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# Neuroprotective effects of minocycline on double-stranded RNA-induced neurotoxicity in cultured cortical neurons

## Key Messages

1. Minocycline, memantine, and glycoconjugate were assessed for their ability to protect cultured primary cortical neurons against double-stranded RNA-induced neurotoxicity.
2. Minocycline but not memantine or glycoconjugate protected cultured cells and warrants further investigation.

## Introduction

Japanese encephalitis virus (JEV) is an RNA virus spread by infected mosquitoes. Patients may develop symptoms such as fever, chills, tiredness, headache, nausea, vomiting, confusion, and agitation. The disease can lead to serious infection in the brain (encephalitis), and in about 30% of patients severe brain damage ensues. There is no specific treatment for the disease, and the role of medication is to relieve severe symptoms.

Few studies have reported on protection of neurons subsequent to viral infection. We hypothesise that neuroprotective drugs can be used to treat Japanese encephalitis patients and safeguard neurons. We tested some neuroprotective drugs from western and Chinese medicine (minocycline, memantine, and glycoconjugate) for treating Japanese encephalitis. The double-stranded RNA analogue, pIpC, is a commonly used analogue mimicking the consequence of virus infection. Cell cultures of neurons were used to investigate whether these three drugs could attenuate any direct toxic effects of pIpC toward neurons. The underlying mechanisms of the neuroprotective effects were also investigated.

Minocycline is a semi-synthetic tetracycline derivative exhibiting biological effects of neuroprotection in different types of neurological disorders including Parkinson's disease, amyotrophic lateral sclerosis, spinal cord injury, and cerebral ischaemia.<sup>1,2</sup> Such neuroprotective effects are largely attributed to the inhibition of microglia and production of inflammatory factors. Minocycline can even attenuate the excitotoxicity of glutamate in the cerebrospinal fluid from amyotrophic lateral sclerosis patients. The pathology of Japanese encephalitis entails severe cerebral inflammation, which may be minimised by minocycline and so protect neurons.

Memantine is a non-competitive NMDA open channel blocker approved by the US Food and Drug Administration for neuroprotection in Alzheimer's disease.<sup>3</sup> Glutamate is usually released from injured or energy deficient neurons in many neurological disorders. It can further depolarise other healthy neurons and the potassium ions released from such depolarised neurons can enhance cerebral inflammation aggravating neuronal death.<sup>4</sup> Memantine may serve as neuroprotective agent to minimise secondary brain damage exerted by glutamate.

Glycoconjugate is extracted from the anti-ageing Chinese medicine *Lycium barbarum*. It is neuroprotective against the toxicity exerted from  $\beta$ -amyloid protein, a toxin found in the Alzheimer's disease, as well as retinal ganglion cells in an animal model of glaucoma.<sup>5</sup> Its cytoprotective properties may extend to other neurodegenerative diseases and even Japanese encephalitis or its dsRNA-elicited neurotoxicity.

