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Original Article

Xenon Confers Neuroprotection in Organotypic Hippocampal Slice Cultures

Fei Lan[1,2], Sheena Sodha[1], Tian-Long Wang[2], and Da-Qing Ma[1]

ABSTRACT

Background: The present study sought to establish the use of a novel model of cerebral ischaemia in which to investigate the neuroprotective effects of xenon based on organotypic hippocampal slice cultures.

Methods: Organotypic hippocampal slices, derived from 7- 10 days post- natal C57B16 mouse pups, were placed and cultured for 10-14 days on membranous inserts. Neuronal injury of cultures was provoked with media deprived with oxygen and glucose (OGD) for 2 hours. Cultures were exposed to 25% xenon or 50% xenon and 20% oxygen and 5% carbon dioxide (CO_2) balanced with nitrogen (N_2) 24 hours before OGD insult (preconditioning) for 2 hours or 75% xenon and 5% CO_2 balanced with oxygen 4 hours after OGD for 2 hours. Cell death was quantified by assessment of propidium iodide (PI) uptake before and after OGD up to 72 hours in the CA1 and the dentate gyrus (DG) of the hippocampus.

Results: Xenon preconditioning at concentrations of 25% and 50% resulted in a significant reduction in PI uptake in the CA1 region of the hippocampus compared with the control group ($P<0.01$). The effect was less pronounced in the DG region with xenon only at a concentration of 25% ($P<0.05$). Xenon 75% treatment 4 hours post-insult significantly attenuated cell death in both the DG and CA1 areas of the hippocampus.

Conclusions: This study corroborated previous data to confirm that xenon is a potent neuroprotectant, and advocated its potential for future use in specific clinical settings.

From the [1]Anaesthetics, Pain Medicine and Intensive Care, Faculty of Medicine, Imperial College London, Chelsea and Westminster Hospital, London, UK; [2]Department of Anesthesiology, Xuanwu Hospital, Capital Medical University, Beijing, China.

Correspondence to Dr. Da-Qing Ma at d.ma@imperial.ac.uk.

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Effective brain functioning relies on a continuous supply of oxygen and glucose, and thus an adequate blood supply. If this blood supply becomes compromised as it does in cerebral ischaemia, glutamate neurotoxicity mediated by a toxic influx of extracellular calcium results in hypoxic-ischaemic (HI) damage to the area downstream within minutes (1). Glutamate exerts its excitatory effects via N-methyl-D-aspartate (NMDA) receptors; excessive activation of these receptors results in excitotoxicty (2), which in turn leads to intracellular swelling and cell death (1). A decline in the oxygen and glucose supply to the brain triggers a complex cascade of cellular events, resulting in both acute neuronal death by necrosis and delayed neuronal death by apoptosis (3).

Brain injury after cerebral ischaemia develops from a complex signalling cascade that evolves in an at least partially unravelled spatiotemporal pattern. Early excitotoxicity leads to fast necrotic cell death which produces the core infarction. This is surrounded by an ischaemic penumbra. In this area, both mild excito-

toxic and inflammatory mechanisms lead to delayed cell death, characteristic of apoptosis (4).

Xenon is thought to exert its anaesthetic and analgesic action by potent non-competitive inhibition of NMDA receptors, with little effect on Gamma-butyric acid type A ($GABA_A$) receptors or non-NMDA glutamatergic receptors (5). In contrast with other anaesthetics that require anaesthetic or supra-anaesthetic doses to act as a neuroprotectant, xenon is effective at significantly subanaesthetic concentrations where anaesthesia is not required or may be detrimental (6). Crucially, it provides marked protection against injury, with half maximal inhibitory concentration (IC_{50}) in some models as low as 20-30% of an atmosphere (7).

Organotypic slice cultures provide an in vitro environment in which cell stoichiometry and the intrinsic synaptic connections found in vivo are preserved, whilst confounding factors are effectively eliminated. Thus the use of organotypic slice cultures as an in vitro model of ischaemia may provide a better correlate of the in vivo situation than dissociated neuronal culture systems which have been used previously (8, 9). The aim of the current study using organotypic slice cultures was to revalidate the xenon's neuroprotective effects.

METHODS

This study conformed to the United Kingdom Animals (Scientific Procedures) Act of 1986 and has been approved by the Home Office (London, UK). Every effort was made to diminish animal suffering and to minimise the number of animals used.

