., Erickson, H.P., and Fässler, R. (2001). Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing hemostasis. Nat. Med. *3*, 324–330.

Schecter, A.D., Giesen, P.L., Taby, O., Rosenfield, C.L., Rossikhina, M., Fyfe, B.S., Kohtz, D.S., Fallon, J.T., Nemerson, Y., and Taubman, M.B. (1997). Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation. J. Clin. Invest. *100*, 2276–2285.

Selvamani, A. and Sohrabji, F. (2010). Reproductive age modulates the impact of focal ischemia on the forebrain as well as the effects of estrogen treatment in female rats. Neurobiol. Aging *31*, 1618–1628.

Shebuski, R.J. and Kilgore, K.S. (2002). Role of inflammatory mediators in thrombogenesis. J. Pharmacol. Exp. Ther. *300*, 729–735.

Simpkins, J.W., Rajakumar, G., Zhang, Y.Q., Simpkins, C.E., Greenwald, D., Yu, C.J., Bodor, N., and Day, A.L. (1997). Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. J. Neurosurg. *87*, 724–730.

Singer, C.A., Rogers, K.L., Strickland, T.M., and Dorsa, D.M. (1996). Estrogen protects primarily cortical neurons from glutamate toxicity. Neurosci. Lett. *212*, 13–16.

Springer, T.A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell *76*, 301–314.

Stewart, G.J., Ritchie, W.G.M., and Lynch, P.R. (1974). Venous endothelial damage produced by massive sticking and emigration of leukocytes. Am. J. Pathol. *74*, 507–532.

Suzuki, S., Brown, C.M., Dela Cruz, C.D., Yang, E., Bridwell, D.A., and Wise, P.M. (2007). Timing of estrogen therapy after ovariectomy dictates the efficacy of its neuroprotective and antiinflammatory actions. Proc. Natl. Acad. Sci. USA *104*, 6013–6018.

Suzuki, S., Brown, C.M., and Wise, P.M. (2009). Neuroprotective effects of estrogens following ischemic stroke. Front Neuroendocrinol. *30*, 201–211.

Taylor Jr., F.B., Chang, A., Esmon, C.T., D'Angelo, A., Vigano-D' Angelo, S., and Blick, K.E. (1987). Protein C prevents the coagulopathic and lethal effects of *Escherichia coli* infusion in the baboon. J. Clin. Invest. *79*, 918–925.

ten Cate, J.W., van der Poll, T., Levi, M., ten Cate, H., and van Deventer, S.J. (1997). Cytokines: triggers of clinical thrombotic disease. J. Thromb. Haemost. *87*, 415–419.

Toung, T.J.K., Traystman, R.J., and Hurn, P.D. (1998). Estrogen-mediated neuroprotection after experimental stroke in male rats. Stroke *29*, 1666–1670.

Varma, M.R., Varga, A.J., Knipp, B.S., Sukheepod, P., Upchurch, G.R., Kunkel, S.L., Wakefield, T.W., and Henke, P.K. (2003). Neutropenia impairs venous thrombosis resolution in the rat. J. Vasc. Surg. *38*, 1090–1098.

Vegeto, E., Benedusi, V., and Maggi, A. (2008). Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. Front. Neuroendocrinol. *29*, 507–519.

Vergouwen, M.D., Anderson, R.E., and Meyer, F.B. (2000). Gender differences and the effects of synthetic exogenous and non-synthetic estrogens in focal cerebral ischemia. Brain Res. *878*, 88–97.

Wagner, D.D. and Burger, P.C. (2003). Platelets in inflammation and thrombosis. Arterioscler. Thromb. Vasc. Biol. *23*, 2131–2137.

Wakefield, T.W., Strieter, R.M., Prince, M.R., Downing, L.J., and Greenfield, L.J. (1997). Pathogenesis of venous thrombosis: a new insight. Cardiovasc. Surg. *5*, 6–15.

Wang, Q., Tang, X.N., and Yenari, M.A. (2007). The inflammatory response in stroke. J. Neuroimmunol. *184*, 53–68.

Weber, C. and Springer, T.A. (1997). Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to αIIbβ3 and stimulated by platelet-activating factor. J. Clin. Invest. *100*, 2085–2093.

White, J.G. (1987). Views of the platelet cytoskeleton at rest and at work. Ann. NY Acad. Sci. *509*, 156–176.

Wilcox, J.N., Smith, K.M., Schwartz, S., and Gordon, D. (1989). Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc. Natl. Acad. Sci. USA *86*, 2839–2843.

Wohner, N. (2008). Role of cellular elements in thrombus formation and dissolution. Cardiovasc. Hematol. Agents Med. Chem. *6*, 224–228.

Wolf, P.A. (1990). An overview of the epidemiology of stroke. Stroke *21*, S114–S116.

Wong, J.H., Dukes, J., Levy, R.E., Sos, B., Mason, S.E., Fong, T.S., and Weiss, E.J. (2008). Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. J. Clin. Invest. *118*, 2969–2978.

Zhang, X., Selli, M.L., Baglioni, S., Hauri, A., Chiari, R., Dottorini, M., Todisco, T., Nenci, G.G., and Gresele, P. (1993). Platelets from asthmatic patients migrate *in vitro* in response to allergen stimulation. Thromb. Haemost. *69*, 1356.

Received February 16, 2012; accepted March 5, 2012

Wendy Jeannette van der Spuy is PhD student with the Department of Anatomy's Division of Histology and Cell Biology at the University of Pretoria, South Africa. She has a passion for electron microscopy, biological research and scientific writing. She is credited as primary or co-author of 14 scientific publications since 2009, with a further number of articles in the peer-review process for 2012. Together with Professor Etheresia Pretorius, her supervisor, she wishes to continue in the field of stroke and inflammatory research.

Etheresia (Resia) Pretorius received her PhD in 1998, and is a Professor in the Department of Physiology, University of Pretoria, South Africa. She has published over 150 peer reviewed manuscripts and book chapters and, since 2000, has also been mentor to 28 successful MSc and PhD students. Her focus is diseases associated with inflammation and she combines ultrastructure and electron microscopy with physiology. Particularly, her focus is the effect of inflammation on platelets, red blood cells and the coagulation system. In December 2011, Resia was named the Southern African Regional winner in the category, Basic Science, Technology and Innovation, of the prestigious African Union Kwame Nkrumah Scientific Awards, which forms part of the African Union Scientific Awards Programme.

