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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 3

General Materials and Methods

3.1 MATERIALS AND METHODS

In addition to information provided in Chapters 4 - 6, many of the materials and methods used in this thesis are provided in this chapter.

3.2 PEPTIDES USED IN THIS THESIS

The peptides used for *in vitro* and animal studies are summarised in Table 3.1. All peptides were purified using high performance liquid chromatography and were subjected to hydrolysis and amino acid liquid chromatography analysis to obtain a precise measurement of peptide content (Mimotopes, Melbourne, Australia). For animal studies, all peptides were prepared in 0.9% sodium chloride for injection (Pfizer, Perth, Australia), aliquoted into a 50 µl volume within 300 µl syringes and stored at -20°C until use.

Table 3.1 Summary of the peptides used in this thesis

Peptide	Sequence*	Arginine residues	Charge at pH 7
R18	H-RRRRRRRRRRRRRRRRR-OH	18	+18
R18D	H-rrrrrrrrrrrrrrrrr-OH	18	+18
JNKI-1-TATD	H-tdqsrpvqpflnlttprkprpp-rrrqrrkkrg-NH_2	9	+12

*T = threonine, D = aspartic acid, Q = glutamine, S = serine, R = arginine, P = proline, V = valine, F = phenylalanine, L = leucine, N = asparagine, K = lysine. Lowercase letters signifies $_D$-enantiomer amino acid.

3.3 P7 RAT MODELS OF HYPOXIC-ISCHAEMIC ENCEPHALOPATHY

3.3.1 Animal ethics approval

All animal surgical procedures and behavioural studies were approved by the University of Western Australia's Animal Ethics Committee (RA/3/100/1329 and RA/3/100/1569), in accordance with the Policies and Guidelines of the National Health and Medical Research Council, Australia. In the design of these studies, every effort was made to minimise the amount of animal suffering and follow Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010), where appropriate. All animal surgical procedures and behavioural studies were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited animal research facility.

3.3.2 Animals numbers used in experimentation

Pregnant nulliparous Sprague-Dawley rats (dam; Animal Resource Centre, Murdoch, Australia) were used for experiments presented in this thesis. For determining a pup's age, day of birth was considered P0. At P2, all litters were counted and where appropriate were culled to 10 – 12 pups per dam; this is to facilitate uniform growth of the pups without littermate competition for feeding. Animals with a weight range of 14.5 ± 1.5 grams were used for studies, with animals falling outside this weight range on day P7 excluded. In addition, while animals were sexed, both male and female pups were used in studies, in a random and blinded fashion. Prior to any surgical procedure, the dam and pups were housed in ventilated cages (Techniplast, Paola, Malta) which were placed in an animal holding room maintained at 25 ± 1°C.

3.3.3 Behavioural assessments

For all behavioural assessments, each animal was given three attempts to complete each behavioural assessment per day, with 5 minutes between each attempt and assessment. All of the assessments used in this thesis are considered highly reproducible throughout the murine pre-weaning period (< P30) and are strain- and sex-independent (Heyser, 2004). In between all behavioural assessments, all animals were placed on a heating pad (37°C) to prevent hypothermia. Forty-eight hours after hypoxia-ischaemia (HI) pups' responses in all 3 behavioural assessments were recorded 3 times.

3.3.3.1 Righting reflex

For the righting reflex, pups were placed on a flat surface covered with a tightly stretched close-knit fabric, to ensure adequate friction. Pups were placed in a supine position and the time recorded for them to rotate to a prone position recorded (in seconds).

The righting reflex required behavioural sensitisation (i.e. pre-training). For three consecutive days before surgery (P4 – 6; inclusive), pups were accustomed to the righting reflex with a mean target inclusion range of ≤ 2 seconds by the day of surgery (P7; behavioural baseline recording measurement). Any animal that did not meet the inclusion criteria was excluded from the study.

3.3.3.2 Negative geotactic response

For the negative geotactic response, animals were placed on a board covered with tightly stretched close-knit fabric, to ensure adequate friction. The board was positioned on a 45° slope

and at the start of assessment, pups were placed facing downslope. The time for the pups to rotate 150° up the 45° slope recorded (in seconds).

The negative geotactic response required behavioural sensitisation. For three consecutive days before surgery (P4 – 6; inclusive), pups were accustomed to the negative geotactic response with a mean target inclusion range of ≤ 30 seconds by the day of surgery (P7; behavioural baseline recording measurement). Any animal that did not meet the inclusion criteria was excluded from the study.

3.3.3.3 Wire-hang assessment

The wire-hang assessment involved suspending pups by their forelimbs on a 2 mm diameter steel wire, suspended 20 cm above a soft foam surface and recording the time taken (in seconds) for the animal to fall to the foam surface.

The wire-hang assessment did not require any behavioural sensitisation and pups' performance was assessed on the day of surgery (P7; behavioural baseline recording measurement) and did not have any study inclusion criteria.

