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Assessment of the neuroprotective efficacy of poly-arginine-18 (R18) peptides in a pre-clinical model of perinatal hypoxic-ischaemic encephalopathy (HIE)

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Chapter 7

General Discussion

7.1 INTRODUCTION

Perinatal HIE occurs when there is a reduction in fetal CBF and/or cerebral oxygen supply, resulting in neuronal and glial cell loss and brain injury. Currently, the most effective clinical therapy to minimise cerebral injury is moderate hypothermia (33.5°C for 72 hours); to be effective, hypothermia needs to be administered within 6 hours of HIE onset. In infants who suffer moderate or severe encephalopathy, hypothermia has been shown to decrease mortality from 40% to 28%, while in surviving infants, neurological morbidity (e.g. cerebral palsy, epilepsy, intellectual disability and autism spectrum disorders) is reduced from 31% to 19% (Jacobs et al., 2013).

Despite the clinical benefits of hypothermia in HIE, its application can be associated with low risk levels of adverse effects such as haemorrhage, infection, impairment of oxygen retention and an increased risk of drug related side-effects (van den Broek, Groenendaal, Egberts, & Rademaker, 2010). Importantly, whilst hypothermia is safe in pre-term infants with necrotising enterocolitis (Hall et al., 2010), it has not been adequately evaluated for use in pre-term infants suffering HIE. Information regarding the safety and efficacy of hypothermia to treat pre-term infants suffering HIE is lacking. In one study, hypothermia in pre-term infants was associated with higher mortality and increased clinical complications when compared to term infants (Rao et al., 2016); and for these reasons it is not routinely used in these patients. In addition, the induction of hypothermia requires specialised equipment, intensive care monitoring and trained staff, which limits its use to centres that have the facilities and medical personnel to undertake the procedure. The transfer of infants to a centre equipped to deliver hypothermia, delays cooling induction, or if induced during

transfer, creates uncertainty regarding the safety and effectiveness of the cooling provided (Committee on Fetus and Newborn et al., 2014). In light of this, the application of a neuroprotective therapeutic agent to HIE patients (e.g. a neuroprotective cationic arginine-rich peptide) prior to transfer to a tertiary hospital may extend the therapeutic time window and effectiveness of hypothermia. In addition, as HIE infant mortality and morbidity are still relatively high following hypothermia treatment (discussed in Chapter 2), there is an urgent need for the development of additional therapeutic agents, which can be used in combination with hypothermia, to further improve outcomes in infants suffering HI. Furthermore, in situations when hypothermia is not available or contra-indicated, an alternative neuroprotective therapy would be highly advantageous.

Recent studies in A/Prof Meloni's laboratory have demonstrated that cationic arginine-rich peptides (CARPs), such as R18, are highly neuroprotective agents both in *in vitro* neuronal and animal models of stroke (Edwards, Anderton, Knuckey, & Meloni, 2016; MacDougall, Anderton, Edwards, Knuckey, & Meloni, 2016; Meloni, Brookes, et al., 2015; Meloni et al., 2017; Meloni, Milani, et al., 2015; Milani, Clark, et al., 2016; Milani et al., 2017; Milani, Knuckey, Anderton, Cross, & Meloni, 2016). As such, previous studies in A/Prof Meloni's laboratory have provided the rationale for this thesis to investigate the application of CARPs as a potential neuroprotective therapy for HIE.

7.2 KEY FINDINGS ARISING FROM THIS THESIS

7.2.1 Cerebral collateral circulation and infarct volume variability

The application of the HIE model using the 7-day-old (P7) rat was first described by Rice et al. (1981), and is often referred to as the Rice-Vannucci model. This is the most commonly used animal model for perinatal HIE neuroprotective and neurodegenerative pre-clinical studies. The model involves the permanent occlusion of the common carotid artery (CCAO) followed by a transient period of hypoxia (e.g. 8% O₂/92% N₂; for 2.5 h). Since its inception, the Rice-Vannucci model is widely reported to be associated with variable outcomes with respect to infarct presence, infarct volume size and by extension, functional outcomes (Ashwal, Tone, Tian, Chong, & Obenaus, 2007; Barks et al., 2017; Hagberg, Gilland, Diemer, & Andiné, 1994; Okusa et al., 2014; Ota, Ikeda, Ikenoue, & Toshimori, 1997; Palmer, Towfighi, Roberts, & Heitjan, 1993; Palmer, Vannucci, & Towfighi, 1990; Rice et al., 1981).

