# Oral feeding of minocycline attenuates glial activation and reductions of tau and drebrin in response to systemically injected cytokines

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## KEY MESSAGES

1. The use of minocycline to regulate the neuropathological effects of systemically administered cytokines was studied.

2. Systemic cytokines cause glial activation, and reduce tau and drebrin in the hippocampus. Minocycline reverses these effects of cytokines, suggesting that it may be neuroprotective against cytokine storm.

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## Introduction

Influenza leads to complications not only in the respiratory tract, but also within the central nervous system (CNS). These influenza-related neurological complications (INCs) include encephalopathy, encephalitis, and seizures, and affect up to 20% of hospitalised children diagnosed with influenza in Hong Kong. Febrile seizure is the most common type of INC.

One process that regulates INCs is the induction of cytokine storm during influenza.[1] A cytokine storm is an over-reactive immune response characterised by drastic elevations of many systemic inflammatory mediators. It has been suggested as the cause of multiple organ failure and high mortality during the 1918 and 2009 influenza pandemics. Infection with influenza viruses has been reported to hyper-induce an array of cytokines, ie IL-1β, IL-6, and TNF-α, IFN-α, and IFN-γ, and chemokines, ie IL-8, MIG, IP-10, MCP-1, and MIP-1α,[2] all of which can potentially influence the brain to affect mood, behaviour, and cognition.[3] Both humoral and/or neural routes are believed to participate in relaying systemic cytokine responses to the brain.[3] We hypothesise that in the course of influenza infection, the generation of cytokine storm can lead to CNS neural and glial changes that may collectively mediate INCs.

Minocycline is a Food and Drug Administration–approved, second-generation, semi-synthetic tetracycline analogue that exerts antimicrobial, anti-inflammatory, and anti-apoptotic properties. It is well tolerated by humans in the treatment of acne vulgaris, rheumatoid arthritis, and certain sexually transmitted diseases. Moreover, it is a strong inhibitor of microglial activity and readily penetrates the blood-brain-barrier. Its efficacy in treating various neurodegenerative disorders has been tested in animal models and clinical trials. Its neuroprotective properties have been reported in patients with cerebral ischaemia, spinal cord injury, multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, and Alzheimer's disease. Nonetheless, conflicting results have also been reported. We aimed to investigate whether minocycline protects against INCs by focusing on (1) the impacts of cytokine storm, which develops during influenza infection, on the brain, and (2) whether minocycline can attenuate or even reverse these changes.

## Methods

This study was conducted from October 2009 to March 2012. We injected a mixture of cytokines (IL-1β, TNF-α, IL-6, IL-8, IFN-α, MCP-1, MIP-1α) intraperitoneally into young male Sprague-Dawley rats to serve as a model of cytokine storm. Minocycline (25 mg/rat) was fed immediately before the injection, and the rats were sacrificed and their brains collected 1 day later to assess whether minocycline could modulate the changes in the hippocampus induced by mixed cytokines.

### Reagents and test substances

All recombinant cytokines, ie rat IL-1β/IL-1F2, rat TNF-α/TNFSF1A, rat IL-6, rat IFN-α, human

CXCL8/IL-8, rat CCL2/JE/MCP-1, and human CCL3/MIP-1α were purchased from R&D Systems (Minneapolis [MN], USA). Minocycline hydrochloride, mouse monoclonal anti-GFAP and monoclonal anti-GAPDH antibodies, and rabbit polyclonal anti-drebrin antibody were from Sigma-Aldrich (St Louis [MO], USA). Rabbit polyclonal antibodies for p-Tau [pS396] and p-Tau [pT231], and Alexa Fluor-488 goat anti-rabbit IgG and Alexa Fluor-568 anti-mouse IgG antibodies were obtained from Invitrogen (Carlsbad [CA], USA). Rabbit polyclonal anti-Iba-1 antibody and mouse monoclonal anti-Tau-5 antibody were purchased from Wako Pure Chemical Industries (Osaka, Japan) and BD PharMingen (California, USA), respectively. Horseradish peroxidase–conjugated goat anti-rabbit and goat anti-mouse antibodies, and anti-fade fluorescent mounting medium were from DAKO (Glostrup, Denmark). The protein content assay kit and polyvinylidene fluoride membrane were obtained from Bio-Rad (Hercules [CA], USA). Enhanced chemiluminescence (ECL) detection kit was from Amersham (Buckinghamshire, UK).

