

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

A STUDY OF BRAIN INJURY

IN

NEW ZEALAND SEA LION PUPS

A thesis presented in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

at

Massey University,
Palmerston North,
New Zealand

Wendi Dianne Roe

2011

ABSTRACT

The New Zealand sea lion (*Phocarctos hookeri*) is a threatened species endemic to New Zealand. The majority of breeding in this species occurs on the Auckland Islands in the sub-Antarctic, and recent population estimates indicate that pup production is declining. Trauma is a significant cause of mortality in New Zealand sea lion pups, and much of this is believed to be caused by adult and subadult males, that bite, crush, shake and throw young pups.

In this thesis, a number of techniques are used to determine the role played by traumatic brain injury in the mortality of NZ sea lion pups. The findings of gross necropsy examinations show that pups have numerous lesions indicative of traumatic brain injury, including skull fractures and subdural haemorrhages, and that pups die due to crushing and impact injuries. Although some pups have gross lesions considered in human paediatric medicine to be indicative of shaking injury, detailed histological and microbiological studies of sea lion pups show that most of these are associated with meningitis due to *Klebsiella pneumoniae*. This bacterium is a common cause of pup mortality.

Immunohistochemical techniques are used to demonstrate that axonal injury is common in sea lion pups, but show that shaking is not a common mechanism of this pathological process. Instead, most axonal injury is found to be due to hypoxia-ischaemia, and evidence that raised intracranial pressure has occurred is comparatively common in dead pups. The combined findings of histological and immunohistochemical studies suggest that lesions such as optic sheath haemorrhage, intracranial subdural haemorrhage, spinal sub-meningeal haemorrhage, and optic nerve axonal injury could be caused by perturbations to vascular, intra-ocular, intracranial and subarachnoid pressure rather than being a direct result of trauma as is proposed in shaken baby syndrome.

ACKNOWLEDGEMENTS

A huge number of people have contributed to this thesis in a huge variety of ways. Firstly, there is no way I could have achieved any of this without the unstinting help of my husband Angus, who fearlessly took on a multitude of child-maintenance tasks during my time in the field, in the lab, and on the computer. He has also earned my undying gratitude for listening to (and pretending to be interested in) many conversations about sea lions and head trauma. Thanks and apologies are also due to Rhiannon and Kristi, my two wonderful daughters, for their long-suffering acceptance of my periodic absences over the past four years. Sorry guys.

Part of the attraction of working with NZ sea lions has been the opportunity to also work with a group of scientists whose enthusiasm and dedication has been infectious, and whose help has been central to this project. Laureline Meynier and Louise Chilvers have given their time and advice freely, and I am forever grateful for their help and friendship. The sea lion team from the 2007/08 season, (Jacinda Amey, Laura Boren, Helen McConnell, and Amelie Auge) kept me entertained during my time on Enderby, were patient with my developing field skills, and uncomplainingly extracted brains and spinal cords for me in rain, hail and sunshine (although not so much of that last one). A vast number of other scientists also were involved with sample collection for this project during several field seasons, and I would like to thank them for their help, which is greatly appreciated: Andy Maloney, Kerri Morgan, Baujke Lenting, Kelly Buckle, Nathan McNally and Katja Geschke. Thanks also to Pdraig Duignan, who introduced me to sea lions in the first place, and to Aurelie Castinel, who provided much advice and encouragement in the early stages of my research.

To all the pathology colleagues who I have picked the brains of, mostly figuratively but sometimes also literally, I would also like to pass on my gratitude. Terry Spraker has

been incredibly supportive, and his generous offer to collect fur seal brains for me on the Pribilof Islands was integral to this thesis. Keith Thompson has been my role model throughout my pathology career, and remained encouraging and positive despite the fact that I spurned production animals in favour of sea lions as the basis of a PhD. To Dani Aberdein, Stuart Hunter and Keren Dittmer, who started out as colleagues and have become friends, thanks for listening to me vent about statistics and lab work over countless coffees and the occasional wine.

