Brainstem pathology in SIDS and in a comparative piglet model

Rita Machaalani
BMedSc. (Hons)

A thesis submitted for the degree of Doctor of Philosophy

Department of Medicine
The University of Sydney
NSW Australia

August 2003

Table of Contents

Chapter 1

Literature review

Chapter 2

Materials and Methods

PART II

Chapter 7

Infant dataset characteristics

Chapter 11

Concluding Remarks

Appendices

Declaration

In accordance with the by laws of The University of Sydney, I declare that this thesis describes original research performed by myself within the Department of Medicine, at The University of Sydney, except where due acknowledgement has been made in the text.

To the best of my knowledge, this thesis does not contain any material previously written or published by another person except where duly referenced.

This work has not been submitted for any other degree at this or any other institution.

Rita Machaalani

August, 2003

Prelude

The first aim of this PhD project was to verify the neuropathological finding of increased apoptosis in the brainstem of SIDS victims as previously observed in a Canadian population by my supervisor (Waters et al., 1999). Subsequent investigations were planned to test for any correlations between the above finding and known SIDS risk factors. Having achieved this, the aim was to proceed with studies to evaluate mechanistic hypotheses regarding such neuronal cell death in SIDS, possibly via an animal model.

To date, most of neuropathological findings in SIDS are based on small infant datasets (20-80 cases in total). In addition, very few studies have correlated the neuropathological abnormalities that have been identified with known clinical or epidemiological risks for SIDS, mostly because this information is often not available.

Here in NSW, Australia, every sudden infant death is investigated by the State Coroner. For infants 1 month to 1 year of age, a death scene investigation is routinely performed, clinical data collected using a standard questionnaire, and an autopsy conducted. Thus, correlations between pathological research and clinical data should be feasible.

The first phase of the current project was the characterization of a large dataset of human infants and subsequent collection of the relevant brainstem tissue from remaining diagnostic blocks. During this time, research on human tissue was suspended for the review of the Human Tissue Act. Since the use of an animal model had always been feasible and contemplated in the planning phase of the project, focus shifted from the infant dataset to an animal model.

A piglet model of intermittent hypercapnic hypoxia (IHH) was being developed in my residing laboratory and so, I moved on to study neuropathological outcomes of this model. The IHH model was designed to mimic the clinical situations of the prone sleeping position with the face down or obstructive sleep apnoea, both of which are risk factors for SIDS. Thus, the aim of my project now was to determine how well this model of IHH was in reproducing the same neuropathological findings sought in SIDS infants, particularly increased neuronal apoptosis.

This shift in focus of my research from the infant dataset to the piglet dataset provided a number of advantages. First, it showed that IHH did induce apoptosis in the brainstem of these piglets, thus supporting the relevance of the model to SIDS. Second, it allowed me to refine new

experimental techniques aimed at determining the mechanism of this increased apoptosis, which would not have been possible in the limited number of human tissue samples that were available. Finally, it provided me with a much broader range of positive studies, three of which have been published in peer reviewed journals.

As I was nearing the end of my studies with the piglet dataset, the original infant tissue dataset became available, with ethics approval to finish studies in the 25 cases for which I had already collected the brain tissue. The results published in this thesis are therefore considered preliminary, but also provide verification of the importance and urgency of undertaking further analysis in larger datasets to definitively conclude the specificity of these findings to SIDS.

The lay out of this thesis reflects the sequence in which my projects were performed. Part I concerns the neuropathology of the piglet model of IHH while Part II concerns the neuropathology of SIDS infants compared to non-SIDS infants, with each part containing several chapters. The final chapter of the thesis highlights the comparative findings of this project between Parts I and Parts II; that is, the findings that are consistent between the piglet model of IHH and SIDS infants, and discusses future research directions.

Abstract

This thesis tests the hypothesis that increased neuronal cell death in SIDS infants is related to the ability of risk factors, such as prone sleeping, to expose infants to intermittent hypercapnic hypoxia (IHH). Based on the hypothesis that the NMDA system is linked to neuronal death, by way of excitotoxicity, correlations were also sought between cell death and changes in NMDA receptor (NR1) expression in brainstem nuclei controlling cardiorespiratory function.