## Methods

This study was conducted from 1 June 2006 to 31 May 2008. Protection of

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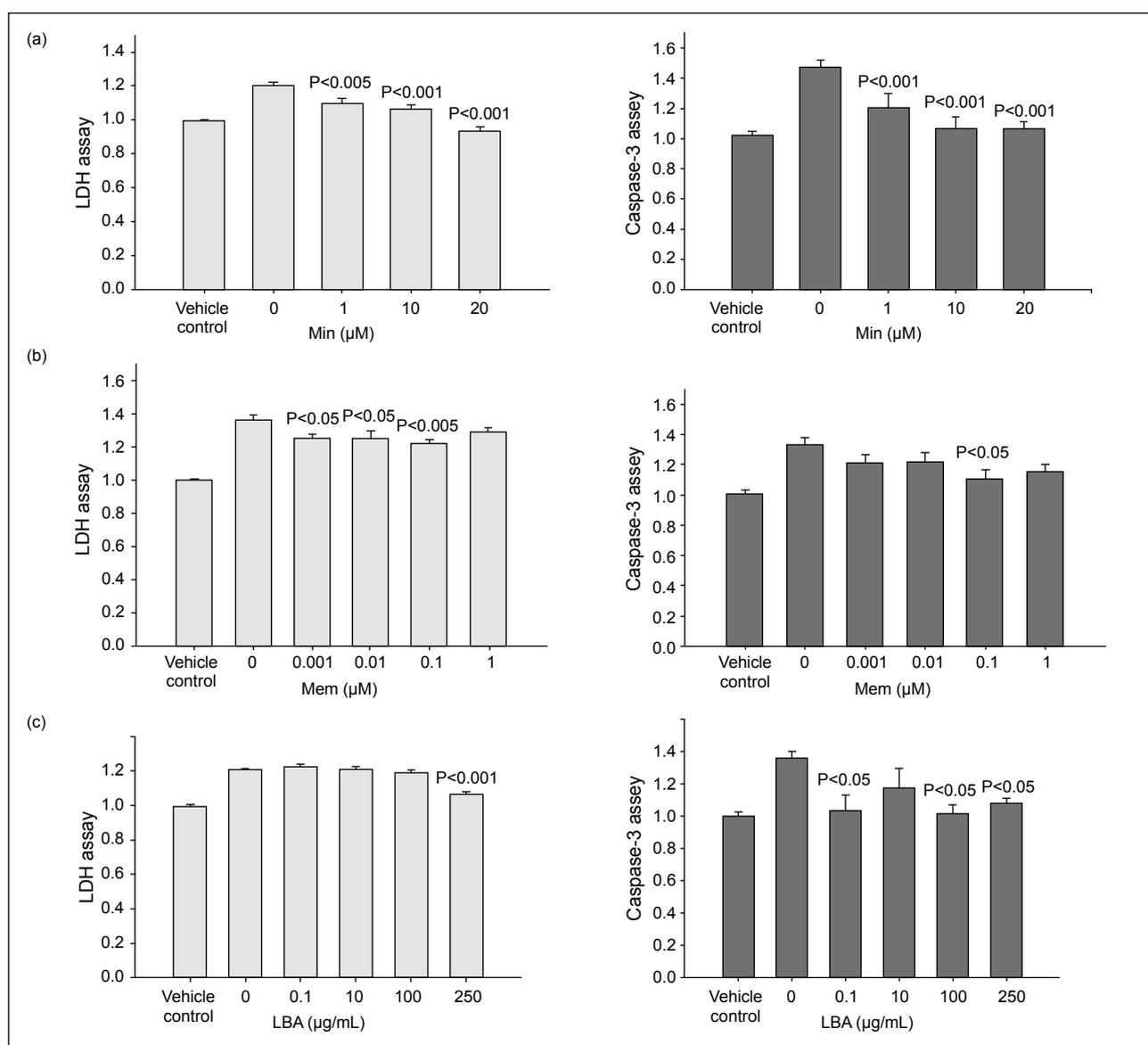
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neurons from the direct toxic effects of pIpC by the three drugs (minocycline, memantine, and glycoconjugate) was examined, using primary cultures of cortical neurons and human neuroblastoma SH-SY5Y cells. Primary cortical neurons were prepared from embryonic day 17 Sprague-Dawley rats (Laboratory Animal Unit, The University of Hong Kong).<sup>6</sup> In brief, cerebral cortices were mechanically dissociated in phosphate-buffered saline with glucose (18 mM). Cells were seeded onto six-well ( $0.8 \times 10^6$  cells/well) or 12-well plates ( $1.0 \times 10^5$  cells/well) pre-coated with poly-L-lysine (25  $\mu\text{g}/\text{mL}$ ). The culture medium consisted of Neurobasal Medium supplemented with 2% B-27 supplement, L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (50  $\mu\text{g}/\text{mL}$ ), and 2-mercaptoethanol. Neurons were maintained at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  and were cultured for 7 days prior to treatment.

To evaluate whether minocycline exerted neuroprotective effects, neurons were pretreated with different dosages of potential drugs for 1 h prior to exposure of pIpC. Neurons were challenged by 20  $\mu\text{g}/\text{mL}$  pIpC with FuGENE 6 reagent. After 48 h, neurons were harvested for assay. General toxicity was determined by measuring lactate dehydrogenase (LDH) activity released into culture medium. Apoptosis was assessed by determining caspase-3 activity. TUNEL and DAPI nuclear staining were used to count the number of apoptotic bodies.