Hippocampal Slice Culture Preparation

Organotypic hippocampal slice cultures were prepared according to the methods previously described (10, 11). Seven to ten days post-natal C57B16 mouse pups were anaesthetised with 0.5% isoflurane and sacrificed by decapitation. The brains were rapidly dissected out and placed into ice-cold dissection medium (composed of 200 ml high sucrose glucose [HSG] solution, 64 ml Hanks balanced salt solution [HBSS] and 0.6 ml antibiotic-antimycotic solution [AAS]); all further manipulations were carried out in cooling conditions.

After careful removal of the meninges and excess brain tissue, 400 μm, sagittal sections were prepared on 0.5% agarose gel using a McIIIwain tissue chopper (Mickle Laboratory, Cambridge, UK). The slices were placed in dissection solution and carefully separated under a microscope (Zeiss W-PI 10X) with a cooling light (KL1500, Schott). Slices containing hippocampi were selected and placed equally spaced onto, semi-porous 0.4 μm, cell culture inserts (Falcon, Becton Dickinson labware, Millipore, Bedford, MA, USA), these were subsequently transferred into 6-well culture trays (MultiwellTM, Falcon, Becton Dickinson labware, Bedford, MA, USA) with 1.5 ml of slice culture medium per well. The slice culture growth medium consisted of 282 ml medium stock (Gibco/Life technologies, Carlsbad, CA, USA), 32 ml horse serum, supplemented with 3.2 ml glutamine and 3.2 ml AAS (Gibco/Life technologies, Carlsbad, CA, USA).

The cultures were maintained at 37℃ in an atmosphere of humidified air and 5% carbon dioxide (CO_2). The slice culture medium was changed twice a week and the slices were kept in culture for 14-16 days before experiment. After about two weeks in vitro, hippocampal slices cultured with this interface method on a semi-porous membrane, reduce from an original thickness of 400 μm to 100-150 μm. These slices can survive for several weeks in vitro with the resulting cultures retaining the characteristic cytoarchitecture of the tissue of origin as well as displaying excellent cellular differentiation (12).

Preconditioning with Xenon

Hippocampal slices were randomly assigned to one of three cohorts. Twenty-four hours prior to induction of the insult, they were placed in an airtight gas chamber (Galaxy R CO_2 incubator, Wolf laboratories) and exposed for 2 hours to various combinations of gases as follows. Cohort 1 was exposed to 25% xenon, 5% CO_2, 50% nitrogen (N_2) and 20% oxygen (O_2). Cohort 2 was exposed to 50% xenon, 5% CO_2, 25% N_2, and 20% O_2. Cohort 3 was assigned as the control group and was exposed to 5% CO_2 and air. All gases were supplied by British Oxygen Company (BOC, Surry, Guildford, UK). All above gases were delivered by pre-calculated

flow meters into air-tight incubator chamber; the gas concentrations in the chamber were stable for up to 4 hours that was confirmed in our previous studies.

Post-Injury Xenon Treatment

Hippocampal slices were randomly assigned to one of two cohorts. All cohorts underwent an insult, oxygen-glucose deprivation (OGD) as described below. Following the insult, cohort 1 was untreated but exposed with 5% CO_2 and air and acted as a positive control; cohort 2 was treated with 75% xenon, 5% CO_2 and 20% O_2, 4 hours after the injury, for 2 hours.

Induction of Cell Injury-OGD

Twenty-four hours after preconditioning with xenon, all the culture slices underwent an identical insult. OGD was achieved by combining anoxia with aglycaemia. The slices were washed twice with HEPES buffer (pH 7.4) (with the following composition: NaCl 120 mmol/l, KCl 5.4 mmol/l, $MgCl_2$ 0.8 mmol/l, $CaCl_2$ 1.8 mmol/l, D-glucose 15 mmol/l, HEPES 20 mmol/l) and once with aglycaemic balanced salt solution (BSS). The cultures (remaining in the aglycaemic BSS) were then placed in the air-tight incubator chamber and exposed to an anoxic atmosphere of 95% N_2 and 5% CO_2 for 2 hours. After the injury the culture tray was removed from the chamber, the anoxic, aglycaemic BSS was aspirated from the wells, and standard (oxygenated) slice culture media was added.