## Animal procedures

All animal procedures were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Briefly, male Sprague-Dawley rats (~250 g) were purchased from the Laboratory Animal Unit of the LKS Faculty of Medicine in the University of Hong Kong and housed with three rats per cage. They were maintained in a temperature-controlled room with a 12-hour light/dark cycle, and were allowed to acclimatise for 3 days before the start of the experiment. On the day of the experiment, the rats were randomly divided into four groups: control (n=6), mixed cytokines alone (n=5), minocycline alone (n=5), and mixed cytokines plus minocycline (n=5). All feeding and injection procedures were performed at 11:00 am. Minocycline hydrochloride was dissolved in distilled water to a concentration of 50 mg/mL. Rats were then orally fed with either 500 µL water or 500 µL of the minocycline solution (ie 25 mg/rat of minocycline), immediately followed by an intraperitoneal injection of 500 µL phosphate buffered saline (PBS) or mixed cytokines (500 ng/kg IL-1β, 100 ng/kg TNF-α, 500 ng/kg IL-6, 500 ng/kg IL-8, 500 IU/kg IFN-α, 20 ng/kg MCP-1, and 10 ng/kg MIP-1α, in PBS). Rats were returned to their home cage, and 24 hours later were sacrificed by an overdose of sodium pentobarbital. They were perfused with saline for 3 minutes, and brains were quickly removed and microdissected on ice. The right brain tissue was snap frozen in liquid nitrogen and stored at -80ºC for western blot analysis. The left brain tissue was fixed in 4% paraformaldehyde at 4ºC for 3 days before tissue processing.

## Tissue processing, immunofluorescence staining, and imaging

After fixation, brain tissue was dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin. It was cut into 6-micron coronal brain sections, dewaxed, and rehydrated. Antigen retrieval was performed by heating in 0.01 M citrate buffer (pH 6.0) with 0.1% Tween-20 at 95ºC for 15 minutes. Subsequently, sections were washed in PBS. Non-specific binding between antibodies and tissues was blocked by 10% normal goat serum in PBS for 1.5 hours. Sections were incubated with primary antibodies for Tau-5 (1:400), drebrin (1:400), Iba-1 (1:500), and glial fibrillary acidic protein (GFAP) (1:500) at 4ºC overnight, washed with PBS, then Alexa Fluor-488 or -568 added for 1.5 hours. Subsequently, sections were washed with PBS, stained with 4',6-diamidino-2-phenylindole (DAPI) for 15 minutes, washed again, and mounted with anti-fade fluorescent mounting medium. Finally, sections were examined using confocal microscopy (Figs 1 & 2) and under a Zeiss Axioplan 2 microscope with a 20X or 40X objective (Figs 3 & 4).

## Western blot analysis

Brain tissue was homogenised in ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM NaCl, 20 mM $Na_4P_2O_7$, 2 mM $Na_3VO_4$, 1% Triton-X-100, 10% glycerol, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail and phosphatase inhibitor cocktail, centrifuged at 14 000 g at 4ºC for 30 minutes, and the supernatant collected. The protein in the cell lysates was quantified using the protein content assay kit. Afterwards, samples from the same group (n=5/6) were mixed, subjected to SDS 10% polyacrylamide gel electrophoresis at 140V, and transferred to polyvinylidene fluoride membranes. Non-specific binding on the membranes was blocked by 5% non-fat milk in TBST (TBS-containing 0.1% Tween-20) for 1 hour. Primary antibodies were diluted in TBST and incubated with the membrane as follows: p-Tau [pT231] (1:3000), p-Tau [pS396] (1:4000), Tau-5 (1:2000), drebrin (1:4000), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:20 000). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour and subsequently developed using the ECL western blotting detection kit. Densitometry measurements were made using Image J software (National Institutes of Health, USA). The band intensities were normalised against GAPDH and results were expressed as fold of control.