Appendix B

Scientific Manuscript in Press

van der Spuy WJ & Pretorius E. A place for ultrastructural analysis of platelets in cerebral ischaemic research. *Microscopy Research and Technique* 2013; DOI 10.1002/jemt.22231

Published Online Prior to Print

MICROSCOPY RESEARCH AND TECHNIQUE 00:00–00 (2013)

A Place for Ultrastructural Analysis of Platelets in Cerebral Ischemic Research

WENDY JEANNETTE VAN DER SPUY,[1]* AND ETHERESIA PRETORIUS[2]
[1]*School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, South Africa*
[2]*Department of Physiology, Faculty of Health Sciences, University of Pretoria, South Africa*

KEY WORDS inflammation; estrogen; stroke; thrombosis

ABSTRACT It is well known that estrogen is neuroprotective through various mechanisms which suggest that sex hormone levels, thrombotic mechanisms, and inflammatory processes are strongly interconnected in predicting the outcome and consequences of cerebral ischemia. Because platelet ultrastructure is altered in conditions like thrombosis and associated with stroke, the question arises whether ultrastructural analyses of platelet morphology may provide further insight into the role of estrogen during ischemic insult. In the current study, a hyperglycemic modification to the two-vessel occlusion model for inducing experimental cerebral ischemia was employed, in order to correlate neural tissue integrity levels between three experimental groups to corresponding platelet ultrastructure so as to determine whether there is an association between cerebral ischemia and the presence of inflammatory or necrotic platelet ultrastructure. It is apparent in the results that under the influence of estrogen in cyclic or intact females, there is lesser neural tissue damage as well as a reduced degree of inflammation evident in platelet activation morphology when compared to males and acyclic or ovariectomized females. It is unmistakable that neural injury is closely shadowed, if not preceded, by inflammatory changes in the coagulation system, particularly manifested in platelet ultrastructure. It is therefore suggested that platelets may indeed be used successfully to follow the progression of events of cerebral ischemia and possibly assist in the assessment of treatment strategies and their effects on hemostasis. *Microsc. Res. Tech. 00:000–000, 2013*.

INTRODUCTION

Cerebral ischemia by mechanism of thrombosis is one of the leading causes of death and/or disability worldwide. Research has shown that there are sex-based differences in both the prevalence and presentation of stroke and thrombosis. Cerebral ischemia triggers a series of events, initiated with cerebral hypoperfusion, and comprising bioenergetic failure of cellular components, excitotoxicity, oxidative stress, microvascular injury, hemostatic activation, inflammation and edema, biphasic dysfunction of the blood-brain barrier (BBB), as well as apoptosis and ultimate necrosis of neuronal, glial and endothelial cells. This cascade of events is dependent on variables such as onset and duration of ischemia, effectiveness of reperfusion, and resultant infarct size or tissue loss (Brouns and De Deyn, 2009; Danton and Dietrich, 2003; Gibson et al., 2005; Saenger and Christenson, 2010).

Thrombosis, the most common cause of stroke, is influenced by factors including endothelial injury, blood stasis or turbulent flow and hypercoagulability of blood (Myers and Wakefield, 2005). Fundamental to the thrombotic process is the functionality of the platelets and its role in the inflammatory process. Platelets are essential in the initial stages of thrombus formation, because they adhere and aggregate at sites of vascular wall injury and then serve as a surface for the coagulation cascade (Wohner, 2008). Thrombosis and inflammation are therefore interrelated (Myers and Wakefield, 2005; Stewart et al., 1974), as inflammation produces local thrombosis and thrombosis can amplify inflammation (Libby and Simon, 2001). In areas of ischemia/reperfusion injury, platelets colocalize with leukocytes; an interaction linking hemostatic thrombotic and inflammatory responses (Libby and Simon, 2001; Ostrovsky et al., 1998).

In a recent review by van der Spuy and Pretorius (2012), the authors discussed the effect of sex hormones on the thrombotic process and in particular the role that platelets play during ischemic insult. Platelet function varies between the sexes, mainly due to the effect of estrogen and interestingly, platelet reactivity is altered in ovariectomized females (Bailey et al., 2009; Leng et al., 2004). Males have been shown to have higher platelet counts and faster clotting times than their female counterparts, thus making them more susceptible to thrombosis; possibly due to the

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: WJ van der Spuy; School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, 2193, South Africa. E-mail: wendy.vanderspuy@wits.ac.za

Received 12 March 2013; accepted in revised form 24 April 2013

Contract grant sponsor: Medical Research Council (MRC) of South Africa (to E.P.).

DOI 10.1002/jemt.22231
Published online in Wiley Online Library (wileyonlinelibrary.com).

differences in growth hormone secretion between the sexes (pulsatile in males, sustained in females), which in turn influence protein production of coagulation and thrombosis regulators (Bailey et al., 2009; Wong et al., 2008). The female sex hormone 17β-oestradiol has been shown to be the principal circulating estrogen protecting the brain from damage by reducing infarct size after experimental cerebral ischemia. It seems that when administered several days before inducing cerebral ischemia, physiological levels of estradiol attenuate brain injury (Jia et al., 2009; Liao et al., 2001; Prewitt and Wilson, 2007; Suzuki et al., 2009).

It is recognized, through research involving experimental animal models of cerebral ischemia that sex hormones, especially estrogen, offer a degree of neuroprotection. Mechanisms of this neuroprotection may be linked to certain anti-inflammatory properties of estrogen, as well as estrogen's regulation of thrombosis through the lowering of coagulation factors, amongst others. It is also understood that sex hormones alter the function and morphology of platelets, and changes in platelet morphology offer one of the earliest confirmations of inflammation. Recently, Pretorius and coworkers (2012) showed that during thromboembolic ischemic stroke in humans, platelet ultrastructure changes—with swelling and membrane tears becoming visible—a typical characteristic of cellular necrosis. Thus sex hormone levels, thrombotic mechanisms, and inflammatory processes are profoundly interconnected in predicting the outcome and consequences of cerebral ischemia.