All results obtained were expressed as mean  $\pm$  standard error from at least three independent experiments. Significant difference between groups was determined by one-way ANOVA, followed by Student-Newman-Keuls as a post hoc test.



**Fig. Effects of minocycline, memantine, and glycoconjugate on pIpC-induced neurotoxicity**

Cultured cortical neurons were treated with (a) minocycline, (b) memantine, or (c) glycoconjugate at different dosages 1 h prior to the exposure of 20  $\mu\text{g}/\text{mL}$  pIpC. Culture medium was taken for lactate dehydrogenase (LDH) assay and cells were harvested for caspase-3 assay 24 h after treatment

## Results and discussion

pIpC exhibits direct neurotoxicity. Increased levels of dsRNA produced from JEV can be toxic to cortical neurons. Dose-dependent and time-dependent experiments were performed using primary cultures of cortical neurons and SH-SY5Y cells as two different models.

Exposure of neurons to 20 µg/mL pIpC resulted in 1.23±0.02-fold increase in the release of LDH when compared to the vehicle control. At higher dosage, the release of LDH decreased to 1.15±0.03-fold. Thus, 20 µg/mL pIpC was chosen for the subsequent experiments. As pIpC could trigger its neurotoxicity via activating caspase-3, a colorimetric caspase-3-like activity assay was carried out for different dosages of pIpC. Exposure of neurons to 20 µg/mL pIpC significantly increased the activity of caspase-3 to 1.33±0.06-fold.

Apart from biochemical assays, neurites were damaged by pIpC, as shown by their morphology. Under a phase contrast microscope, the cell bodies of cultured neurons in the control group appeared round and dark with a network of process. After exposure to pIpC, cultured neurons were damaged and showed fragmented neurites.

Exposure of neurons to 20 µg/mL pIpC for 48 h resulted in 1.2±0.03-fold increase in the release of LDH when compared to the vehicle control. There was a slight decrease of LDH release at 72 h. The colorimetric caspase-3-like activity assay was also carried out after different durations of exposure to pIpC. Exposure of neurons to 20 µg/mL pIpC for 48 h significantly increased the activity of caspase-3 to 1.43±0.03-fold. Similar to the results of the LDH release assay, caspase-3-like activity decreased at 72 h. As the maximum increase of LDH release and caspase-3 was at 48 h, this duration was chosen for the subsequent experiments.

Exposure of human dopaminergic SH-SY5Y cell neurons to 20 µg/mL pIpC resulted in 1.50±0.09-fold increase of LDH release when compared to that of the vehicle control. Exposure of neurons to 20 µg/mL pIpC significantly increased the activity of caspase-3 to 1.29±0.03-fold. These results provided further proof of the toxicity of pIpC, using SH-SY5Y cells as second cell culture model.

pIpC induced neuronal apoptosis can be confirmed by recognised methods. Treatment with pIpC for 48 h

increased the number of apoptotic bodies (typical nuclear condensation and fragmentation) as indicated by the increased number of TUNEL-positive neurons. TUNEL assay relied on the detection of fragmented DNA strands. In control cultures treated with vehicle, only few TUNEL-positive cells were observed.

Minocycline exhibited the best neuroprotective effect on neurons based on the LDH assay; 10 or 20 µM exerted similar neuroprotection on caspase-3 activity (Fig a). We could not further increase the concentrations of minocycline as this would exceed the normal dosage for treatment and exert side-effect in patients. Memantine significantly reduced the toxicity of pIpC (both LDH release and caspase-3 activity), but its neuroprotective effect was marginal. In contrast to memantine, minocycline elicited significant neuroprotective effects on cortical neurons against direct pIpC toxicity (Fig b). Glycoconjugates from *L barbarum* elicited cytoprotective effects; *L barbarum* attenuated caspase-3 activity triggered by pIpC (Fig c). Nonetheless, only the highest dosage of *L barbarum* could inhibit LDH release triggered by pIpC.

Of the three different compounds, minocycline exhibited significant neuroprotective effects against direct pIpC neurotoxicity and warrants further investigation.

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