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Optimizing the Utility of Sex-Specific Organotypic Hippocampal Sliced Cultures for *In Vitro* Ischaemia Studies

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Abstract

In vitro models of ischaemia are commonly used in neuroscience research yet can be difficult to establish in a reproducible manner due to lack of experimental details commonly reported in publications. This study describes a series of optimization steps in order to produce a reliable model of organotypic hippocampal sliced cultures used for *in vitro* ischaemia studies. In addition, we describe the process by which sex-specific cultures were produced. Optimization data is provided regarding the age of animals used, time course of culture maintenance *in vitro* and optimal time of Oxygen and Glucose Deprivation (OGD) in order to produce a sufficient level of cell death. In addition, we demonstrate the effect of the age and sex of the pups on the amount of cell death produced. This study provides detailed optimization steps required to produce a reliable and reproducible sex-specific organotypic hippocampal sliced culture model which would then be suitable for exposure to OGD in order to model *in vitro* ischaemia and assess potential neuroprotective compounds.

Keywords: Hippocampus; Ischaemia; Organotypic; Optimization; Sex

Introduction

Numerous experimental approaches, including *in vitro*, *ex vivo* and *in vivo*, have been developed in order to model the impact of cerebral ischaemia. Experimental cerebral ischaemia studies allow the characterisation of the underlying pathophysiological mechanisms and the investigation of potential protective strategies. *In vitro* models offer high throughput and a model in which mechanisms of injury and/or potential therapies can be investigated. Such *in vitro* models are largely formed from primary cell cultures involving dissociated cells, slice cultures or acute brain slices. The majority of dissociated culture models consist of one cell type obtained from embryonic or postnatal tissue with the majority of ischaemic models being based on cortical or hippocampal tissue.

In terms of trying to model ischaemia, the hippocampus is a common target [1], as it is an area of the brain most susceptible to brain injury [2] and due to its characteristic anatomy, easy to localise. The most widely used *in vitro* approach to model ischaemic conditions is exposure to Oxygen and Glucose Deprivation (OGD) although other approaches include glutamate-mediated excitotoxicity [3], hypoxia [4] and hypoglycaemia [5]. Organotypic Hippocampal Sliced Cultures (OHSCs) are commonly used to study the mechanisms induced following ischaemia [6], as OHSCs exposed to OGD results in both necrotic and apoptotic cell death as seen following *in vivo* ischaemia [7]. Advantages of this model over other single-cell culture models include the maintenance of experimental conditions over a longer period of time and the ability to maintain two-dimensional neuronal architecture of the hippocampus along with neuron-glia interactions [8].

There are important sex differences in a number of neurodevelopmental disorders (e.g. autism, attention deficit hyperactivity disorder) and those disorders resulting from CNS injury and/or neurodegeneration such as ischemic stroke and Alzheimer's disease [9-11]. However, for research which utilises *in vitro* CNS models they tend to report cultures derived from tissues pooled from male and female pups within the same litter to increase yield, in particular when utilising embryonic or postnatal neurons. Here we report the optimization of a sex-specific model of OHSCs. Although the use of OHSCs for *in vitro* ischaemia studies is well established the majority of studies only give brief experimental details and don't provide sufficient information regarding the optimization of such a model. This study describes a series of experiments aimed at optimizing the use of OHSCs for *in vitro* ischaemia studies and gives details of the effect of various factors including age of animals, time of culture maintenance and duration of OGD exposure.

Material and Methods

Animals

This study was conducted in accordance with the UK Animals Scientific Procedures Act, 1986. *In vitro* cultures were prepared from 4-9 days old mouse C57/Bl6 pups. Sex of the pups was determined possibly by visible inspection of anogenital distance or the distance between the mouse's genital area and anus as female mice have a genital area much closer to the anus compared to male mouse. Also, pigmented cells on the scrotum are visible to the naked eye on the day of birth in male animals compared to female mice in C57BL/6J pups and female mice have 10 nipples compared to male mice that do not have nipples [12].