In this study, we therefore employed a hyperglycemic two-vessel occlusion model for induction of experimental cerebral ischemia, in which the effect of estrogen on cerebral ischemia could be determined. Furthermore, we compared neural ischemic tissue injury levels between groups to corresponding platelet ultrastructure, to determine if there is a correlation between cerebral ischemia and presence of inflammatory or necrotic platelet ultrastructure.

MATERIALS AND METHODS

A total of 79 age-matched, outbred Sprague Dawley rats, consisting of 26 males and 53 females were allocated for the purposes of this study. One male and two female groups were allocated: sexually mature intact males (MM), sexually mature intact or cyclic females (MF) and sexually mature ovariectomized or acyclic females (OVX). Females of the group OVX underwent bilateral ovariectomy two weeks prior (Jia et al., 2009; Selvamani and Sohrabji, 2010) to commencement of experimental procedures. The three groups were further divided into four subgroups of six animals each: 0 h or preischemic controls, 2 h postreperfusion for immediate injury determination, 24 h postreperfusion for determination of maximal tissue compromisation (Jia et al., 2009), and 48 h postreperfusion for analysis around the onset of possible regeneration (Gibson et al., 2005).

All experimental procedures were carried out in strict accordance with the requirements of the South African National Standard (SANS 10386:2008) pertaining to the care and use of animals for scientific purposes. These guidelines were enforced by both the University of Pretoria's Animal Use and Care Committee and Tshwane University of Technology's Animal Research Ethics Committee where the study was conducted.

Experimental Cerebral Ischemia

Transient experimental cerebral ischemia was induced by occlusion of bilateral common carotid arteries in spontaneously breathing rats in an acute systemic hyperglycemic state. Mild hyperglycemia is known to attenuate cerebral ischemia in experimental models (Dietrich et al., 1993; Pulsinelli et al., 1982). Ketamine/Xylazine anesthesia is proven to cause acute hyperglycemia in fed but not fasted Sprague Dawley rats, with glucose levels remaining elevated for approximately 3 h postanesthesia (Saha et al., 2005). The classical two-vessel occlusion model performed in rats is accompanied by either hypoxia, or hypotension to attenuate cerebral ischemia by preventing collateral blood supply to the forebrain during the procedure (Eklof and Siesjo, 1972; Eklof and Siesjo, 1973; Hoyte and Buchan, 2009; McBean and Kelly, 1998). To assess the effects of systemic hyperglycemia in exclusivity, a modified two-vessel occlusion model was employed, anesthetizing fed-state rats with a Ketamine/Xylazine (Ketamine 100 mg/kg and Xylazine 10 mg/kg IP) combination, without inducing hypoxia or hypotension. Experimental cerebral ischemia was therefore performed in all but the 18 control animals belonging to the 0 h or preischemic termination subgroups. Subsequent to anesthesia, hyperglycemia was confirmed by tail prick (blood glucose >150 mg/dL) using a portable blood glucose meter. Bilateral common carotid arteries were occluded with microsurgical clips and ischemia induced for 15 min, after which reperfusion was confirmed by visualizing the return of pulsations to the artery beyond the point of occlusion. Neck incisions were sutured closed and animals allowed to survive for a maximum of 48 h with free access to food and water.

Monitoring and Termination Procedures

Animals were observed for signs of abnormal behavior, by qualified personnel, on a daily basis for the duration of the study. Monitoring logged included, but was not limited to, weights, habitus, appetite, respiratory pattern and locomotive behavior. At the conclusion of each scheduled phase, animals were anesthetized with Ketamine (100 mg/kg IP) and terminated by cardiac puncture. Blood and whole brains were collected individually.

Tissue and Blood Processing

Neural Tissue Injury Analysis. Whole brains were removed from each individual animal at the termination intervals of 0 h, 2 h, 24 h and 48 h. Upon removal, the cerebellum and olfactory bulbs of each brain were carefully dissected off and discarded. Cerebrums were weighed and sectioned into 2 mm coronal sections using an adult rat coronal brain matrix submerged in chilled 0.1 M phosphate buffered saline. To quantify ischemic damage, sections were stained with 0.2% of the mitochondrial stain 2,3,5-Triphenyltetrazoliumchloride (TTC) (Joshi et al., 2004). Subsequent to staining procedures, the TTC solution was removed from the container, sections washed, a solvent of 50:50 Ethanol/Dimethylsulphoxide (Sladowski et al., 1993) added to the container at 20 mL/g of tissue (Xue et al.,

2004) and allowed to extract the TTC formazan product from the tissue. At conclusion of extraction, the container was briefly shaken and a small amount of the extract drawn out for absorbance measurement of each brain at a wavelength of 485 nm (Preston and Webster, 2000; Xue et al., 2004). The percentage of tissue injury could then be calculated for each of the animals using the equation (Preston and Webster, 2000; Xue et al., 2004):

$$\%\,\text{Tissue Injury} = 100 \times \left[1 - \left(\text{Absorbance}_{\text{Injury}} / \text{Absorbance}_{\text{Control}}\right)\right]$$

Platelet Ultrastructural Analysis. Platelet smears from each individual animal from groups were prepared on Millipore membranes following techniques previously described by Pretorius and colleagues (2011) and blindly analyzed with a ZEISS ULTRA plus FEG Scanning Electron Microscope (Carl Zeiss Pty Ltd., Randburg, South Africa), with accelerating voltage set at 1 kV for high quality surface analysis. Platelets were imaged with the In-lens secondary electron detector.

Statistical Analysis

Data processing and analysis was performed using SAS® (Version 9.3). The three groups of rats (MM, MF and OVX) were compared statistically with respect to neural tissue injury variables within four termination subgroups (times 0 h, 2 h, 24 h, 48 h). Six repeats (absorbance readings) were recorded for each of the animals resulting in $n = 324$ observations of calculated neural tissue injury. Using a multiple analysis of variance (ANOVA) group effect and interaction between group and time were assessed followed by General Linear Model procedure. Specific differences were tested for using Fisher's Least Squares Means to assess pairwise differences. Results are presented as LS means and standard error with significance accepted at the level of $P < 0.05$.