Assessment of Cell Death Using Propidium Iodide (PI)

Cell death was assessed by using the fluorescent exclusion dye PI (sigma, Poole, UK). This is a highly polar substance which is usually excluded from cells with an intact cell membrane. If however, membrane integrity is disrupted, PI enters the cells and upon binding to exposed DNA becomes highly fluorescent. PI has been shown to be non-toxic to neurons and has been used in many studies to ascertain cell viability (13). In addition, measurement of PI fluorescence intensity in slice cultures has been shown to correlate well with results from counting dead cells (14, 15). The bound form shows increased emission at 619 nm (red light) when measured with an excitation wavelength of 536 nm (green light). The PI fluorescence was visualised with an IX70 inverted fluorescence microscope (Olympus, Southall, UK), using the ×4 objective lens.

Photomicrographs of the fluorescent images were obtained using the camera (Nixon Coolpix, MDC lens) mounted on the fluorescence microscope. Images were digitised, stored and subsequently analysed using 'Image J 1.33' analysis software (Wayne Rasband, NIH).

Prior to experimental treatment (preconditioning and OGD), slices were incubated in culture medium without antibiotics supplemented with 2 μg/ml PI for 1 hour and baseline photomicrographs of PI uptake were taken. Slices were imaged prior to preconditioning and OGD and 72 hours after OGD, as preliminary studies have observed that maximum post-OGD death occurred at this time (16).

PI Image Analysis

The intensity of PI fluorescence in the CA1 and dentate gyrus (DG) subfields of the hippocampus was used as an index of cell death. For quantification, a region of interest (ROI) was selected from the image of the slice and serial measurements of PI fluorescence were made in a predefined area (manually outlining DG and CA1 separately with an interactive drawing tool) using NIH Image J software, thus cell death was followed in the same regions of each slice after simulated ischaemia. Computer analysis of each image was performed by subtracting the background PI intensity from both the baseline and 24 or 72 hours post-OGD images. Cell death was calculated from each ROI as follows: (Fexp-Fmin)/Fexp, where Fexp is the fluorescence of the test condition and Fmin is the baseline fluorescence (prior to preconditioning and OGD).

Statistical Analysis

All results were expressed as means and standard deviation. Statistical analysis of the three groups comprised a non-parametric Kruskall-Wallis (one way analysis of variance) (SPSS 12.0 for windows) comparison of group means followed by Mann-Whitney U test of each pair (and Bonferroni correction) for multiple comparisons across groups. A $P<0.05$ was considered to be statistically significant.

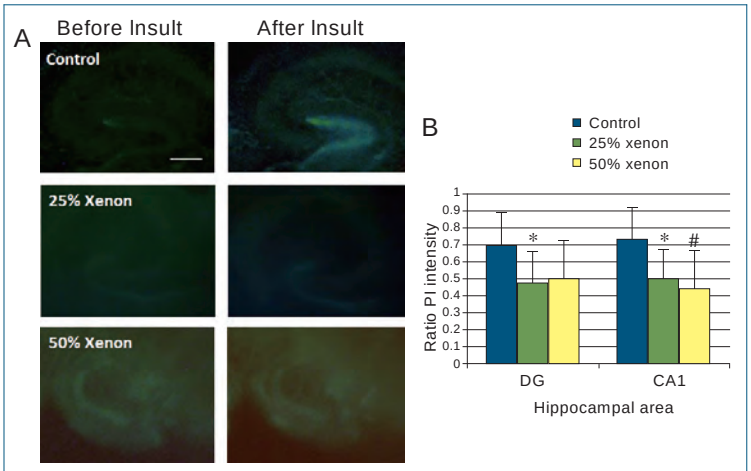

Figure 1. Xenon Given before Insult Protected Neuronal Death in the Organotypic Hippocampal Slice Culture.
Slice cultures, derived from 7-10 days post-natal C57B16 mouse pups, were exposed to 25% xenon or 50% xenon, 20% oxygen and 5% CO_2 balanced with nitrogen or air with 5% CO_2 (control) 24 hours before oxygen glucose deprivation (OGD) insult for 2 hours. Cell death was quantified by the assessment of propidium iodide (PI) uptake before and 72 hours after OGD in the CA1 and the dentate gyrus (DG) of the hippocampus, and presented as example images (A) and mean data (± SD; N=7-9) (B). *P<0.05; #P<0.01. Bar=50 µm.

RESULTS

Xenon Neuroprotection at Preconditioning Settings

Preconditioning with xenon 25% and 50% significantly reduced cell death in the CA1 area of the hippocampus as evidenced by a significant reduction in PI uptake in the CA1 region of the hippocampus compared with the control group (N=7-9, P<0.05 or P<0.01), respectively. The preconditioning effect was less pronounced in the DG region with results only showing significant reduction of cell death in the xenon 25% treatment group (P<0.045) (Figure 1).