The first aim of this study was to verify that increased neuronal cell death occurs in SIDS infants. To verify a piglet model of SIDS risk factors, brainstem changes were examined in piglets exposed to IHH, and comparisons were made to changes seen in SIDS infants. The NMDA receptor was characterised in controls for both the human infant and the piglet groups. Comparisons of neuronal changes were made with SIDS infants, and piglets exposed to IHH.

Non-radioactive in-situ hybridisation and immunohistochemistry were performed on formalin fixed and paraffin embedded brainstem tissue to identify markers of cell death (caspase-3, active caspase-3, and TUNEL), and to examine NR1 mRNA and protein expressions. Staining was quantified using computerised image analysis software. Eight nuclei from the brainstem medulla (caudal in piglets, and mid in infants), and two nuclei from the rostral pons (infants) were studied.

The first dataset included human infants aged 1-6 months with a diagnosis of SIDS (n=15) or non-SIDS (n=10). The second dataset comprised developing piglets aged 13-14 days, with controls (n=6), against those exposed to IHH for 2 (n=6) or 4 (n=5) days. Increased neuronal cell death was not verified in the SIDS infants, but abnormalities in NR1 expression were present in selected nuclei of the medulla. Piglets exposed to IHH had increased neuronal cell death and changes in NR1 in selected nuclei of the medulla. There was also a positive correlation between increased cell death and high NR1 levels. Preliminary data showed that SIDS infants who usually slept prone had some differences in NR1 compared to those who did not usually sleep prone.

From these findings, it was concluded that IHH may underlie the abnormalities in NMDA receptor expression that are present in the brainstem of SIDS infants. Although IHH can induce an increase in neuronal cell death, its significance in the aetiology of SIDS is not known. In piglets, IHH induced cell death correlated with high NMDA expression in some brainstem nuclei, supporting the hypothesis that excitotoxicity may be involved in the mechanism for cell death. Moreover, this thesis presents for the first time, ‘preliminary pathological proof’ of an association between prone sleeping and abnormal NMDA receptor expression in SIDS infants.

Acknowledgments

Although many say a PhD is hard work and very testing, for me, it did not seem true. Mostly because of all those who played a hand in helping me through my PhD, be it by teaching me, advising me, supporting me, encouraging me, and/or just being there for me in general. I would now like to take the opportunity to acknowledge and thank them.

To Dr Karen Ann Waters, my supervisor. From the day I meet Karen and all throughout my time as her student, she has provided me with much support, encouragement and terrific guidance. She has always been there for me when I needed someone to sound off my ideas and thoughts, and has always treated me as an equal, which I am so grateful for. In many respects, Karen has travelled this journey with me and taken all the big and small steps that were taken to make this project run smoothly. I am much indebted to Karen who has made this professional milestone, a great and enjoyable one. She is highly recommendable as a supervisor.

To Kellie Tinworth, my laboratory co-worker. It was Kellie who first introduced me to the laboratory and to Karen. From problems with computers to general laboratory issues, Kellie has always provided me with the assistance to overcome them. She has also been kind in helping me with presentations and in proof reading my written works. The animals used in this thesis were from an initial project conducted in the laboratory and, Kellie has been the sole person responsible for their care and handling.

To Dr. Bronwyn Relf, who has become an invaluable friend and research colleague. Together, Bronwyn and I set up the *in situ* hybridisation technique, and all the time doing so, she taught me all that I needed to know about it. When it was up and running and I was left to it on my own, she was always reachable and willing to help trouble shooting despite being at home and caring for two children, or being overseas. I thank her so very much for always being happy to assist me and being available if needed because on many occasions, and even till today, I have called upon that assistance and she has never failed me.