RESULTS

Experimental cerebral ischemia was successfully implemented utilizing the hyperglycemic two-vessel occlusion model. Neural tissue injury (NTI) was evident in all experimental animals when compared to group-matched controls. Variability within the model was calculated to be 63% and the P-value obtained was <0.0001. When only groups effects are accounted for statistically (standard error of 0.619), males (MM, NTI = 23.987%) display significantly more neural tissue injury overall than acyclic females (OVX, NTI = 13.466%) which in turn also display more neural tissue injury overall than cyclic females (MF, NTI = 9.217%). In Figure 1, where group and time effects are both accounted for statistically (standard error of 1.073), it is clear that 2 h postreperfusion, neural tissue injury in MM-2 (NTI = 21.045%) is significantly higher than that in MF-2 (NTI = 14.798%) which is also significantly higher that the injury in OVX-2 (NTI = 11.621%). Importantly at 24 h postreperfusion, there were no significant differences in tissue injury found between MM-24 (NTI = 21.348%) and OVX-24 (NTI = 22.864%), and both were significantly higher in percentage of injury than MF-24 (NTI = 2.780%), validating that estrogen does indeed play a role in tissue survival subsequent to ischemic injury by the point when maximal injury is expected. By 48 h postreperfusion, we see the same trend as with the 2 h subgroups,

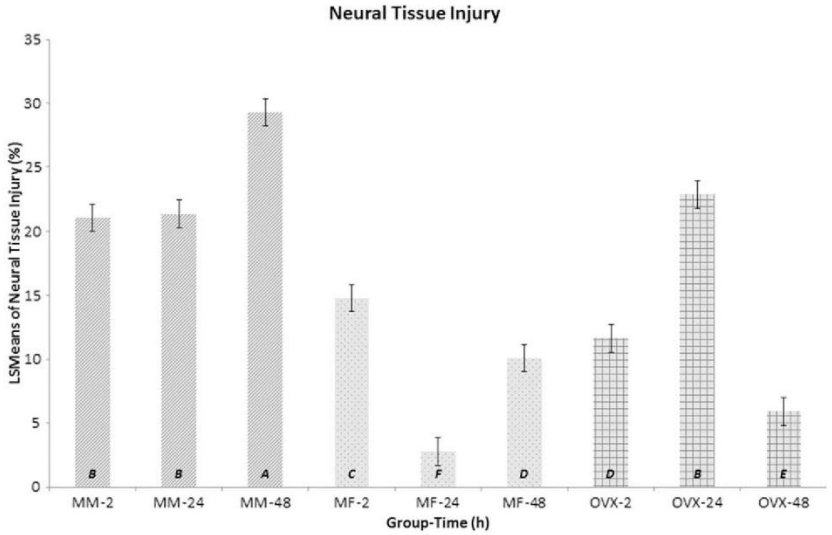


Fig. 1. Percentage neural tissue injury analysis where group and time effect are accounted for. Absorbance values per animal in each subgroup were used to calculate neural tissue injury against absorbance values of animals allocated as controls to that specific group. Italicized letters of the same value at the base of bars indicate no significant difference between the bars so annotated, at a confidence level equal or better than 95% (see Supporting Information). Variability within the model was calculated to be 63% and the P-value obtained was <0.0001 both for the model and the group-time effect set. Group MM = Males, Group MF = Cyclic females, Group OVX = Acyclic females. Time 2 = 2 h postreperfusion, Time 24 = 24 h postreperfusion, Time 48 = 48 h postreperfusion.

in that MM-48 (NTI = 29.299%) displays significantly more injury than MF-48 (NTI = 10.071%) which is also significantly higher in injury percentage than OVX-48 (NTI = 5.911%). MM-48 displays the highest level of neural tissue injury amongst all group-time comparisons and MF-24 displays the lowest level of tissue injury, closely followed by OVX-48. Therefore, it seems that though the lack of estrogen in acyclic females definitely lessens neuroprotection, there is still some degree of inherent protection from the insult of mild cerebral ischemic injury in these ovariectomized females when compared to age-matched males.

All animals within core-specific subgroups displayed similar platelet morphology and figures presented here are representative of a typical platelet as viewed overall per group. Figures 2A–2D shows representative platelets for MM in the four termination subgroups. Platelets for preischemic MM-0, display typical minimal pseudopodia formation and smooth membrane surfaces (Fig. 2A). At 2 h postreperfusion, MM-2 platelets all present with extensive formation of pseudopodia – typically seen in activated platelet states. This is to be expected, as the insult would have triggered the inflammatory response and the coagulation process would now be exposed to the initial stages of equilibrium disturbances (Fig. 2B). By 24 h postreperfusion in MM-24, the impact of the insult has a profound effect on the coagulation system and platelet swelling is visible with breaks in the membrane and reduced pseudopodia formation compared to 2 h after insult (Fig. 2C). These morphological changes—swelling and membrane breakage—are typical of necrosis and were present in 70% of MM-24 platelets. Interestingly, necrotic platelets were also previously noted in humans 24 h after stroke (Pretorius et al., 2012). Figure 2D shows a platelet for 48 h postreperfusion in MM-48. Here it appears that the coagulation systems response has stabilized and that the recovery process has been commenced. Platelets are no longer necrotic in appearance, although the contours of the membrane and the extent of pseudopodia formation still suggest an activated state. We do not suggest that necrosis is reversible but rather that the coagulation system recovers in such a way that necrotic platelets have been cleared and that platelets of activated morphology are once again visible.