Xenon Post-Injury Neuroprotection

Xenon treatment for 2 hours after an insult of OGD significantly reduced PI uptake, represented by a significant attenuation of cell death in the DG and CA1 regions of the hippocampus assessed at both 48 and 72 hours after injury (N=3, Figure 2).

DISCUSSION

Excitotoxicity, through over activation of NMDA receptors (2), is thought to play a key role in the development of neuronal degeneration following a HI insult (1, 17). Xenon, an NMDA receptor antagonist, has been shown to be neuroprotective in several paradigms of neuronal injury in both in vitro and in vivo models (18-21). In vitro models, it offers the ability to strictly control the microenvironment and easily quantify cell death; however the major disadvantage is a loss of synaptic connections. Whilst in vivo models in contrast, it represents an intact multicellular system in which intrinsic synaptic connectivity is maintained, these however are subject to systemic vascular influences which represent difficult factors to control and standardise.

Organotypic slice cultures offer several advantages, which make them an attractive alternative for investigating ischaemic preconditioning (IPC) by xenon. They survive for weeks and can therefore be observed for long periods of time (22). Furthermore, the cells continue to differentiate and mature, resulting in a preparation where the phenotypic morphology of neurons and the tissue organisation are very similar to those in vivo (10, 22). Consequently the present study sought to establish this model as a novel in vitro method in which to assess the neuroprotective efficacy of xenon.

This study has shown that preconditioning neuroprotection imparted by xenon can be replicated in the organotypic hippocampal slice culture model, particularly in the most vulnerable CA1 region. In addition, post-injury treatment with xenon 2 hours after a HI insult was also shown to be neuroprotective; again this effect was most marked in the CA1 region of the hippocampus. The neuroprotection afforded by xenon preconditioning and post-injury treatment in the organotypic hippocampal slice model, corroborated previous data from a variety of other in vivo and in vitro studies (18-21), hence validating the use of this model for further studies of xenon neuroprotection.

IPC was first described in cardiac tissue where a sublethal ischaemic insult rendered the tissue tolerant to a subsequent insult (23). Similarly a tolerant state can be induced by pharmacological agents (pharmacological preconditioning) including xenon as shown in the present study. The mechanisms underlying these phenomena are

not fully understood. However it is thought that both ischaemic and pharmacological preconditioning trigger complex signalling cascades and induce the intracellular transduction of protein kinase C (PKC) and the nuclear transcription factor cAMP response element binding protein (CREB) (24, 25). Activation of PKC appears to be a crucial intermediate in preconditioning since pharmacological inhibition of it abolishes protection (26). In particular, PKC is an essential component of the signalling pathways associated with preserving cellular viability (27). In addition, NMDA antagonists, increase PKC activity (28) and xenon as an NMDA antagonist, is thought to induce cardioprotection by pharmacological preconditioning via activation of the PKC isoform PKC-ε (25). PKC is responsible for the activation of the nuclear transcription factor CREB by phosphorylation.

Xenon preconditioning may activate a putative CREB-mediated transcription system which is thought to play a critical role in neuronal survival via the expression of various neuroprotective proteins including brain derived growth factor (BDNF) and the anti-apoptotic protein Bcl-2 (24). Previous studies have shown that CREB plays a key role in cell survival (29, 30) and is in particular relevance to the model being used in the present study, the selectively vulnerable CA1 pyramidal cells, which underwent delayed neuronal death following mild HI, showed a loss of CREB and phosphorylated CREB (pCREB) following HI (31). In contrast the resistant dentate granule cells produced a bimodal increase in pCREB (31). Furthermore a transient increase in CREB activation after preconditioning ischaemia is thought to play a key role in induction of ischaemic tolerance in the CA1 neurons (24).

HI brain damage is an evolving process, which begins during the insult and extends into the recovery period. Tissue injury takes the form of either selective neuronal necrosis or infarction. When infarction occurs, the penumbral region consists of neurons undergoing either necrosis or apoptosis, and it is the penumbral region that appears most amenable to reversal of cellular injury through therapeutic intervention (32).

The therapeutic window is the interval after resuscitation from hypoxic- ischaemia, during which an intervention might be efficacious in attenuating the severity of the ultimate brain injury. Due to the slow process of neuronal necrosis and apoptosis in adults, the therapeutic window can extend for several hours; however the process of cellular destruction is much more rapid in perinatal subjects, thus shortening the therapeutic window (32). Therefore, interventions implemented later than two hours after the termination of the HI injury are unlikely to be efficacious in reducing the severity of the injury (32). The data presented here, suggested that xenon, particularly in one of the most vulnerable regions of the brain can exert its neuroprotective effects up to 4 hours after injury. If treatment can be accomplished within this window of opportunity, the putative effects of xenon neuroprotection may be realised.

