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A novel method for inducing focal ischemia *in vitro*

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ABSTRACT

Current *in vitro* models of stroke involve applying oxygen-glucose deprived (OGD) media over an entire brain slice or plate of cultured neurons. Thus, these models fail to mimic the focal nature of stroke as observed clinically and with *in vivo* rodent models of stroke. Our aim was to develop a novel *in vitro* brain slice model of stroke that would mimic focal ischemia and thus allow for the investigation of events occurring in the penumbra. This was accomplished by focally applying OGD medium to a small portion of a brain slice while bathing the remainder of the slice with normal oxygenated media. This technique produced a focal infarct on the brain slice that increased as a function of time. Electrophysiological recordings made within the flow of the OGD solution ("core") revealed that neurons rapidly depolarized (anoxic depolarization; AD) in a manner similar to that observed in other stroke models. Edaravone, a known neuroprotectant, significantly delayed this onset of AD. Electrophysiological recordings made outside the flow of the OGD solution ("penumbra") revealed that neurons within this region progressively depolarized throughout the 75 min of OGD application. Edaravone attenuated this depolarization and doubled neuronal survival. Finally, synaptic transmission in the penumbra was abolished within 50 min of focal OGD application. These results suggest that this *in vitro* model mimics events that occur during focal ischemia *in vivo* and can be used to determine the efficacy of therapeutics that target neuronal survival in the core and/or penumbra.

1. Introduction

During an occlusive stroke, blood flow and critically important supplies of oxygen, glucose, and key nutrients to an area of the brain is blocked. In the context of this ischemic event, neurons receiving blood supply from this vessel begins to die due to processes mediated by the ischemic cascade (Lipton, 1999; Brouns and De Deyn, 2009). This initial area of neuronal death is focal in nature and is termed the "core" region and recovery of affected cells in this region is negligible and thought to be non-existent (Beilharz et al., 1995). As neurons within the core region die primarily via necrosis, they release their intracellular contents which diffuse into the surrounding extracellular space. This disruption in ionic equilibrium, combined with the continued hypoxia and hypoglycemia, leads to a secondary stage of neuronal damage that progressively spreads to neurons located adjacent to, and well outside of, the core region (Lipton, 1999; Zheng et al., 2003; Brouns and De Deyn, 2009). This region is called the "penumbra" and is characterized by metabolic arrest, cellular stress and eventual death of neurons primarily via apoptosis (Astrup et al., 1981; Hossmann, 1994; Lipton, 1999). Together, the core and penumbra continue to grow in volume as long as the ischemia is maintained.

There is great interest in using stroke models to study and search for compounds capable of reducing the rate at which damage spreads subsequent to a focal ischemic insult (Durukan and Tatlisumak, 2007). However, in order to efficiently characterize such compounds, it is desirable to have a model that allows researchers to rapidly determine efficacy as well as to determine mechanism of action of the compounds. The most common models currently used in stroke research include: *in vivo* focal ischemia models (Weng and Kriz, 2007; Saleh et al., 2009), *in vitro* dissociated cell models (Larsen et al., 2005; Ye et al., 2009) and *in vitro* brain slice models (Garcia de Arriba et al., 1999; Jarvis et al., 2001). *In vivo* focal ischemia models (whole animal models) are used extensively to study stroke and involve invasive surgery to expose and occlude a cerebral artery (Saleh et al., 2009). Although these *in vivo* models are the most physiologically accurate, they have a very low throughput, are technically demanding, and require the use of large numbers of animals, all of which results in a very high cost per data point (Graham et al., 2004). As well, using this type of model, it is difficult to determine the mechanism of action of therapeutic interventions. The use of *in vitro* models where oxygen-glucose deprivation (OGD) is used to mimic stroke (Jarvis et al., 2001; Wise-Faberowski et al., 2009; Ye et al., 2009) overcomes many of these problems. Of the *in vitro* models, the dissociated cell models are popular for screening

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due to their high throughput, low cost and ease of use. Unfortunately, these models are also the least physiologically accurate because the cells are dissociated through enzymatic and mechanical treatments and hence are not at all similar to being in their normal environment. Consequently, neurons in culture lack normal synaptic contacts and interactions with other neurons as well as with other cerebral cell types (i.e. glial cells; Lossi et al., 2009). The *in vitro* brain slice model may be lower throughput than the cell culture model, however, it is far more physiologically accurate. Within each slice, cytoarchitecture is maintained and thus many of the cell-to-cell interactions and neuronal networks remain intact (Gahwiler et al., 1997; Noraberg et al., 2005; Lossi et al., 2009). Hence, this model is well suited for physiological experiments to assess mechanism of action of drugs as well as to study neurophysiological changes that occur with stroke.

One limitation of current *in vitro* slice models of stroke is that OGD media is applied to the entire brain slice and thus all of the cells in the slice are subject to the same ischemic condition (Jarvis et al., 2001; Wise-Faberowski et al., 2009). This is referred to as global ischemia and thus the entire slice is representative of the "core". Although these models are used to characterize ischemia-induced neuronal death (Martínez-Sánchez et al., 2004) and have utility in identifying compounds that are neuroprotective (Lipski et al., 2007) or toxic (Bonde et al., 2003), they do not mimic the focal nature of stroke or the ability to study the events that occur within the penumbra. Consequently, using the existing global models, it is difficult to properly characterize the processes involved in the spread of cell death from the core to adjacent healthy tissue. Since, clinically speaking, the majority of strokes are focal in nature, there remains a need for a reliable *in vitro* focal ischemic model. Thus, our aim was to develop a novel *in vitro* brain slice model of stroke that would mimic focal ischemia and thus allow us to study events occurring within the penumbra.