This thesis seemed to generate an incredible number of samples of varying kinds, and numerous people have helped out with tissue processing and laboratory procedures. I would like to thank Evelyn Lupton, Eugene Ndeke, Elaine Booker and Nicola Wallace for cutting all those thousands of histo slides, and for repeatedly tracking down missing blocks. I would especially like to thank Mike Hogan, the world's best post mortem technician, for his sense of humour, his calmness, and his inexhaustible ability to find places to store fish bins. Thanks also to: Lynn Rogers, a veritable 'Girl Friday', who is a wonderful microbiologist, and turned her hand to whatever else was needed at the time; to Janis Bridges and Jonathan Marshall, who provided much appreciated statistics advice; to Laryssa Howe and Sharifah Syed-Hussain for de-mystifying Western blot; to Sandra Forsyth and Sue Pannifer for running assays for me; to the imaging team (Devon Thompson, Nicki Moffat, Sharon Bray and Anna Swainson) for their radiological and CT assistance; and to Matthew Perrott, for guidance with immunohistochemistry.

Finally, I have been incredibly lucky to have such a wonderful supervisory team. Joe Mayhew was encouraging from the very outset, and skillfully and calmly guided me through this project. I could not have asked for a better chief supervisor. I began my undergraduate pathology training with Bob Jolly, and it has been a huge pleasure to also have him involved with my PhD. Bob has kept me focussed, and his wealth of experience and knowledge have been invaluable. Christine Thomson has been a great mentor, and rounded out my supervisory team perfectly.

TABLE OF CONTENTS

1 Prologue

1.1 Problem statement.....	1
1.2 Research aim.....	3
1.3 Overview of thesis structure.....	4
1.4 Presentation of thesis.....	5
1.5 References.....	6

2 Background and literature review

2.1 Introduction.....	7
2.2 New Zealand sea lions.....	8
2.2.1 General information.....	8
2.2.2 Evidence of traumatic brain injury in NZ sea lion pups.....	10
2.3 Neonatal abuse and infanticide in pinnipeds.....	10
2.3.1 The influence of social behaviour and reproductive biology on neonatal trauma.....	11
2.3.2 Nature and severity of neonatal trauma.....	12
2.3.3 Role of head trauma in pup morbidity and mortality due to attacks by adult otariids	14
2.4 The nature of brain injury.....	15
2.4.1 Hypoxic and ischaemic brain injury.....	16
2.4.2 Traumatic brain injury.....	18
2.4.3 Clinical correlates of diffuse axonal injury.....	23
2.4.4 Pathogenesis of traumatic axonal injury.....	23
2.4.5 Assessing severity of CNS injury using post mortem tissues.....	27
2.5 Paediatric traumatic brain injury.....	27
2.5.1 Comparative anatomy and pathology: adult versus infant humans.....	28
2.5.2 Inflicted head injury and shaken baby syndrome.....	29
2.6 Animal models of traumatic brain injury.....	31
2.7 Extrapolating data from human and experimental animal traumatic brain injury.....	32
2.8 Non-experimental traumatic brain injury in animals.....	34
2.9 References.....	37

3 Preliminary investigations of traumatic brain injury in NZ sea lion pups

3.1	Introduction.....	45
3.2	Chapter aim.....	48
3.3	Materials and methods.....	48
3.3.1	Mechanisms of injury.....	48
3.3.2	Nature and prevalence of gross lesions of traumatic brain injury.....	50
3.4	Results.....	51
3.4.1	Mechanisms of traumatic brain injury.....	51
3.4.2	Review of necropsy records 1998/99 to 2006/07.....	53
3.5	Discussion.....	55
3.6	Summary.....	57
3.7	References.....	58

4 The role of traumatic brain injury in NZ sea lion pups, 2007/08

4.1	Introduction.....	61
4.2	Investigation of freeze-thaw artefact.....	63
4.2.1	Materials and methods.....	63
4.2.2	Results.....	64
4.2.3	Discussion.....	73
4.3	Gross, histological and microbiological analysis of tissues from 2007/08.....	79
4.3.1	Introduction	79
4.3.2	Materials and methods.....	79
4.3.3	Results.....	82
4.3.4	Discussion.....	90
4.4	Summary.....	95
4.5	References.....	97