Figures 3A–3D shows representative platelets for animals from group MF. The typical control platelet at 0 h for MF-0 is similar to that of the MM-0, with some pseudopodia present and a smooth membrane surface (Fig. 3A). Platelets for MF-2, at 2 h postreperfusion also show extensive pseudopodia formation due to initial inflammation (Fig. 3B)—noted in the activated platelet state of the males. Figure 3C shows a platelet from MF-24, 24 h postreperfusion, with platelets already returning to a preinflammatory state without

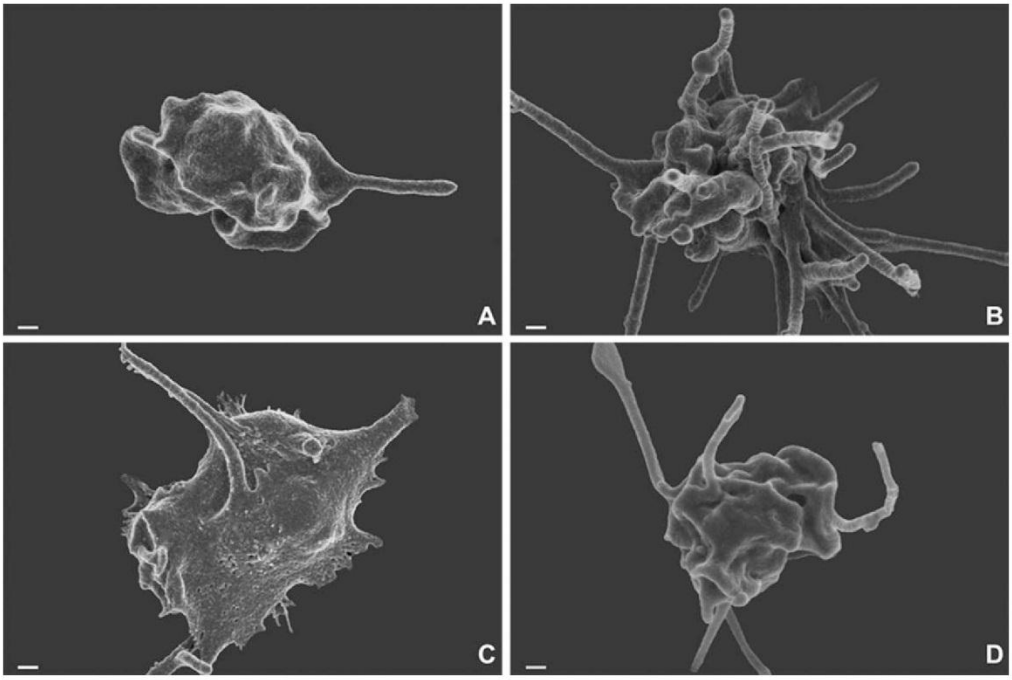


Fig. 2. Platelet morphology representation of males in group MM. A: Platelet from representative male 0 h preischemia (MM-0), showing typical pseudopodia formation and smooth membrane surfaces. B: Platelet from representative male 2 h postreperfusion (MM-2), showing extensive pseudopodia formation – typically seen in activated platelet states. C: Platelet from representative male 24 h postreperfusion (MM-24), here platelet swelling is visible with breaks in the membrane and reduced pseudopodia formation compared to 2 h after insult. This is typical of necrosis. D: Representative platelet 48 h postreperfusion (MM-48). Recovery begins, and platelets do not have a necrotic appearance any more, although pseudopodia formation suggests an activated state. Scale = 200 nm.

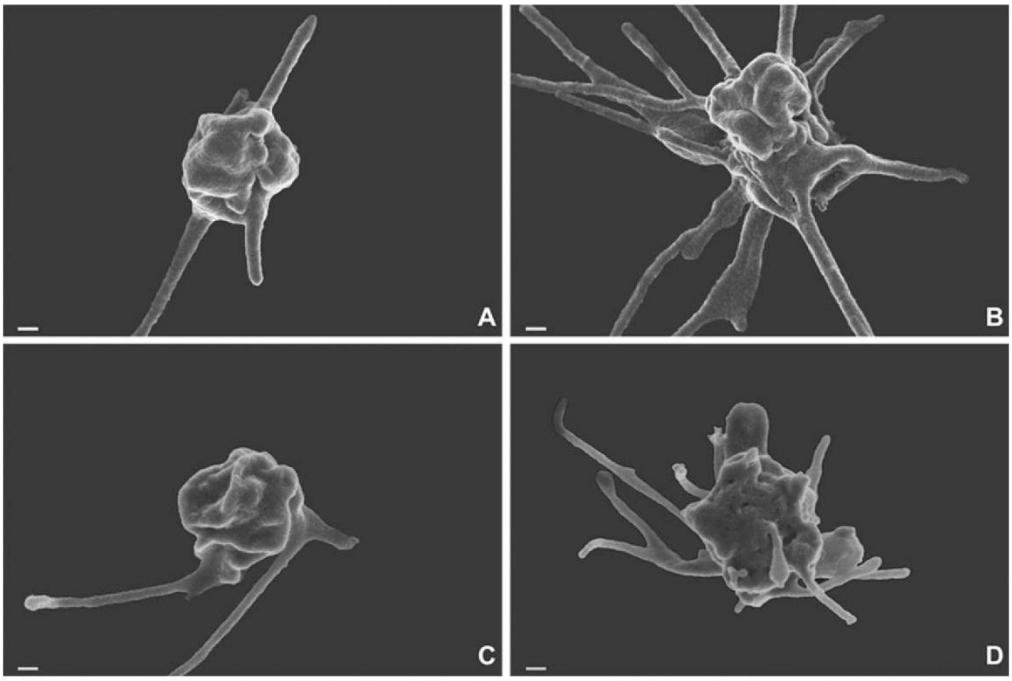


Fig. 3. Platelet morphology representation of cyclic females in group MF. A: Representative platelet preischemia (MF-0), showing typical pseudopodia formation and smooth membrane surfaces. B: Representative platelet 2 h postreperfusion (MF-2), showing extensive pseudopodia formation—typically seen in activated platelet states. C: Representative platelet 24 h postreperfusion (MF-24), here platelets are returning to a preinflammatory state with little pseudopodia formation. D: Representative platelet 48 h postreperfusion (MF-48), pseudopodia formation typical of activation is visible. Scale = 200 nm.

many pseudopodia, similar to group-matched controls. Interestingly, no necrotic platelet ultrastructure was noted in MF. This suggests that estrogen may indeed have a protective effect as previously suggested in the literature, exerting anti-inflammatory effects and delaying apoptosis not only in neural tissue but also at the level of blood hemostasis. At 48 h postreperfusion in MF-48, platelets again appear more activated, but not necrotic (Fig. 3D). This morphology is similar again to MM-48. This reactivation of platelets in MF-48 may possibly be due to the second phase of BBB disruption which again changes the balance in hemostasis; thus evidencing that male platelets which at 48 h were also still in an activated state due to this very reason, though they were reverting to normal morphology when compared to the necrotic platelets in MM-24.