2. Materials and methods

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care and under an approved protocol by the University of Prince Edward Island Animal Care Committee.

2.1. Brain slice preparation

In vitro focal ischemia studies were performed on freshly prepared rat brain slices. Methods for preparing the brain slices were similar to those previously published (Saleh et al., 1997). Briefly, male Sprague-Dawley rats (100–125 g; Charles River, Montreal, PQ, Canada) were anaesthetized with isoflurane vapour (Isoflo™; Abbott Laboratories, Saint-Laurent, PQ) and then decapitated. Brains were rapidly removed and immersed in ice-cold (2–3 °C) artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 145 NaCl, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 1.3 $MgCl_2$, 2.5 $CaCl_2$ (pH 7.4, osmolarity of 295–305 mOsmol/L, continuously bubbled with 95% O_2 and 5% CO_2). The brain was then mounted in a vibratome (VT 1000S, Leica) and cut coronally into 400 μm thick slices while submersed in ice-cold aCSF. Prior to the initiation of experiments, slices were incubated for at least 1 h in aCSF at room temperature.

2.2. Brain slice experimental conditions

For experimentation, an individual slice was transferred to an experimental chamber and viewed under low magnification using an upright microscope (BX51WI, Olympus Canada Inc., Markham, ON, Canada). aCSF was superfused at 3 mL/min and the bath temperature was continuously monitored and maintained at 34 ± 1 °C

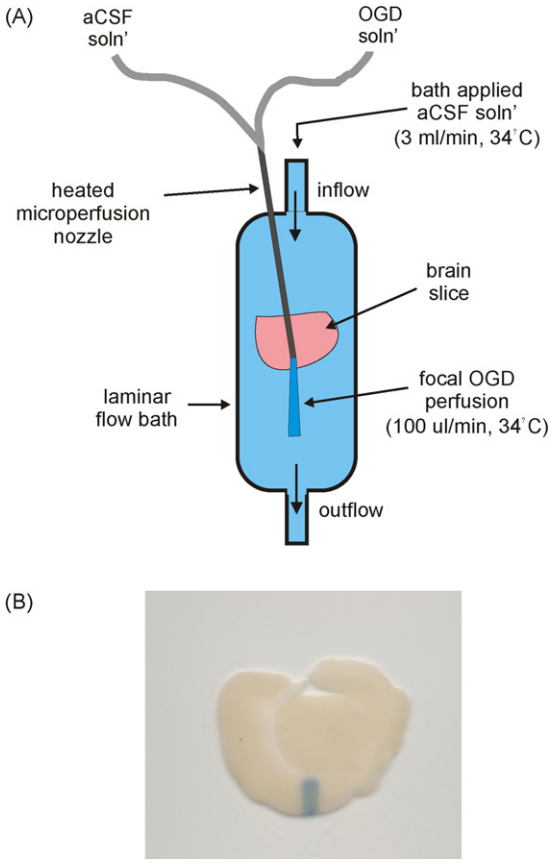

Fig. 1. Experimental design of the *in vitro* focal ischemia model. (A) Representative drawing of the experimental setup. (B) Chicago sky blue tissue stain was focally applied to the cortex of a brain slice. The dark band on the slice represents the area of focal solution application. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

using a temperature control unit (TC-344B, Warner Instruments, Hamden, CT, USA). Bath level was controlled and perfusate was removed via a suction tube located at the opposite end to the inflow of the chamber. The slice was suspended on a mesh insert placed within the bath and was secured in place by a slice anchor hold-down apparatus (thin wire loop with fine thread spaced approximately 1 mm apart). Subsequent to a 30 min equilibration period, drugs were added to the bath solution to allow for pre-treatment. Drugs were also added to the bath and focal perfusion solutions throughout the experiment.

2.3. Focal OGD application

In order to mimic *in vivo* focal ischemia in the present *in vitro* system, OGD medium was focally applied to a small area (0.5–1 mm wide) of the cortex within a prefrontal brain slice. The composition of this OGD medium was the same as that of the aCSF solution except for the following two variables: (1) glucose was excluded and replaced with 10 mM D-mannitol and (2) oxygen was displaced (verified using an O_2 sensing electrode) from this media by bubbling continuously with 95% N_2 and 5% CO_2. The experimental setup used to achieve focal perfusion is illustrated in Fig. 1A. A microperfusion system driven by syringe infusion pumps (Physio 22 pump, Harvard Apparatus, Holliston, MA, USA) was used to deliver aCSF or OGD solution. The nozzle of the microperfusion system was made from a 23 gauge stainless steel tube and was mounted to a micromanipulator in order to ensure accurate placement. Using the micromanipulator, the nozzle was lowered until it was just above the surface of the slice (at an angle of approximately 30°). A heating