To Dr. Jane Radford, who also has become an invaluable friend and research colleague. Jane is responsible for introducing me to the world of immunohistochemistry. I am very thankful for her experience, knowledge, and willingness to help me at the start and then throughout the course of my project especially by providing me with the assistance needed in troubleshooting, and/or in interpreting results.

To Drs. Bronwyn Relf, Jane Radford and Tina Hinton. Thank you for proof reading my thesis and the useful comments provided.

To Mr. Dennis Dwarte, Mrs. Ellie Kable, and the Electron Microscopy Unit, at The University of Sydney. I thank Dennis for his advise and assistance in setting up the image analysis programs for quantitative measurements, and Ellie for her technical help. The Electron Microscopy Unit were kind in allowing me to use their facilities without any compensation.

To Professor Colin Sullivan, my associate supervisor who always provided encouragement and enthusiasm for my research, and allowed me the use of the facilities at the David Read Laboratories, at The University of Sydney.

To Mr. John Reynolds, and the Institute of Respiratory Medicine (IRM), at The University of Sydney. John was the "computer guy" who, during my 3.5 years of PhD, always made sure I had the best in a computer & answered my distress calls when something did not work. The IRM were kind in allowing me to use their printing facilities during the course of my PhD.

To Community Health and Tuberculosis Australia (CHATA), who provided me with a scholarship to carry out this PhD. If it was not for their financial assistance, this project would not have been possible. My sincerest thanks to CHATA.

To my family. Firstly to my parents Samir and Vilma. How does one start to thank those who have done so much for them and continue to do so unconditionally? Words cannot express how grateful and indebted I am to my parents. They made me the person I am and have always encouraged me in whatever I chose to do. It is their upbringing that has given me the confidence to pursue challenging things and their courage that aspires me to do the best I can in everything. Their love and support is phenomenal. Secondly, to my siblings, Kathy, Charbel and Jessica. Being the big sister, you had to endeavour my bossing you, advising you, spoiling you, caring for you and loving you. I know I have set the bar high for you guys to reach in your lives when it comes to our parents, but there is no doubt that you will make it and will be successful no matter what paths you decide to take. Remember, I'm always here for you, when needed.

Finally, but most importantly, to God, my creator and saviour. For giving me life, and being with me every moment since then. You have never abandoned me, and my completing this thesis is proof of that. Thank you for your unconditional love and for giving me purpose in life.

Publications arising from this thesis

Machaalani R., K.A. Waters, (2002) The distribution and quantification of NMDA R1 mRNA and protein in the piglet brainstem and effects of intermittent hypercapnic hypoxia (IHH), *Brain Research* 951: 293-300.

Machaalani R., K.A. Waters, (2003) Correlations between brainstem NMDA receptor changes and active neuronal cell death after intermittent hypercapnic hypoxia in the developing piglet, *Brain Research* 975 (1-2): 141-148.

Machaalani R., K.A. Waters, (2003) Increased neuronal cell death after Intermittent Hypercapnic Hypoxia in the developing piglet brainstem, *Brain Research* 985: 127-134.

Machaalani R., K.A. Waters, (2003) NMDA Receptor 1 expression in the brainstem of human infants and its relevance to the Sudden Infant Death Syndrome (SIDS), *Journal of Neuropathology and Experimental Neurology* 62(10): 1076-1085.

Machaalani R., K.A. Waters, (2002) Neuropathology of piglets as a model of SIDS, *International Journal of Developmental Neuroscience* 19 (8): P74 (Abstract publication).

Publications related to this thesis

Relf B.L., **R. Machaalani**, K.A. Waters, (2002) Retrieval of mRNA from paraffin-embedded human infant brain tissue for non-radioactive in situ hybridization using oligonucleotides. *Journal of Neuroscience Methods* 115:129-36.

Waters K.A., **R. Machaalani**, (2003) NMDA receptors in the developing brain and effects of noxious insults. *Neurosignals*, In press.

Abstracts arising from this thesis

Machaalani R., K.A Waters, (2003) Implication of NMDA receptors in IHH induced neuronal death. *Australian Neuroscience Society.* 23rd Annual Meeting, Adelaide, SA, Australia.