Whilst several sources of variability in the Rice-Vannucci model have been described (see Table 1, Chapter 4), an additional source of variability in both perinatal and adult ischaemic brain injury models can be attributed to the presence of cerebral collateral circulation via the circle of Willis and/or cerebral anastomoses; stabilising CBF following vessel occlusion and/or haemodynamic compromise. However, prior to this thesis the impact of collateral circulation as a source of variability in the Rice-Vannucci model had not been investigated.

In an attempt to minimise the known variability associated with the Rice-Vannucci model of HIE, a modified Rice-Vannucci model was developed (Chapter 4). The surgical procedure involves the permanent ligation of both the right common carotid artery (CCA) and external carotid artery (ECA) followed by hypoxia (8% O₂/92% N₂; for 2.5 h). The modified Rice-Vannucci model demonstrated a more reproducible cerebral infarct (infarct presence and reduced standard deviation), a larger infarct lesion, and more severe behavioural deficits when compared to the original Rice-Vannucci model.

In addition, magnetic resonance angiography phase-contrast velocity encoding and pulsed arterial spin labelling analysis in animals subjected to the original Rice-Vannucci model (right CCA occlusion only) demonstrated residual blood flow in the right internal carotid artery; an effect that would help preserve CBF to tissue affected by the CCA occlusion. In contrast, animals subjected to the modified Rice-Vannucci model (right ECA/CCA occlusion) demonstrated complete cessation of blood flow within the right internal, external and common carotid arteries, and a significant overall reduction in CBF to the right hemisphere. The source of cerebral collateral and anastomotic circulation is detailed diagrammatically in Figure 1, Chapter 4.

As a consequence of the modification to the original Rice-Vannucci surgical procedure, the model now offers a more reliable and reproducible cerebral infarct and was used for all animal experimental studies in this thesis (Chapters 5 & 6).

7.2.2 Effectiveness of R18 and R18D in a perinatal HIE model

One of the primary aims of this study was to identify a potential lead neuroprotective poly-arginine peptide and an effective peptide dose. Based on previous *in vitro* (Meloni, Brookes, et al., 2015) and adult animal studies in stroke related brain injury models (Milani, Clark, et al., 2016; Milani et al., 2017; Milani, Knuckey, et al., 2016), two peptides were selected; R18 (L-enantiomer) and R18D (D-enantiomer). The initial study examined the dose response effectiveness of R18 and R18D when administered immediately after HI in the modified Rice-Vannucci model. The CARP, JNKI-1-TATD (also referred to as XG-102) at the 1000 nmol/kg dose (equivalent to the highest dose of R18 and R18D examined), was used as a positive control and benchmark (Nijboer et al., 2010).

This thesis demonstrated that both R18 and R18D at all doses examined reduced cerebral infarct volume, and at several doses improved functional outcome measures, as well as improving weight gain in animals. In contrast, JNKI-1-TATD treatment resulted in a non-significant reduction in cerebral infarct volume and did not significantly improve any functional outcomes or weight gain. The efficacy of both R18 and R18D over a wide dose range (30 – 1000 nmol/kg) is highly significant and could ultimately translate to clinical efficacy at low doses, which would have the advantage of reducing the risk of side effects due to the peptide. Interestingly, a wide neuroprotective dose-response range has been observed previously for JNKI-1-TATD following intraperitoneal administration in adult mice (Wiegler, Bonny, Coquoz, & Hirt, 2008) and P14 rats (Vaslin, Naegel-Tollardo, Puyal, & Clarke, 2011) subjected to a middle cerebral artery occlusion.