Representative platelets are shown in Figures 4A–4D for animals of group OVX. Here, OVX-0 before ischemic insult shows more extensive pseudopodia formation than seen in the typical morphology noted in both MM-0 and MF-0 controls (Fig. 4A). This could be due to the state of thrombotic preparedness (inherent inflammation due to heightened fibrinogen activity and altered platelet activity caused by increased signaling) previously seen in menopausal women (Pretorius et al., 2010). Platelets from 2 h postreperfusion in OVX-2 presented with less pseudopodia than MM-2 and MF-2 (Fig. 4B). This could be due to the fact that with the presence of higher levels of inflammation after cerebral ischemia, the state of thrombotic preparedness is soothed to some extent. Platelets at 24 h postreperfusion in OVX-24 showed platelet swelling with membrane breaks and reduced pseudopodia formation. This ultrastructure is similar to that of MM-24 and is typical of necrosis (Fig. 4C), which in OVX-24 was evident in 60% of platelets. At 48 h after the insult, platelets from OVX-48 show a degree of recovery—similar to males at this time—platelets no longer have a necrotic appearance, although extensive pseudopodia formation once again suggests that the second phase of BBB disruption is in play (Fig. 4D), challenging the balance in hemostasis as in males and cyclic females at 48 h. There is also a possible reversion to morphology typical of thrombotic preparedness at this time.

DISCUSSION

Several experimental animal models exist to study the consequences and mechanisms of cerebral ischemia. These models provide an opportunity to investigate the interaction between estrogen, thrombosis, and inflammation. Understanding the mechanisms of these interactions serve to enable the development of improved treatment regimens for patients presenting with cerebral ischemia. Because platelet ultrastructure is changed in conditions like thrombosis and associated with stroke, the question arises whether

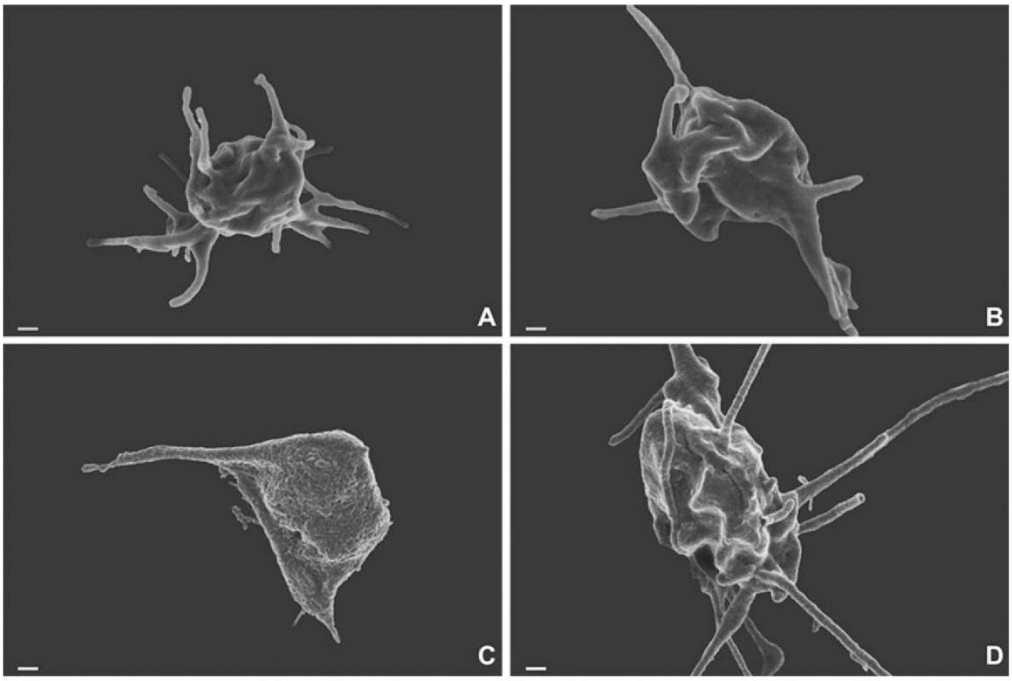


Fig. 4. Platelet morphology representation of acyclic females in group OVX. A: Representative platelet from acyclic female, preischemia (OVX-0). Pseudopodia formation is visible. B: Representative platelet from acyclic female, 2 h postreperfusion (OVX-2). Pseudopodia formation is visible. C: Representative platelet from acyclic female, 24 h postreperfusion (OVX-24). Here platelet swelling is visible with breaks in the membrane and reduced pseudopodia formation compared to 2 h after insult. This is typical of necrosis. D: Representative platelet from acyclic female, 48 h after ischemic (OVX-48) insult. Recovery begins, and platelets do not have a necrotic appearance any more, although pseudopodia formation suggests an activated state. Scale = 200 nm.

ultrastructural and morphological analyses of platelets may provide insight into further roles of estrogen during phases subsequent to ischemic insult.

The ischemic cascade explained earlier, leads us to expect a series of events subsequent to cerebral ischemia. After cerebral hypoperfusion, oxidative stress peaks rapidly and then progressively lowers after a few hours. Inflammation should be evident very early as hemostatic activation takes place within minutes of insult, and is indeed shown to peak early and progressively lessen after a number of hours, with minimal inflammation still present after a few days. Dysfunction of the BBB is biphasic, the second peak being substantially higher than the first. The initial peak is evident within hours of ischemia and the second peak, which is apparent within days, is most likely the period after which secondary and possibly more static cerebral damage becomes apparent.