5 The role of meningitis in subdural haemorrhage in NZ sea lion pups

5.1	Introduction.....	99
5.2	Materials and methods.....	100
5.3	Results.....	104
5.3.1	General and microbiological findings.....	104
5.3.2	Gross lesions in pups with meningitis.....	106
5.3.3	Histopathology.....	108
5.4	Comparison of lesions in pups with meningitis and without meningitis.....	117
5.4.1	Frequency of meningitis in pups with subdural haemorrhage.....	117
5.4.2	Intracranial dura mater and cervical spinal histology.....	118

5.4.3	Temporal pattern of meningitis deaths.....	121
5.5	Discussion.....	121
5.5.1	Cause of meningitis	121
5.5.2	Clinical syndrome.....	122
5.5.3	Role of meningitis in subdural haemorrhage.....	124
5.5.4	Other causes of subdural haemorrhage.....	125
5.5.5	Importance of meningitis in morbidity and mortality.....	126
5.6	Summary.....	128
5.7	References.....	129

6 β -amyloid precursor protein as a marker of axonal injury in NZ sea lion pups

6.1	Introduction.....	133
6.2	Materials and methods.....	135
6.2.1	Sample population.....	135
6.2.2	Examination of fixed brain and spinal cord.....	135
6.2.3	Examination of eyes.....	137
6.2.4	Assessment of β APP immunoreactivity.....	137
6.2.5	Comparisons between groups of pups.....	142
6.2.6	Review of H&E sections: evidence of hypoglycaemia or hypoxia-ischaemia.....	142
6.2.7	Glucose assays.....	143
6.2.8	Brain swelling.....	143
6.2.9	Statistical analysis.....	143
6.3	Results.....	144
6.3.1	β APP immunohistochemistry.....	144
6.3.2	Assessment of intracranial pressure.....	151
6.3.3	Assessment of hypoglycaemia and hypoxia-ischaemia.....	156
6.3.4	Eyes.....	157
6.4	Discussion.....	159
6.5	Summary.....	165
6.6	References.....	167

7 MAP2 as a marker of ischaemia

7.1	Introduction.....	171
7.2	Immunohistochemical validation and optimisation of MAP2 in NZ sea lion brain.....	172

7.3	Pilot Study: Effect of post mortem interval on MAP2 expression.....	173
7.3.1	Materials and Methods.....	173
7.3.2	Results.....	175
7.3.3	Analysis and conclusions.....	177
7.4	Effect of post mortem interval in northern fur seals.....	178
7.4.1	Materials and methods.....	178
7.4.2	Results.....	179
7.4.3	Discussion.....	182
7.5	Evaluation of MAP2 expression in 2007/08 field cases.....	183
7.5.1	Materials and methods.....	183
7.5.2	Results.....	183
7.5.3	Discussion.....	185
7.5.4	Summary.....	186
7.6	References.....	188

8 General discussion and conclusions

8.1	The pathogenesis of subdural haemorrhages in NZ sea lion pups.....	191
8.2	Importance of brain injury in pup mortality and morbidity.....	195
8.3	Does shaking alone lead to pup mortality?.....	196
8.4	The role of attacks by subadult males in pup mortality.....	198
8.5	Study limitations.....	199
8.6	Conclusions and future directions.....	200
8.7	References.....	202

LIST OF FIGURES

<i>Figure 2.1.</i> Map showing location of Auckland Islands.....	9
<i>Figure 3.1.</i> The sea lion breeding environment at Sandy Bay, Enderby Island.....	49
<i>Figure 3.2.</i> A NZ sea lion breeding harem at Sandy Bay, Enderby Island.....	49
<i>Figure 3.3.</i> NZ sea lion pup being shaken by a subadult male.....	52
<i>Figure 3.4.</i> Seasonal distribution of traumatic brain injury lesions in pups from 1998/99 to 2006/07.....	54
<i>Figure 3.5.</i> Frequency of selected traumatic brain injury-like lesions.....	55
<i>Figure 4.1.</i> Gross lesions present in pinnipeds that have been frozen and thawed....	67
<i>Figure 4.2.</i> A pseudo-contusion of the cerebral cortex from a frozen-thawed fur seal.....	70
<i>Figure 4.3.</i> Cracks in the cerebellum of a frozen-thawed fur seal.....	70
<i>Figure 4.4.</i> Free erythrocytes in the subarachnoid space of the cerebral cortex in a frozen-thawed fur seal.....	71
<i>Figure 4.5.</i> Disrupted blood vessels in the subarachnoid space in a pseudo-contusion.....	71
<i>Figure 4.6.</i> A pseudo-contusion from the ventrum of a frozen-thawed fur seal.....	72
<i>Figure 4.7.</i> A true (ante-mortem) bruise after freezing and thawing.....	72
<i>Figure 4.8.</i> The renal capsule of a non-frozen fur seal.....	73
<i>Figure 4.9.</i> The renal capsule of a frozen-thawed fur seal.....	73
<i>Figure 4.10.</i> Posterior calotte of an eye from a NZ sea lion pup.....	80
<i>Figure 4.11.</i> Frequency of lesions of traumatic brain injury over time.....	85
<i>Figure 4.12.</i> Gross lesions seen in pups necropsied at Sandy Bay in 2007/08.....	87
<i>Figure 4.13.</i> Gross lesions consistent with traumatic brain injury in pups, 2007/08.....	87