However, the inferior efficacy observed with JNKI-1-TATD in HIE may reflect its lower arginine content (R = 9) and cationic charge (+12), compared to R18 and R18D (R = 18, net charge +18). In support of this, previous studies from our laboratory have confirmed that the neuroprotective efficacy of CARPs increases with increasing peptide arginine content and peptide positive charge (Edwards et al., 2016; Meloni, Brookes, et al., 2015).

One of the key principals to developing a therapeutic intervention for HIE is the concept of a delayed secondary phase of brain injury. In infants suffering from a cerebral HI event, the development of secondary brain injury is associated with mitochondrial energy failure, cytotoxic oedema, cell death and the presence of clinical seizures. The secondary injury phase typically occurs 6 – 15 hours following HI onset, and thus provides a therapeutic window for intervention to reduce further brain injury. Therefore, a subsequent aim of this thesis was to determine if the R18 peptide was effective when administered at delayed time points following hypoxia in the modified Rice-Vannucci model. While R18 and R18D demonstrated similar efficacy when administered immediately after hypoxia, the R18D peptide was selected for the delayed administration studies in view of the known resistance of _D-enantiomer peptides to enzymatic degradation (Weinstock, Francis, Redman, & Kay, 2012), which would potentially extend peptide half-life and tissue bioavailability. Furthermore, due to the acute and ongoing nature of brain injury following HI, delayed administration would expose the peptide to increasing levels of proteolytic enzymes activated in the extracellular matrix and released from dead and dying cells in the brain. Hence, the use of a _D-enantiomer peptide (R18D), as opposed to a _L-enantiomer peptide (R18) is potentially likely to be more effective in this situation.

The therapeutic window study revealed that R18D at all the doses examined significantly reduced cerebral infarct volume when administered 30 or 60 minutes after hypoxia (3 and 3.5 h after the commencement of HI, respectively), and at several doses improved functional outcomes. As with the dose response study, the efficacy of R18D over a wider dose range (10 – 1000 nmol/kg) when administered after HI was shown to be highly significant and demonstrated neuroprotective efficacy at even lower doses down to 10 nmol/kg.

7.3 LIMITATIONS OF THIS THESIS

While this thesis has confirmed the neuroprotective efficacy of R18 and R18D in a rat model of HI, and the potential application of the peptides as a novel therapeutic to treat HIE, there are several limitations associated with the study. A 48-hour endpoint was used in the HI studies (Chapters 5 and 6), however, to confirm that neuroprotection provided by R18 and R18D is maintained longer-term, extended time points after HI (e.g. 4 – 7 weeks) need to be assessed. In addition, the use of an extended study endpoint would allow for the assessment of R18 and R18D neuroprotective effects following cell death that occurs beyond 48 hour time point, as well as examine additional behavioural assessments (e.g. gait analysis, rotarod and Barnes maze) to provide a more comprehensive and sensitive measure of functional outcomes and therapeutic benefit.

Whilst the R18D peptide was chosen for the delayed treatment studies due to its potentially greater resistance to proteolytic degradation, it remains to be determined if the R18 peptide is less, more or equally effective than R18D. The efficacy of delayed

administration of R18 will be important to determine, as the use of an L-enantiomer peptide may be more suitable for repetitive dosing due to less likelihood of the accumulation of the peptide in affected brain tissue, which at high concentrations may be toxic to already vulnerable neurons and glial cells.

Histologically, the P7 rat brain corresponds with a late pre-term human (34 weeks gestation), while growth/proliferation, persistence of a periventricular germinal matrix, neurochemical and metabolic data, EEG pattern, synapse formation and patency of the blood brain barrier of the P7 rat correspond well with the full term human (37 weeks gestation) (Hagberg, Bona, Gilland, & Puka-Sundvall, 1997). Despite the physiological similarities, to confirm R18 and R18D neuroprotective efficacy in term humans, pre-clinical efficacy in P10 rodents would need to be assessed.