Cell Culture Preparation

Cell culture cabinets and all materials were sterilized with 70% Industrial Methylated Spirit (IMS). Dissecting tools were sterilized in pure ethanol and then heated with a gas flame to ensure sterility. Organotypic hippocampal cultures were prepared according to the methods of with some modifications [13]. Brains were removed from animals and placed in sterile petri dishes containing ice cold dissecting buffer. The hemispheres were separated using a scalpel and the hippocampus dissected and sliced (350µm) using a tissue chopper (McIlwain tissue chopper). The slices were carefully separated using sterile syringe needles and transferred onto Millicell membrane inserts (0.4 µm, MilliPore) using a plastic Pasteur pipette. These inserts were placed into six well plates and cultured in growth medium. Cultures were maintained in a humidified incubator with 5% CO_2 at 37°C and culture medium was changed every 3 days. It is important to note that culture medium should be changed on time as any delays resulted in poor growth of cells having an impact on the number of slices available for subsequent experiments. Cultures were observed under the microscope every few days to monitor growth. For the normoxic experiments, which aimed to establish the optimal time course for culture maintenance, independent cultures were maintained 7, 10, 14, 18 and 21 days.

In Vitro Ischaemia

Hippocampal slice cultures were exposed to Oxygen-Glucose Deprivation (OGD) at day 14, a time point selected as this has been shown previously to be sufficient for the slices to have recovered from microglia activation caused by the slicing procedure [14]. OGD medium containing 75% MEM, 25% HBSS, 1mM glutamine and 3.75 µg/ml amphotericin B was bubbled for 30 minutes with 5% CO_2 and 95% N_2. After two washes with the OGD medium, 1ml of OGD medium was placed in the well and plates were transferred to an anoxic chamber. The chamber was sealed and pumped with 5% CO_2 and 95% N_2 for 10 minutes then placed in an incubator at 37°C for 2 hours, 3 hours or 4 hours of OGD. The cultures were returned to oxygenated serum-free culture medium and placed back in the incubator for a further 24 hours.

Assessing Neuronal Cell Death

In order to assess cell death, 30 minutes prior to the termination of experiments, the fluorescent cell death marker propidium iodide (PI, 5 µg/ml) and Hoechst (5 µg/ml) were added to the medium. At the end of the experimental time, slices were fixed with 4% paraformaldehyde at 4°C for 2 hours. Previous studies [15], report using fixation times of 4 hours but longer fixation times can result in higher levels of background staining thus we compared fixation times of 1 hour, 2 hours and 3 hours and found 2 hours to be optimal. Following fixation, the cultures were briefly washed in Phosphate Buffered Saline (PBS). The slices were removed from the membrane inserts and mounted onto glass slides in PBS and glycerol solution and imaged using a Nikon epifluorescence microscope. For each hippocampal slice, images were taken, using a Nikon microscope, from two randomly selected regions. For each region, two images were taken, one image allowing visualization of PI-labelled cells and the other showing Hoechst-labelled cells. The number of cells in each image was counted manually. Cell counting was performed by an individual blinded to experimental condition. To obtain the percentage cell death, cells labelled with PI (i.e. number of dying cells) were divided by the total number of cells, as indicated by the Hoechst labelling, which labels all cell nuclei.

Data Analysis

All data are reported as mean ± standard error of the mean and were analyzed using GraphPad Prism version 7.0 for windows (GraphPad Software, San Diego, USA). Normality tests were carried out prior to comparative statistical analysis using D'Agostino and Pearson normality test. For datasets not normally distributed Kruskal Wallis analysis was used with Dunn's multiple comparisons test. For normally distributed data student's t-tests

were used to compare between two sets of data whereas Analysis Of Variance (ANOVA) with Turkey's multiple comparisons test, if relevant, was used to analyse differences between more than two groups. The criterion for statistical significance was P < .05. Slices were prepared from 2-4 pups (of each sex) per experiment and the n value is shown as the number of independent wells with each well having three slices.

Results

Initially cultures were prepared from Postnatal Day 4 (P4) mouse pups and in order to maximize survival the hippocampus was removed within minutes and the dissecting medium kept on ice at all times in order to maintain a low temperature during the process of dissection and slice preparation. To optimize the time, course of culture maintenance, cell survival of cultures was assessed following 7, 10, 14, 18 and 21 days *in vitro* (Figure 1). Previous reports have used cultures at 14 days *in vitro*, a time point that has been shown to be sufficient for the slices to have recovered from microglia activation caused by the slicing procedure [14]. In the current study assessment of cell viability showed that a significant increase in cell death was observed following 21 days *in vitro* (24.7 ± 1.2%, P < .0001, Figure 1) which was not observed between days 7 and 18 suggesting cultures may be maintained for up to 18 days *in vitro* without any increase in cell death occurring.