From neural tissue injury results we can deduce that subsequent to cerebral ischemia, neural tissue in males progressively deteriorates from 2 h to 48 h due to the absence of sufficient levels of estrogen, and therefore, the inability to curb the inflammatory response and suppress apoptosis. Cyclic female neural tissue presents with initial or primary injury at 2 h though less injury is apparent than in males. By 24 h regeneration variables come into play, with estrogen possibly compensating to curb the peak in inflammation and initiate recovery by stalling apoptosis and necrosis. At 48 h, where the second and highest peak of BBB dysfunction is conceivably in play, secondary neural loss becomes evident. Ovariectomized or acyclic females seem to display a smaller degree of primary injury than cyclic females, which could be due to the inherent state of inflammation found in menopausal females—termed thrombotic preparedness (Pretorius et al., 2010)—and thus the inflammatory shock to the hemostatic system is lessened initially. By 24 h, acyclic females present with greatest neural tissue injury, due to the absence of sufficient levels of estrogen and therefore the inability to suppress necrosis and initiate recovery. At 48 h however, the neural tissue of acyclic females seemed to have recovered to a great extent, almost similar to control levels. Return to the state of thrombotic preparedness may potentially have curbed the injury initiated by the second peak of BBB dysfunction. It would be of value to assess 72 h post-ischemic subgroups to determine true cerebral tissue loss, after the ischemic cascade has reached its conclusion and hemostasis has truly stabilized.

Platelet ultrastructure followed the same trends realized in the neural tissue injury results. Cyclic females were perceived to be the gold-standard in assessing the progression of events subsequent to cerebral ischemia due to their high levels of endogenous estrogen available for protection against cerebral ischemic consequences. At 0 h preischemia, platelets

displayed ideal control morphology, with smooth membranes and the extension of few pseudopodia. Upon initial injury assessment at 2 h postischemia, platelets demonstrated typical inflammatory morphology, with the extension of a multitude of pseudopodia. At 24 h postischemia, where maximal damage is expected, estrogen plays its most significant role in that platelets return to preinflammatory morphology due to the noteworthy anti-inflammatory mechanisms exerted by the presence of high levels of estrogen. In these females, 24 h was indeed the point when the smallest neural tissue injury percentage was apparent. At 48 h postreperfusion, platelets appeared more activated once again, though less so than platelets at 2 h postreperfusion. This reactivation may have been due to the second phase of BBB disruption which would again change the balance in hemostasis to a more inflammatory state. It is important to note here that though early inflammation responses (as at 2 h) may potentiate ischemic injury, late inflammatory responses (as at 48 h) may be beneficial to recovery and the repair of ischemic lesions (Wang et al., 2007). In males and acyclic females, insignificant concentrations of estrogen are present and unable to protect/mask the coagulation system from hemostatic disturbance subsequent to cerebral ischemia. Therefore, at 24 h postreperfusion, both these groups expressed a prominent necrotic platelet ultrastructure which did not exist in cyclic females who had full access to the protective effects of estrogen. In males as well as acyclic females, platelet recovery or return to the direction of normalized morphology was noted at 48 h postreperfusion. This corresponds to what was seen in the neural tissue assessment of acyclic females and correlates therefore perfectly with the ultrastructure of the platelets. In the males, however, 48 h is the point of maximal neural tissue damage. Thus commencement of recovery in the males, though not immediately present in neural tissue integrity seems to be evident in platelet morphology.

It has been well established that estrogen is neuroprotective through various actions (Demopoulos et al., 1972; Dračа, 2009; Gibson et al., 2005; Singer et al., 1996), including roles in the regulation of thrombosis and inflammation, targeting neural cells by stabilizing neurotransmission, inhibiting apoptosis, reducing cerebral edema and exerting anti-inflammatory and antioxidant effects (Dračа, 2009; Manthey and Behl, 2006). Our results support these findings and strongly suggest that estrogen is indeed neuroprotective through various actions including roles in the regulation of thrombosis and inflammation, targeting neural cells, inhibiting apoptosis and exerting anti-inflammatory and antioxidant effects. It is evident that under the influence of estrogen in cyclic females, there is reduced neural tissue injury as well as a smaller degree of inflammation evident in platelet activation morphology when compared to males and acyclic females. Furthermore, it is unmistakable that neural injury is closely shadowed, if not preceded, by inflammatory changes in the coagulation system, particularly manifested in platelet ultrastructure. It is therefore suggested that platelets may indeed be used successfully to follow the progression of events of cerebral ischemia and possibly assist in the assessment of treatment strategies and their effects on hemostasis.

ACKNOWLEDGMENTS

The authors declare no conflict of interest in the work presented here. The authors wish to thank Dr Daan Goosen and Dr Mike van der Linde for their veterinary and statistical expertise respectively, as well as Dr Nanette van der Merwe for her laboratory assistance.

REFERENCES

Bailey AL, Scantlebury DC, Smyth SS. 2009. Thrombosis and antithrombotic therapy in women. Arterioscler Thromb Vasc Biol 29:284–288.

Brouns R, De Deyn PP. 2009. The complexity of neurobiological processes in acute ischemic stroke. Clin Neurol Neurosur 111:483–495.

Danton GH, Dietrich WD. 2003. Inflammatory mechanisms after ischemia and stroke. J Neuropath Exp Neur 62:127–136.

Demopoulos HB, Milvy P, Kakari S, Ransohoff J. 1972. Molecular aspects of membrane structure in cerebral edema. In: Reulen, H, Shurmann, K, editors. Steroids and brain edema, New York: Springer-Verlag. pp. 29–39.

Dietrich WD, Alonso O, Busto R. 1993. Moderate hyperglycaemia worsens acute blood-brain barrier injury after forebrain ischemia in rats. Stroke 24:111–116.

Dračа S. 2009. Are female sex steroids neuroprotective in experimental stroke. Med Hypotheses 73:1051–1052.

Eklof B, Siesjo BK. 1972. The effect of bilateral carotid artery ligation upon the blood flow and energy state of the rat brain. Acta Physiol Scand 86:155–165.