## Statistical analysis

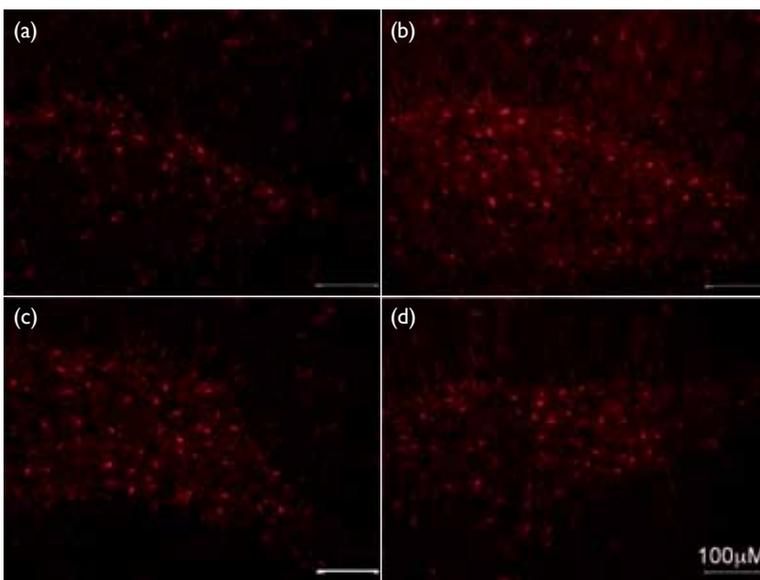Normalised band intensities were analysed by one-way ANOVA, followed by Student-Newman-

FIG 1. Fluorescent images of GFAP positive astrocytes 1 day post-treatment, in the dentate gyrus of rats treated with (a) control, (b) mixed cytokines, (c) 25 mg minocycline, and (d) 25 mg minocycline plus mixed cytokines. Minocycline attenuates the increase in GFAP immunoreactivity by mixed cytokines.
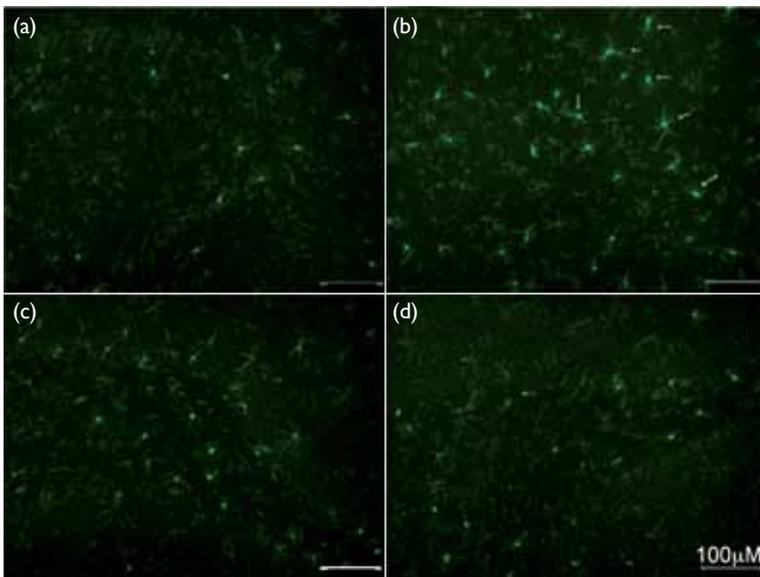


FIG 2. Fluorescent images of Iba-1 positive microglia 1 day post-treatment, in the dentate gyrus of rats treated with (a) control, (b) mixed cytokines, (c) 25 mg minocycline, and (d) 25 mg minocycline plus mixed cytokines. Arrows in (b) indicate activated microglia, ie hypertrophy of cell body, thickening of processes, in the mixed cytokine-treated rats. Minocycline reduces activation of microglia by mixed cytokines.