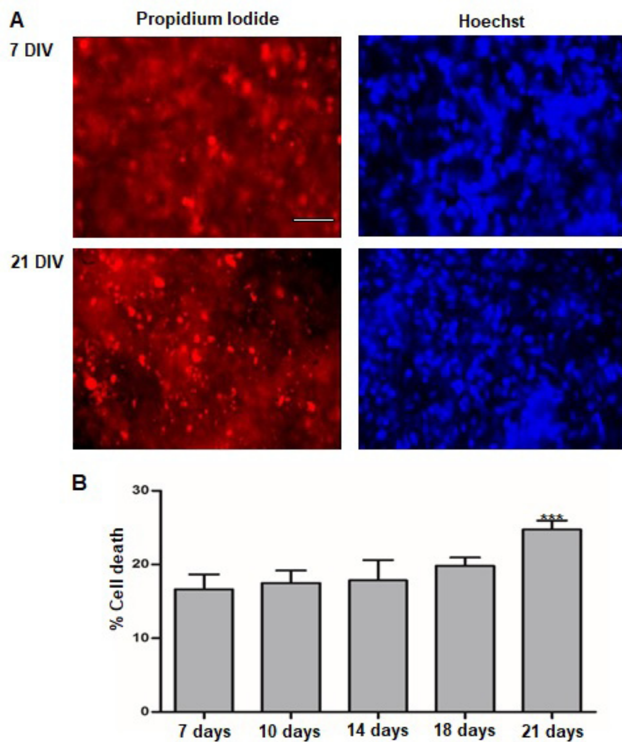

Figure 1: Representative images of hippocampal slices exposed to normoxic conditions and maintained for either 7 or 21 Days *In Vitro* (DIV). All cells are shown by Hoeschst staining and cell death is shown in images stained with PI. Cell death was analysed at different time points and found to significantly increase at 21 days compared to 7, 10 and 14 days *in vitro* (***P<0.001). Data are expressed as mean ± SEM, n = 20-34 per time point, scale bar represents 20 υm.

Experiments were conducted to establish an optimal time period of OGD in order to produce a sufficient level of cell death to assess potential neuroprotective compounds (Figure 1). For each time point, cultures exposed to OGD were compared to cultures exposed to normoxic conditions. Normoxic cultures also underwent a media change to ensure the only experimental condition differing between the two groups was the presence or absence of OGD. The amount of cell death significantly increased as the duration of OGD lengthened (P < 0.001, Figure 2). Following OGD cell death was significantly increased compared to normoxic controls (17.8 ± 2.8%) after 2 hours (34.6 ± 3.1%), 4 hours (60.0 ± 3.2%) and 6 hours (71.5 ± 4.0%) OGD.