<i>Figure 4.14.</i> Seasonal distribution of intracranial subdural haemorrhage and spinal sub-meningeal haemorrhage.....	89
<i>Figure 5.1.</i> Trimming sites used for collection of brain tissues.....	103
<i>Figure 5.2.</i> Cases of bacterial infection in pups, 2007/08 season.....	107
<i>Figure 5.3.</i> Gross lesions seen in pups with meningitis.....	108
<i>Figure 5.4.</i> Microscopic appearance of exudate and intracellular bacteria in a pup with meningitis.....	112
<i>Figure 5.5.</i> Inflammatory exudate extending alongside the fornix.....	113
<i>Figure 5.6.</i> The ependyma and choroid plexus of the third ventricle in a pup with meningitis.....	113
<i>Figure 5.7.</i> Arterioles within the spinal subarachnoid space of a pup with meningitis.....	114
<i>Figure 5.8.</i> Haemorrhages in the sclera of a sea lion pup.....	115
<i>Figure 5.9.</i> Histopathology of the intracranial dura mater	115
<i>Figure 5.10.</i> Histopathology of the intradural unlined channels.....	116
<i>Figure 5.11.</i> Intradural haemorrhage surrounding spinal nerves.....	117
<i>Figure 5.12.</i> Spinal dura mater containing unlined channels.....	117
<i>Figure 5.13.</i> Anatomical location of sub-meningeal spinal cord haemorrhages.....	118
<i>Figure 5.14.</i> Association between meningitis and sub-meningeal haemorrhages in pups from the 2007/08 season.....	120
<i>Figure 5.15.</i> Prevalence of infection and meningitis in NZ sea lion pups, 2005/06 to 2009/10.....	125
<i>Figure 6.1.</i> Examples of β APP immunoreactivity scoring.....	140
<i>Figure 6.2.</i> An example of a β APP map using greyscale values.....	141
<i>Figure 6.3.</i> Normal structures showing immunoreactivity for β APP in the brains of NZ sea lion pups.....	147
<i>Figure 6.4.</i> Immunoreactive profiles present within brain sections.....	148
<i>Figure 6.5.</i> Patterns of axonal injury recognised in sea lion pups.....	149
<i>Figure 6.6.</i> Relative distribution of axonal injury for pups with subdural haemorrhage compared with pups with neither meningitis nor subdural haemorrhage.....	154

<i>Figure 6.7.</i> Relative distribution of axonal injury for pups with meningitis compared with pups with neither meningitis nor subdural haemorrhage.....	155
<i>Figure 6.8.</i> Relative distribution of axonal injury for pups with meningitis compared with pups that have both meningitis and subdural haemorrhage.....	156
<i>Figure 6.9.</i> Relative distribution of axonal injury for pups with haemorrhage of the cerebellar vermis and pups without haemorrhage of the vermis.....	141
<i>Figure 6.10.</i> Microscopic appearance of acidophilic neurons (H&E).....	159
<i>Figure 6.11.</i> Groups of β APP immunopositive axons in the optic nerve of a sea lion pup.....	160
<i>Figure 7.1.</i> Anti-MAP2 immunostaining in sections of brain from a dog and a subadult male Northern fur seal.....	160