Keuls test using SigmaStat (Jandel Scientific [CA], USA). Results were considered to be significantly different if P<0.05.
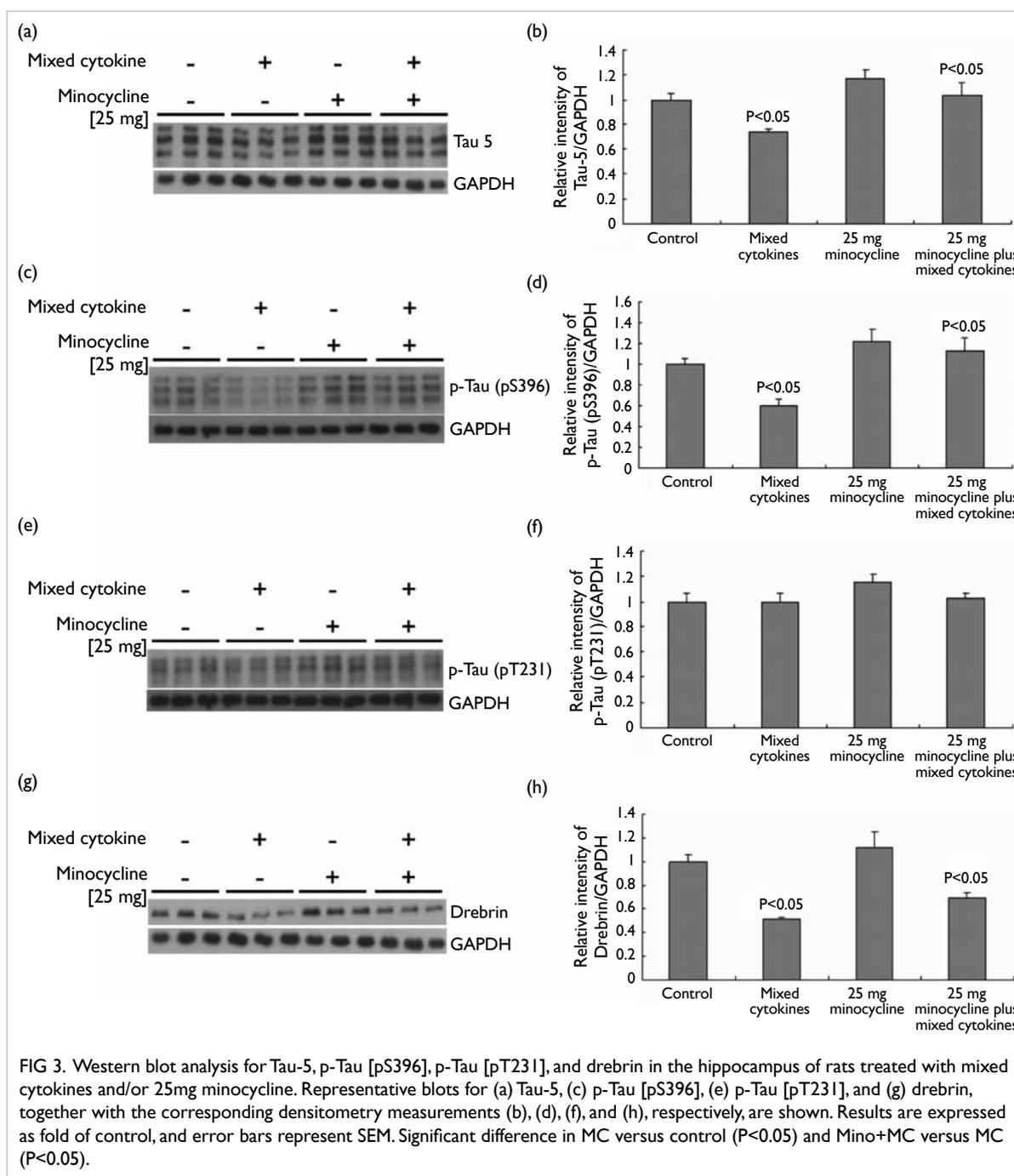
# Results