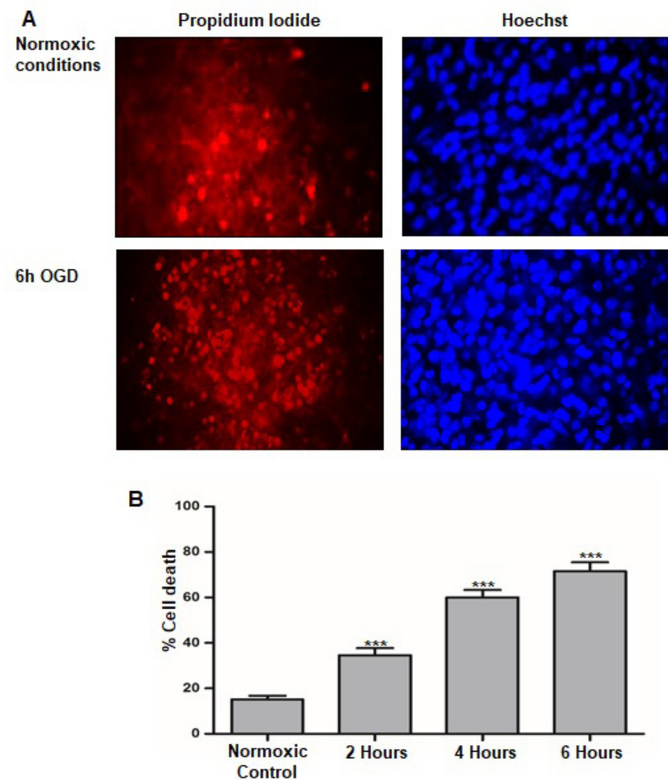

Figure 2: Representative images of hippocampal slices showing cell death stained with PI following exposure to normoxic conditions or 6 hours OGD. The amount of cell death was significantly increased following 2, 4 or 6 hours of OGD compared to normoxic conditions (***P < 0.001). Data are expressed as mean ± SEM, n = 22-27 slices.

In order to examine the effect of the age and sex of pups on the amount of cell death cultures were prepared separately from male and female pups at P4, P6, P7 and P9. Cultures were exposed to 4 hours OGD and compared to normoxic controls. Following

OGD there was no significant difference in the amount of cell death according to the age of the pups in cultures derived from either male (P = 0. 52) or female (P = 0.65) pups (Figure 3). There was a significant increase in the amount of cell death following OGD compared to normoxia in the CA1 region of the hippocampus in both males (P < 0.001) and females (P < 0.001). There was significantly less cell death following OGD exposure in female-derived cultures (27.36 ± 2.01%) compared to male-derived cultures (45.36 ± 2.34%, P < 0.001, Figure 4).

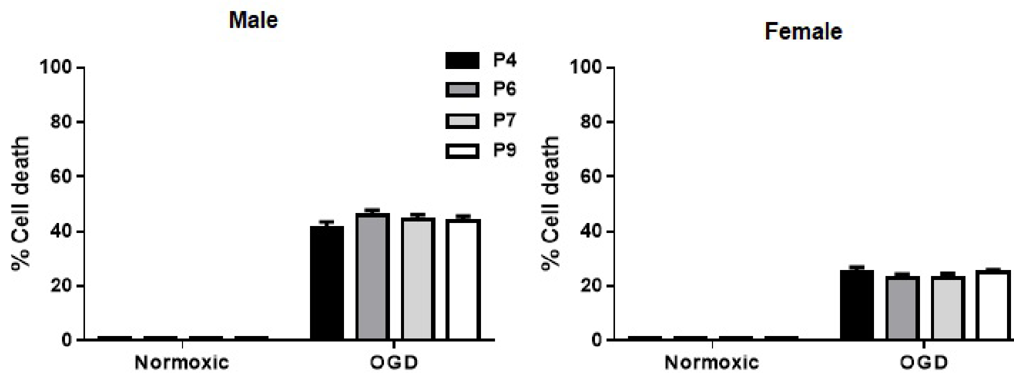

Figure 3: The amount of cell death, following OGD, was not significantly different according to the age of the pups (between P4 - P9) in both male- and female-derived cultures. Data are expressed as mean ± SEM, n = 6-8 independent wells.

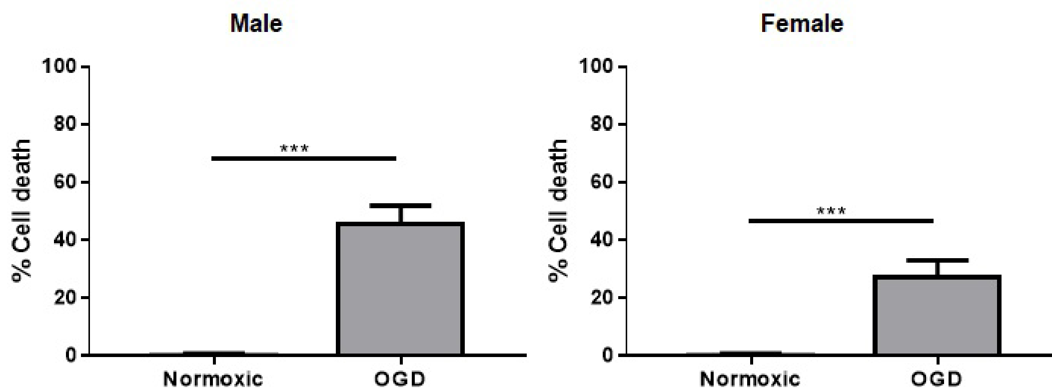

Figure 4: There was a significant increase, in both male (***P < 0.001) and female (***P < 0.001) cultures, in the amount of cell death following OGD compared to normoxia in the CA1 region of the hippocampus. Data are expressed as mean ± SEM, n = 6-8 independent wells.