The current clinical ‘gold standard’ to treat HIE is hypothermia. Consequently, in the event that a R18 peptide progresses to clinical trials, it would have to be assessed alongside or in combination with hypothermia. Therefore, it will be essential for pre-clinical studies to determine if the addition of R18 has synergistic effects or can extend the therapeutic time window of hypothermia. Initial preclinical studies could be examined in the rodent HI model, and if positive, could then be extended to the fetal sheep or piglet HI models. To this end, previous studies have been performed examining adjunct therapies with hypothermia in rodent, sheep and piglet animal models of HI (Davidson et al., 2015; Faulkner et al., 2011; Liu, Dingley, Scull-Brown, & Thoresen, 2015).

It is acknowledged that an intravenous, rather than intraperitoneal, route of administration would be favoured in a clinical setting. Owing to the difficulty for intravenous administration of rodent pups due to their small size, assessment of the intravenous route for peptide delivery would be more appropriately assessed in a larger animal model of HIE, such as the piglet or lamb. It should also be noted that following intraperitoneal injection, the first circulatory pass of the peptide would be through the hepatic portal vein, which would likely reduce the concentration of the peptide available reaching the brain. An additional benefit of using a larger animal model is the assessment of R18 efficacy in an animal with a gyrencephalic brain, which more closely resembles the human brain.

It is widely accepted that temperature maintenance in rodent neuroprotective studies is paramount. Despite surface temperatures of the animals routinely examined until the end of experiment and animals being housed in a ventilated warming cabinet ($26^{\circ}\text{C} \pm 1^{\circ}\text{C}$) throughout the experimental period, it is possible animal temperatures can vary. Alternative telemetric rectal probes are inappropriate for long-term temperature monitoring in this model as the leads cannot be incorporated into the sealed hypoxic chamber and maternal grooming would result in the removal of the probes. Additional implantable wireless temperature probes are not appropriate for the current studies as this would require extended surgical times and isoflurane exposure to allow for intraperitoneal implantation.

Finally, as a typical dose-response pattern of cerebral infarct volume reduction was not observed in this thesis, it would be worthwhile examining a wider dose range of

both R18 and R18D in future pre-clinical studies. The assessment and identification of an R18 peptide dose, lower than the lowest dose used in this thesis may prove beneficial in terms of reducing potential side effects of the treatment.

7.4 SUMMARY OF IMPORTANT FINDINGS OF STUDIES

The findings presented in this thesis have for the first time confirmed the neuroprotective properties of the poly-arginine peptide R18 and its _D-enantiomer R18D in a modified and more consistent rat model of perinatal HIE. Although additional studies are required to elucidate the full neuroprotective potential of the R18 peptides as a neuroprotective therapy in this model of HIE, the present findings do suggest that CARPs are an exciting and promising new class of neuroprotective agents. Further evaluation of CARP neuroprotective mechanisms of actions are warranted, as well as the assessment of neuroprotective efficacy in other animal models of HIE.

Although the present studies have provided clear evidence of neuroprotective efficacy of the R18 peptides in a rodent model of HIE, a number of crucial questions will need to be resolved before consideration is given to their evaluation in a human clinical trial. Firstly, their efficacy will next need to be demonstrated in a larger gyrencephalic animal model (e.g. the piglet or lamb), which have longer but clearly defined gestation times and whose brain and cerebrovascular anatomy is closer to that of the human. Furthermore, as therapeutic hypothermia is considered ‘standard care’ for infants suffering from HIE, it is paramount that R18 peptides are assessed as an adjunct to hypothermia in a larger animal model of HIE, to ensure safety and to identify any synergistic effects associated with hypothermia. Moreover, as there is currently no

available therapeutic agent to reduce the severity of brain injury in the pre-term infants with HIE, and as HIE in pre-term and term infants share pathophysiological similarities relating to the mechanism of action of R18, it is important that R18 peptides are also assessed in pre-clinical animal models of pre-term HIE, both to ensure safety and the capacity to reduce brain injury and functional impairment.