Mixed cytokines led to activation of microglia and astrocytes at the dentate gyrus, and treatment with minocycline appeared to reduce these phenomena. Moreover, mixed cytokines reduced both protein expression level and immunoreactivity of the cytoskeleton-associated proteins tau and drebrin, and decreased the phosphorylation of tau at serine-396 but precipitated no change at threonine-231. Minocycline significantly reversed, if not totally, the decrements in total tau, p-Tau (pS396), and drebrin by mixed cytokines. These results suggest that oral administration of minocycline can likely reverse the impact of cytokine storm on glia and the neuronal cytoskeleton in the brain. Future studies are encouraged to verify whether minocycline will be useful in alleviating INCs.

## Minocycline suppressed mixed cytokine-mediated glial activation

Systemic injection of mixed cytokines led to signs of astrocyte activation (ie increased GFAP immunoreactivity and astrocyte number versus the control) and microglial activation (ie cell body hypertrophy, and thickening of processes versus the control) within the dentate gyrus (Fig 1). Minocycline alone did not induce any observable differences in cell morphology or cell numbers of astrocytes and microglia (Figs 1 and 2). When rats were co-treated with mixed cytokines and minocycline, there was decreased astrocyte and microglia activation compared with the mixed cytokine group (Figs 1 and 2).

## Minocycline reversed the reduction in Tau-5 and p-Tau [pS396] immunoreactivities caused by mixed cytokines

We performed western blot analysis (Fig 3) and immunofluorescence staining (Fig 4) for Tau-5, which serves as an index for total Tau. Mixed cytokines reduced Tau-5 protein immunoreactivity to 0.74±0.02-fold (P<0.05 versus control group). Although minocycline alone did not cause any change in Tau-5 protein immunoreactivity, eg 1.17±0.07-fold, it did attenuate the reduction in Tau-5 mediated by mixed cytokines to 1.03±0.1-fold (P<0.05 versus mixed cytokines group). Such changes in protein expression were accompanied by a similar change in Tau-5 immunoreactivity in the CA3 region. Mixed cytokines reduced Tau-5 immunoreactivity compared with that of the control. Minocycline alone did not appear to affect Tau-5 immunoreactivity, but did reduce the drop in immunoreactivity when it was co-treated with mixed cytokines. Moreover, it should be noted that in both the minocycline alone group and the co-treatment group, there was increased Tau-5 immunoreactivity

FIG 3. Western blot analysis for Tau-5, p-Tau [pS396], p-Tau [pT231], and drebrin in the hippocampus of rats treated with mixed cytokines and/or 25mg minocycline. Representative blots for (a) Tau-5, (c) p-Tau [pS396], (e) p-Tau [pT231], and (g) drebrin, together with the corresponding densitometry measurements (b), (d), (f), and (h), respectively, are shown. Results are expressed as fold of control, and error bars represent SEM. Significant difference in MC versus control (P<0.05) and Mino+MC versus MC (P<0.05).

in the cell bodies of parenchymal cells.

We further verified if there was any change in the phosphorylation of Tau at serine-396 and threonine-231. Similar to the trend in Tau-5, mixed cytokines decreased the p-Tau [pS396] level to 0.60±0.07-fold (P<0.05 versus control group). Minocycline alone did not elicit any effect, ie 1.22±0.11-fold, but could reverse the reduction in p-Tau [pS396] level when it was co-treated with mixed cytokines (1.13±0.13-fold, P<0.05 versus mixed cytokines group). There was no significant

difference in the p-Tau (pT231) level amongst all groups.