Eklof B, Siesjo BK. 1973. Cerebral blood flow in ischaemia caused by carotid artery ligation in the rat. Acta Physiol Scand 87:69–77.

Gibson CL, Constantin D, Prior MJW, Bath PMW, Murphy SP. 2005. Progesterone suppresses the inflammatory response and nitric oxide synthase-2 expression following cerebral ischemia. Exp Neurol 193:522–530.

Hoyte LC, Buchan AM. 2009. Animal models of stroke. In: Hof PR, Mobbs CV, editors. Handbook of the neuroscience of aging, Italy: Elsevier Inc. pp. 575–582.

Jia J, Guan D, Zhu W, Alkayed NJ, Wang MM, Hua Z, Xu Y. 2009. Estrogen inhibits Fas-mediated apoptosis in experimental stroke. Exp Neurol 215:48–52.

Joshi CN, Jani SK, Murthy PSR. 2004. An optimised triphenyltetrazolium chloride method for identification of cerebral infarcts. Brain Res Protoc 13:11–17.

Leng XH, Hong SY, Larrucca S, Zhang W, Li TT, Lopez JA, Bray PF. 2004. Platelets of female mice are intrinsically more sensitive to agonists than are platelets of males. Arterioscler Thromb Vasc Biol 24:376–381.

Liao SL, Chen WY, Kuo JS, Chen CJ. 2001. Association of serum estrogen level and ischemic neuroprotection in female rats. Neurosci Lett 297:159–162.

Libby P, Simon DI. 2001. Inflammation and thrombosis: The clot thickens. Circulation 130:1718–1720.

Manthey D, Behl C. 2006. From structured biochemistry to expression profiling: Neuroprotective activities of estrogen. Neuroscience 138:845–850.

McBean DE, Kelly PAT. 1998. Rodent models of global cerebral ischemia: A comparison of two-vessel occlusion and four-vessel occlusion. Gen Pharmacol 30:431–434.

Myers DD, Wakefield TW. 2005. Inflammation-dependent thrombosis. Front Biosci 10:2750–2757.

Ostrovsky L, King AJ, Bond S, Mitchell D, Lorant DE, Zimmerman GA, Larsen R, Niu XF, Kubes P. 1998. A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process. Blood 91:3028–3036.

Preston E, Webster J. 2000. Spectrophotometric measurement of experimental brain injury. J Neurosci Methods 94:184–192.

Pretorius E, Engelbrecht MJ, Duim W. 2012. Thromboembolic ischemic stroke and the presence of necrotic platelets: A scanning electron microscopy investigation. Ultrastruct Pathol 36:19–22.

Pretorius E, Swanepoel AC, Oberholzer HM, van der Spuy WJ, Duim W, Wessels PF. 2011. A descriptive investigation of the ultrastructure of fibrin networks in thrombo-embolic ischemic stroke. J Thromb Thrombolysis 31:507–513.

Pretorius E, Oberholzer HM, van der Spuy WJ, Meiring JH. 2010. Age-related changes in fibrin networks and platelets of individuals over 75: A scanning electron microscopy study showing "thrombotic preparedness." J Thromb Thrombolysis 29:271–275.

Prewitt AK, Wilson ME. 2007. Changes in estrogen receptor-alpha mRNA in the mouse cortex during development. Brain Res 1134:62–69.

Pulsinelli WA, Waldman S, Rawlinson D, Plum F. 1982. Moderate hyperglycaemia augments ischemic brain damage: A neuropathological study in the rat. Neurology 32:1239–1246.

Saenger AK, Christenson RH. 2010. Stroke biomarkers: Progress and challenges for diagnosis, prognosis, differentiation, and treatment. Clin Chem 56:21–33.

Saha JK, Xia J, Grondin JM, Engle SK, Jakubowski JA. 2005. Acute hyperglycaemia induced by ketamine/xylazine anaesthesia in rats: Mechanisms and implications for preclinical models. Exp Biol Med 230:777–784.

Selvamani A, Sohrabji F. 2010. Reproductive age modulates the impact of focal ischemia on the forebrain as well as the effects of estrogen treatment in female rats. Neurobiol Aging 31:1618–1628.

Singer CA, Rogers KL, Strickland TM, Dorsa DM. 1996. Estrogen protects primarily cortical neurons from glutamate toxicity. Neurosci Lett 212:13–16.

Sladowski D, Steer SJ, Clothier RH, Balls M. 1993. An improved MTT assay. J Immunol Methods 157:203–207.

Stewart GJ, Ritchie WGM, Lynch PR. 1974. Venous endothelial damage produced by massive sticking and emigration of leukocytes. Am J Pathol 74:507–532.

Suzuki S, Brown CM, Wise PM. 2009. Neuroprotective effects of estrogens following ischemic stroke. Front Neuroendocrin 30:201–211.

van der Spuy WJ, Pretorius E. 2012. Interrelation between inflammation, thrombosis, and neuroprotection in cerebral ischaemia. Rev Neurosci 23:269–278.

Wang Q, Tang XN, Yenari MA. 2007. The inflammatory response in stroke. J Neuroimmunol 184:53–68.

Wohner N. 2008. Role of cellular elements in thrombus formation and dissolution. Cardiovasc Hematol Agents Med Chem 6:224–228.

Wong JH, Dukes J, Levy RE, Sos B, Mason SE, Fong TS, Weiss EJ. 2008. Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. J Clin Invest 118:2969–2978.

Xue Q, Yu B, Wang Z, Chen H. 2004. Effects of ketamine, midazolam, thiopental, and propofol on brain ischemia injury in rat cerebral cortical slices. Acta Pharm Sinic 25:115–120.

Appendix C

Scientific Manuscripts in Preparation

van der Spuy WJ, Goosen DJ, Pretorius E. Hyperglycaemic modification to classical two-vessel occlusion for inducing simplified, repeatable transient cerebral ischaemia

van der Spuy WJ, Bester MJ, Pretorius E. The role of 17β-oestradiol and neural oestrogen receptors in neural ischaemic injury

van der Spuy WJ and Pretorius E. Interaction of Red Blood Cells Adjacent to and Within a Thrombus in Experimental Cerebral Ischaemia