LIST OF TABLES

<i>Table 3.1.</i> Summary of recorded gross lesions from pup necropsies conducted between 1998/99 and 2006/07.....	53
<i>Table 4.1.</i> Comparison of gross lesions from fur seals that were chilled or frozen and thawed prior to necropsy examination.....	65
<i>Table 4.2.</i> Cause of death and concurrent syndromes diagnosed in pups found dead in the 2007/08 breeding season.....	83
<i>Table 4.3.</i> Pups with lesions indicative of inertia injury.....	99
<i>Table 5.1.</i> Suppurative lesions and microbiological findings in pups with meningitis.....	107
<i>Table 5.2.</i> Gross lesions present in the skull, brain, eyes and cervical spinal regions of pups with meningitis, 2007/08.....	109
<i>Table 5.3.</i> Details of haemorrhagic and inflammatory meningeal lesions in the intracranial dura mater and cervical spinal tissues of pups from the 2007/08 breeding season.....	121
<i>Table 6.1.</i> Key for semi-quantitative scoring of anti- β APP immunoreactivity in brain sections.....	140
<i>Table 6.2.</i> Details of β APP immunoreactivity and cause of death for 2007/08 late season pups.....	152
<i>Table 7.1.</i> Counts of the percentage of Purkinje cells immunopositive for MAP2 antigen in dog brain tissue collected at differing post mortem intervals.....	179
<i>Table 7.2.</i> MAP2 scores in Northern fur seal brains following various post mortem intervals.....	183
<i>Table 7.3.</i> Results of manual, semi-quantitative and qualitative analyses of MAP2 immunoreactivity in Northern fur seals.....	1683

GLOSSARY

<i>acceleration</i>	the change in velocity over time. Can be positive or negative (deceleration)
<i>AMPA/KA receptors:</i>	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainic acid receptor. A membrane-bound glutamate receptor
<i>ATP:</i>	adenosine triphosphate. An energy-containing nucleotide produced by ATP-synthase
<i>axotomy:</i>	physical separation/tearing of an axon
<i>biomechanics:</i>	the study of mechanical forces as they apply to biological systems
<i>blood-brain barrier:</i>	the anatomical structures responsible for prevention of uncontrolled movement of fluid and solute between the cerebral blood vessels and the brain parenchyma
<i>brain herniation:</i>	movement of brain tissue from one anatomical compartment into another
<i>brain swelling:</i>	enlargement of the brain that occurs due to increased intravascular or extravascular fluid (e.g. cerebral oedema), resulting in flattening of gyri, shallow sulci, and herniation of brain tissue

<i>bridging veins:</i>	the veins that pass from the leptomeningeal surface through the subarachnoid space and dura mater, to empty into the intradural venous sinuses
<i>calvarium:</i>	the domed upper part of the skull comprising the frontal, parietal and occipital bones
<i>cerebral autoregulation:</i>	the maintenance of cerebral blood flow within homeostatic limits in response to changes in central venous pressure, intracranial pressure and metabolic demands
<i>cerebral oedema:</i>	accumulation of excessive amounts of extravascular fluid within brain tissue
<i>compressive strain:</i>	a force that results in tissues or structures being moved closer together
<i>concussion:</i>	transient loss of consciousness following a head injury
<i>contre-coup contusion:</i>	a contusion occurring remote to the site of impact. Historically these were said to occur opposite to the site of impact, but in human beings most occur on the frontal and temporal lobes, irrespective of impact site. The pathogenesis may involve tearing of parenchyma as it moves against bony protuberances or meningeal structures such as the falx cerebri
<i>contusion:</i>	disruption of the brain parenchyma following trauma, leading to haemorrhage and necrosis
<i>coup contusion:</i>	a contusion that occurs at the site of impact

<i>cytoskeleton:</i>	the microtubules, neurofilaments and microfilaments that make up the structural elements of a cell
<i>cytotoxic oedema:</i>	swelling of glial cells that results from damage to the energy-requiring membrane ion pumps, with subsequent influx of ions and fluid
<i>depressed fracture:</i>	the result of a focal impact where the outer table of the skull is depressed below the surface of the unaffected bone, and the inner table of the skull is pushed into the parenchyma
<i>diffuse axonal injury:</i>	both a neuropathological and clinical entity. The clinical syndrome injury involves immediate loss of consciousness followed by prolonged coma in the absence of a mass lesion. The neuropathological entity comprises widespread damage to axons throughout the parasagittal cortex, corpus callosum, internal capsule and long tracts of the brainstem
<i>dynamic loading:</i>	rapid application of a force, usually over a period of between 2 and 25 milliseconds
<i>excitotoxicity:</i>	excessive activation of excitatory glutamate receptors in the brain, resulting ultimately in cell death
<i>fast axonal transport:</i>	rapid (up to 400mm per day) transport of intracellular substances along microtubules
<i>global ischaemia:</i>	inadequate blood supply to the entire brain
<i>hypoxia:</i>	decrease in availability of oxygen to a tissue