## Minocycline attenuated the decrease in drebrin protein expression and immunoreactivity induced by mixed cytokines

Mixed cytokines led to a significant decrease in the expression of drebrin (Fig 3) to 0.51±0.02-fold (P<0.01 versus control group). Minocycline *per se* did not change expression of drebrin, ie 1.12±0.12 fold. However, co-treatment of mixed cytokines
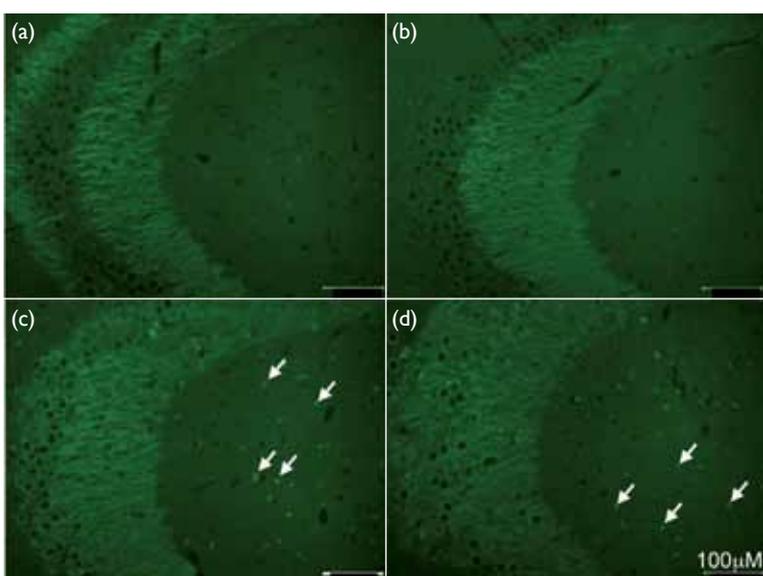
FIG 4. Fluorescent images of Tau-5 immunofluorescence staining, 1 day post-treatment, in the CA3 region of rats treated with (a) control, (b) mixed cytokines, (c) 25 mg minocycline, and (d) 25 mg minocycline plus mixed cytokines. Arrows indicate increased number of cells having Tau-5 immunoreactivity in the cell body of the Mino group and MC+Mino group. Minocycline reverses the drop of Tau-5 immunoreactivity by mixed cytokines.
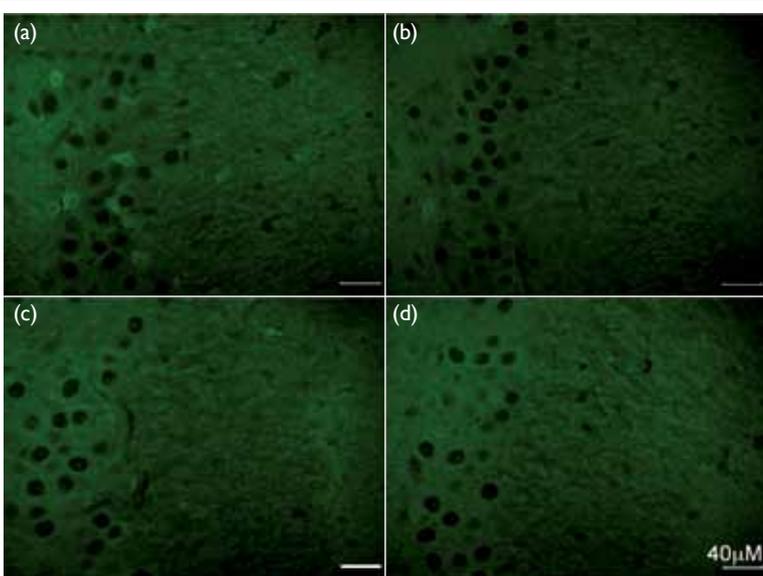


FIG 5. Fluorescent images of drebrin immunofluorescence staining, 1 day post-treatment, in the CA3 region of rats treated with (a) control, (b) mixed cytokines, (c) 25 mg minocycline, and (d) 25 mg minocycline plus mixed cytokines. Minocycline hinders the decrease in drebrin puncta number and immunoreactivity by mixed cytokines.

with minocycline could partially suppress the reduction of drebrin protein triggered by mixed cytokines to $0.69\pm0.05$-fold (P<0.05 versus mixed cytokines group). Such changes in drebrin protein expression were associated with a similar pattern of immunofluorescent staining at the CA3 region (Fig 5). Mixed cytokines reduced the abundance of drebrin puncta and its immunoreactivity. Minocycline alone did not seem to alter these two parameters. When co-treated with mixed cytokines, minocycline abolished the drop in the abundance of drebrin puncta and its immunoreactivity by mixed cytokines.