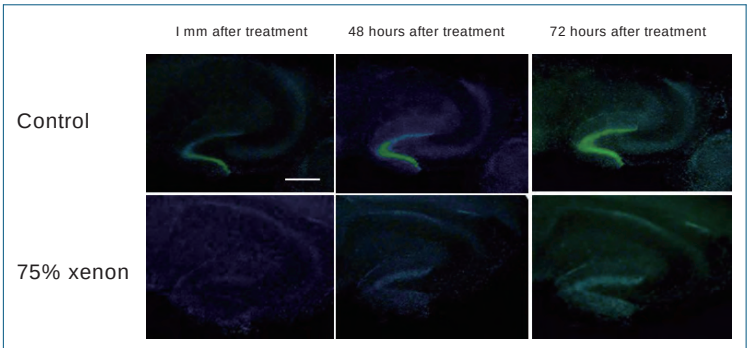

Figure 2. Xenon Given after Insult Protected Neuronal Death in the Organotypic Hippocampal Slice Culture.
Slice cultures, derived from 7- 10 days post- natal C57B16 mouse pups, were injured by media being deprived with oxygen and glucose (OGD) for 2 hours. Four hours after insult, they were treated with 75% xenon, 20% oxygen and 5% CO_2 or air with 5% CO_2 (control) for 2 hours. Cell death was assessed by propidium iodide (PI) uptake before and immediate (I mm), 24 or 72 hours after treatment in the CA1 and the dentate gyrus (DG) of the hippocampus. Images were the representatives from three independent experiments (N=3). Bar=50 μm.

Our data showed a partial region selective neuroprotective effect in the pyramidal cell layer containing CA1 neurons, illustrated by neurodegeneration being attenuated by 25% and 50% xenon preconditioning and by xenon treatment 4 hours after injury. It is well known that ischaemia selectively damages hippocampal subregions, CA1 is the most vulnerable, while CA3 and the DG are relatively resistant to ischaemic insult (CA1>CA3>DG) (33, 34).

Understanding the molecular and cellular ba-

sis of this vulnerability is of significance in interpreting our results. One explanation is due to the distribution of glutamate receptors which also shows regional specificity, with the highest density of NMDA receptors being found in the CA1 region whereas greater expression of α-amino-3-hydroxy-methyl-4-isoxazole propionic acid (AMPA) and metabotropic glutamate receptors being found in the CA3 pyramidal and the DG neurons (35, 36). These findings indicate that intrinsic differences in the expression of glutamate receptors linked to calcium influx may account for selective neuronal vulnerability. In addition, previous data indicated that NMDA receptors may be involved when the severity of the insult is above a certain threshold whereas AMPA receptors mediate a more protracted insult at lower intensities, suggesting that the events leading to granular cell death in the DG are likely to be different from those in the CA1 region. Conversely, our data did show some neuroprotection in the DG region, which may be explained by the fact that hypoxia-induced neuronal damage in the DG involves in part, the activation of NMDA receptors (37). Hypoxic-ischaemia triggers a substantial increase in membrane permeability. A putative factor contributing to neuronal injury is the consequent profound depolarisation of neurons. Simultaneous recordings of the extracellular potential in CA1 and DG during hypoxia reveal that CA1 always depolarises before DG (33) which may also explain the differences in injury of the two regions.

In addition, a combination of several other factors is likely to account for the vulnerability of the pyramidal neurons in CA1 and the relative resilience of DG neurons to HI injury. The neurons in the CA1 are smaller and more tightly packed than those in the CA3 and DG regions. As a consequence, even identical increases in HI-induced ion conductances may produce larger changes Nernst and transmembrane potentials in CA1 neurons when compared to the other neurons, thereby leading to increased depolarisation, associated swelling and neurodegeneration in CA1 neurons (38).

In conclusion, the data reported here showed robust neuroprotection against OGD in organotypic hippocampal slices following preconditioning or post-injury treatment with xenon. The neuroprotection afforded by xenon in this model corroborates findings of in vivo and in vitro studies, validating the slice culture model for future studies of xenon neuroprotection.

The authors have no potential conflicts of interest for this work.

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