Machaalani R., K.A Waters, (2002) NMDA receptor 1 (NR1) mRNA is increased in the mid medulla of SIDS infants. *College of Health Sciences and Medical Foundation Research Conference- FROM CELL TO SOCIETY 3*, Blue Mountains, NSW, Australia.

Machaalani R., K.A Waters, (2002) Increased NMDA (NR1) expression in SIDS open medulla. *17th SIDS International conference*, Florence, Italy.

Machaalani R., G. Velingos, K.A.Waters, (2002) Piglet as a model of SIDS: Neuronal apoptosis induced by intermittent hypercapnic hypoxia. *17th SIDS International conference*, Florence, Italy.

Machaalani R., K.A.Waters, (2002) Neuropathology of Piglets as a model of SIDS. *14th Biennial meeting of the International Society for Developmental Neuroscience ISDN*, Sydney, NSW, Australia. (Note. Further reported by Laura Spinney on BioMedNet (www. bmn.com) Feb 2nd, 2002)

Machaalani R., K.A Waters, (2001) The use of non-radioactive in situ hybridization to localize and quantify the mRNA of NMDA in piglet brainstem. *International Society for Neurochemistry Rio-Satellite Symposium: "Cell Communication in the Nervous System-Function and Dysfunction"*, Rio de Janeiro, Brazil.

Machaalani R., K.A Waters, (2000) Characteristics of a dataset for studying the neuropathology of SIDS. *College of Health Sciences and Medical Foundation Research Conference- FROM CELL TO SOCIETY 2*, Blue Mountains, NSW, Australia.

Abbreviations used often

5HT	Serotonin
ABC	Avidin Biotin Complex
Ach	Acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionate
AN	Arcuate nucleus
APTES	3-aminopropyltriethoxysilane
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BR	Blocking reagent
CASP3	Caspase-3
CNS	Central nervous system
Cun	Cuneate nucleus
DAB	Diaminobenzidine
DB	Digoxigenin buffer
DDB	Digoxigenin detection buffer
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMNV	Dorsal motor nucleus of the vagus
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAA	Excitatory amino acids
EDTA	Ethylenediamine tetra-acetic acid
Epi	Epinephrine
Glu	Glutamate
Gr	Gracile nucleus
IHC	Immunohistochemistry
IHH	Intermittent hypercapnic hypoxia
ISH	*In situ* hybridisation
ION	Inferior olivary nucleus
KA	Kainate
LC	Locus coeruleus
LRt	Lateral reticular nucleus
ME	Met enkephalin
mRNA	Messenger ribonucleic acid

NBT	Nitroblue tetrazolium
NDS	Normal donkey serum
NE	Norepinephrine
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NR1	NMDA receptor 1
NRS	Normal rabbit serum
NSS	Normal sheep serum
NSTT	Nucleus of the spinal trigeminal tract
NTS	Nucleus of the tractus solitarus
OD	Optical density
OR	Odds ratio
OSA	Obstructive sleep apnoea
PaO_2	Mean arterial oxygen tension
$PaCO_2$	Mean arterial carbon dioxide tension
PBS	Phosphate buffered saline
Pon	Pontine nuclei
RIA	Radioimmunoassay
RISH	Radioactive *in situ* hybridisation
RLB	Radioactive ligand binding
RR	Relative risk
RVLM	Rostral ventral lateral medulla
SIDS	Sudden Infant Death Syndrome
SP	Substance P
SSC	Standard saline citrate
TBS	Tris buffered saline
TdT	Terminal deoxynucleotidyl transferase
TE	Tris EDTA buffer
TEA	Triethanolamine
Tris	Tris[hydroxymethyl]aminomethane
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling
URTI	Upper respiratory tract infection
Vest	Vestibular nucleus
v/v	Volume per volume
w/v	Weight per volume
XII	Hypoglossal nucleus

List of Tables

List of Figures