3.3.4 Original Rice-Vannucci HI surgical procedure

Anaesthesia was induced using a facemask with 5% isoflurane in 100% O_2, and once the animal was anaesthetised, the isoflurane concentration was reduced and maintained at 1.5 ± 0.5%; the duration of anaesthesia from induction to beginning of recovery did not exceed 10 minutes.

Through a 1 cm mid-line ventral incision, the right common carotid artery (CCA) was exposed and carefully dissected from the vagus nerve, venous circulation and carotid body. The CCA was permanently ligated using a 6-0 silk suture. A diagrammatic description of this surgical procedure is presented in Chapter 3, Figure 1. The wound was closed using Vetbond (3M, Maplewood, USA) and the animals recovered on 100% O_2 for 5 minutes. Sham-operated animals underwent the same operative procedure, except the exposed CCA was not ligated.

Following surgical recovery, pups were returned to their dam for approximately 1 hour prior to hypoxia, which consisted of placing between 4 and 6 pups into an airtight container (approximate volume: 4 litres) and gassing the container with warm humidified gas (8% O_2/92 % N_2; 3 litres/minute) for 2.5 hours. During the gassing procedure, the container was housed in an incubator maintained at 35°C. Following hypoxia, pups were removed from the container and immediately administered peptide or saline (IP bolus; 50 µL), then placed back with the dam. For delayed post-hypoxia treatment administration, following hypoxia, pups were immediately returned to their dam and at the appropriate time injected with peptide or saline. Sham-operated animals remained with the dam at all times after the surgical procedure.

3.3.5 Modified Rice-Vannucci HI surgical procedure

For the modified Rice-Vannucci model, the surgical procedure was the same as described above for the original Rice-Vannucci model, except both the CCA and external carotid artery (ECA) were permanently occluded using a 6-0 silk suture. A diagrammatic description of this surgical procedure is presented in Chapter 3, Figure 1.

3.3.6 Post-surgical analgesia, animal body temperature monitoring and housing

All animals were administered pethidine analgesia (5 mg/kg; intraperitoneally) immediately before the cessation of anaesthesia. Animal surface body temperature was monitored periodically throughout the surgical and hypoxia procedures and immediately post-hypoxia using an infrared thermometer. To avoid animals becoming hypothermic during the surgical procedure and when separated from the dam during behavioural assessments, animals were placed on a heating pad maintained at 37°C. During the surgical procedure, if the animal's body temperature decreased (< 36.5°C) a heating fan would be used to maintain a surface body temperature of 37°C. In addition, from the conclusion of surgical anaesthesia to the experimental endpoint, all animals (pups and dam) are housed at 27 ± 1°C in a Ventilated Warming Cabinet (Techniplast, Paola, Malta).

3.4 *EX VIVO* ASSESSMENT OF BRAIN INJURY

3.4.1 Tissue sectioning and triphenyl tetrazolium chloride (TTC) staining

Forty-eight hours after HI, animals were euthanised by lethal intraperitoneal injection of pentobarbitone (50 mg/kg; intraperitoneally). Infarct volume measurement was performed using coronal brain slices stained with TTC. The brain was carefully removed from the skull and placed in 0.9% sodium chloride and cooled for 15 minutes at -80°C before being placed in an adult mouse brain matrix (Note: the adult mouse brain matrix is a suitable size for a P9 rat brain). Razor blades were inserted into the matrix to generate 2 mm coronal slices. The brain slices were incubated for 20 minutes at 37°C in a 3% TTC (w/v; in 0.9% sodium chloride) to stain viable non-infarcted tissue, then fixed by placing in 4% formalin for at least 24 hours.

3.4.2 Measurement of infarct volume from TTC stained coronal slices

Digital images of coronal brain slices (not including cerebellum) were acquired with a colour scanner and analysed using ImageJ software (National Institute of Health, Maryland, United States of America) to calculate an infarct volume. The calculation accounts for changes in hemisphere size due to oedema and is presented below and in Chapter 3:

$$Corrected\ infarct\ volume = \left(\frac{Contralateral\ hemisphere\ volume}{Ipsilateral\ hemisphere\ volume}\right)(Infarct\ volume)$$

Once the infarct area for all sections was defined, the total infarct volume was presented as a percentage of total brain volume.

3.5 STATISTICAL ANALYSIS

Data from mean total infarct volume measurements were evaluated by analysis of variance (ANOVA) followed by Fisher's *post hoc* analysis. Data for behavioural assessments were analysed as described in Chapters 4 - 6.

3.6 REFERENCES

Heyser, C. J. (2004). Assessment of Developmental Milestones in Rodents. In *Current Protocols in Neuroscience* (Vol. Chapter 8, p. Unit 8.18). Hoboken, NJ, USA: John Wiley & Sons, Inc. https://doi.org/10.1002/0471142301.ns0818s25

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