Discussion

The aim of this study was to optimize the experimental parameters required to establish a suitable and reliable sex-specific model of *in vitro* ischemia. Initially, the current experiments used animals on P4 however, in subsequent experiments we used animals aged between P4-P9 for slice preparations and found that age did not affect the rate of cell death observed following either normoxic or OGD conditions. Neonatal slices appear to be more suitable for experiments requiring longer-term maintenance of cultures whereas slices prepared from older animals, which degenerate quicker, may be more suitable for short-term electrophysiological studies [7].

In order to model cerebral ischaemia it is necessary to quantitatively assess the amount of cell death induced. Several studies have demonstrated a linear relationship between the fluorescence intensity of propidium iodide and cell death, where an increase in cell death over a period of time has shown to correspond with an increase in propidium iodide uptake [6]. However, the fixation time is important as longer fixation periods can result in a significant diffusion of propidium iodide throughout the tissue, which increases background fluorescence and makes it difficult to visualise individual cells [15]. In the current study we observed that fixation times of 2h were ideal as longer fixation times were associated with increased background staining due to the diffusion of propidium iodide, which made it difficult to visualise individual

cells. Advantages of using propidium iodide as a cell death marker include that is a simple method for providing quantitative data, cell death can be visualised and quantified in the same cultures over time, and distinct patterns of cell death can be identified [16]. Although propidium iodide cannot stain dead cells with denaturation of intracellular nucleic acid this can be overcome by using in combination with other fluorescent dyes, such as Hoechst, which stains dead, dying and living cells.

It is important the cultures are maintained for a period of time under *in vitro* conditions to allow them to recover from any trauma associated with slice preparation but also for the recovery of synaptic activity. During the period of maintenance, cultures undergo reorganization and form new neuronal networks and continue to develop to mimic the developmental processes which take place *in vivo*. It has been reported cultures maintained for 2-3 weeks are able to recover synapses at a similar level to that observed *in vivo* [17]. The majority of other studies maintain cultures *in vitro* for 10-14 days whereas the present study shows that cell viability is unaffected after 18 days *in vitro* but is detrimentally affected if cultures are maintained for 21 days *in vitro*.

Studies attempting to model *in vitro* ischaemia have used varying lengths of OGD exposure, from 30 minutes to 3 hours [18]. The time point of OGD exposure is largely dependent on the culture system i.e. dissociated or organotypic cultures as this will impact on the severity of injury. Data presented here indicate that 2 hours of OGD was a suitable time point to induce cell death at a level which could be subject to (e.g. pharmacological) manipulation. In addition, 2h OGD induced cell death uniformly across the slice whereas, in agreement with other studies [19], shorter OGD times tend to induce cell death in restricted regions of the hippocampus.

There is increasing awareness of sex differences in terms of incidence, pathology and response to treatment for a number of CNS disorders. Utilising sex-specific *in vitro* models allows sex differences to be investigated both in terms of the development of the CNS [20] and mechanistic investigation of therapeutic targets for certain disorders. Here we describe that sex-specific OHSCs can be generated from as young as P4 pups with sufficient yield and ability to anatomically distinguish between the sexes. It would be interesting to explore the feasibility of such cultures from an earlier postnatal stage when anatomical discrimination between the sexes is still feasible.

Although, *in vivo* models of ischaemia represent a closer comparison to humans, *in vitro* models hold several advantages as they permit investigation under a controlled environment avoiding the confounding effects of temperature and vascular components. In addition, the lack of blood-brain barrier permits direct access to the cellular compartments. These advantages of *in vitro* model permit the investigation of neuroprotective agents without the influence of other, potentially confounding factors. Thus, it is important that such *in vitro* models are well characterized and validated in order to ensure they are reproducible.

Conflict of interest

The authors have no conflict of interest to declare.

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