<i>impact loading:</i>	the force resulting from direct contact between a tissue and a rigid object
<i>impulse loading:</i>	the force resulting from sudden acceleration or deceleration of tissue
<i>inertia:</i>	acceleration or deceleration forces without impact
<i>intracranial pressure:</i>	pressure within the cranial cavity, dependent upon central venous pressure, cerebral perfusion pressure and soft tissue/fluid volume of the brain and vasculature
<i>intradural haemorrhage:</i>	haemorrhage that originates within the dense fibrous layer of the dura mater
<i>ischaemia:</i>	lack of blood supply to a tissue relative to its needs
<i>lamina cribrosa:</i>	part of the sclera that is penetrated by axons of the optic nerve
<i>mechanical loading:</i>	the application of force(s) to a tissue
<i>mechanoporation:</i>	rapid movement of calcium ions into a neuron following transient membrane depolarisation which occurs as a result of mechanical deformation of the neuronal soma or axon
<i>microtubule:</i>	polymers of tubulin that make up part of the neuronal cytoskeleton and play a role in intra-cellular transport

mitochondrial membrane permeability transition pore:

a protein-lined 'hole' (pore) in the inner membrane of the mitochondria, which can open following traumatic brain injury and allow flux of ions across the membrane

neurocranium:

the portion of the skull that encloses the brain, including the calvarium as well as the temporal and basisphenoid bones.

neurofilament:

the class of intermediate filament found in neurons

NMDA receptors:

N-methyl *D*-aspartate receptor. A voltage-dependent membrane-bound glutamate receptor

node of Ranvier:

unmyelinated portions of axons found between myelinated internodes

otariids:

eared seals (fur seals and sea lions)

pan-necrosis:

Death of neurons as well as other cellular elements in an area of brain affected by focal ischaemia

phocids:

'true' seals. These species do not have pinnae

pinnipeds:

a suborder of carnivorous marine mammals with limbs specialised as flippers

polygynous:

an hierarchical social system whereby mature males defend a territory within which a group of females is sequestered. Females are comparatively free to enter and leave the territory

pond fracture: similar to a depressed fracture, but with no displacement of the inner table of bone

rotational acceleration: acceleration about an axis

selective neuronal necrosis: necrosis of specific populations of neurons that are particularly susceptible to ischaemia. Occurs with global ischaemia

shaken baby syndrome: a syndrome seen in human infants with inflicted brain injury proposed to be due to shaking. The typical syndrome comprises encephalopathy with subdural and retinal haemorrhages

shear forces: sliding forces involving differential movement between layers of tissue

skull: the bones of the head exclusive of the mandibles but including the neurocranium, zygomatic processes and the facial, maxillary, and palatine bones

spinal sub-meningeal haemorrhage:

haemorrhage between the spinal dura mater and the spinal cord. Can involve haemorrhage between the dura mater and the parietal layer of the arachnoid mater (subdural haemorrhage) or haemorrhage between the parietal layer of the arachnoid mater and the spinal cord parenchyma (subarachnoid haemorrhage)

<i>static loading:</i>	the gradual application of a force over a period of greater than 200 milliseconds
<i>strain:</i>	deformation of tissue resulting from the application of a force
<i>stress:</i>	application of force to a tissue
<i>subdural haemorrhage:</i>	haemorrhage into the subdural compartment, which is made up of loose dural border cells at the inner aspect of the dura mater
<i>tensile strain:</i>	a force that results in tissues or structures being moved further apart
<i>translational acceleration:</i>	acceleration in a linear plane
<i>vasogenic oedema:</i>	loss of intravascular fluid into surrounding parenchyma following damage to cerebral blood vessel walls