## Discussion

In order to mimic cytokine storm, we injected intraperitoneally a mixture of cytokines comprised of IL-1β, IL-6, and TNF-α, IFN-α, IL-8, MCP-1, and MIP-1α. We chose to inject these cytokines because they have demonstrated an increase in blood and in nasal fluids during influenza infection. We also used nanogram levels of cytokines because in the presence of influenza infection, these cytokines exist at concentrations of pg/ml in blood.[2] Based on the assumption that their *in-vivo* half-life ranges from minutes to hours, these cytokines would be able to last for hours *in-vivo* before they return to basal levels. We focused on any change that occurred within the hippocampus because damage in this region has been frequently reported in several models of seizure,[4] ie the most common neurological complication in patients with influenza.

Neuroinflammation is a common phenomenon that occurs in acute neurological complications, eg epilepsy, encephalitis, and encephalopathy, and in chronic neurodegenerative diseases, eg Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Activation of astrocytes and microglia leads to production of inflammatory mediators such as cytokines, chemokines, and prostaglandins. These inflammatory mediators not only alter neurotransmitter, synaptic plasticity, neurotrophin signalling, and the cytoskeleton, but also influence mood, behaviour, and cognition.[3] We showed that systemic injection of mixed cytokines induced signs of astrocytic and microglial activation within the dentate gyrus. Oral feeding of minocycline attenuated this activation. These results imply that cytokine storm may cause glial activation, and may be inhibited by minocycline.

Tau protein associates with microtubules and stabilises them. In mature neurons, tau protein is predominately located along axons and is required for axonal growth and transport.[5] The binding of tau protein to microtubules is in turn regulated by its phosphorylation. We asked whether mixed cytokines could alter the level of total tau protein and/or its phosphorylation at the residues serine 396 and threonine 231, and if yes, could minocycline hinder such changes? To our surprise, mixed cytokines decreased the amount of total tau, as revealed by decreases in Tau-5 immunoreactivity and protein

expression. Minocycline treatment restored Tau-5 protein immunoreactivty and expression. Yet, it is notable that minocycline treatment, ie in both the minocycline group and co-treatment group, appeared to increase Tau-5 immunoreactivity in the cell bodies of parenchymal cells. Such disorganisation of tau protein into the cell body by minocycline was rather unexpected. Although the mis-sorting of tau protein into the cell body of neurons has been previously reported with beta-amyloid treatment, and it is thought to be undesirable,[5] we are not sure whether this indicates a negative impact of minocycline in our model, and have not validated the cell type of these cells. In addition, mixed cytokines reduced the p-Tau [pS396] level and minocycline was able to attenuate it. Based on these results, we suggest that in our model mixed cytokines may have affected the dynamics of tau protein, and minocycline may have hindered these effects.

Apart from tau protein, we also studied whether mixed cytokines altered drebrin, which is a binding protein of F-actin, the major cytoskeleton in dendritic spines.[6] Drebrin regulates spine plasticity: overexpression of drebrin leads to elongation of dendritic spines in mature neurons,[7] whereas down-regulation of drebrin expression by antisense oligonucleotides reduces the width and density of filapodia-spines in developing neurons.[8] Our results showed that mixed cytokines decreased both drebrin immunoreactivity at the CA3 region and protein expression. Minocycline was able to attenuate such decrements induced by mixed cytokines. Such data raise the possibility that cytokine storm could modulate synaptic plasticity and spine morphogenesis, and minocycline may remove this modulation.

Therefore, it could be seen that mixed cytokines induced activation of glia, and changes in the cytoskeleton-associated proteins tau and drebrin in the hippocampus 1 day post-treatment. An oral dose of minocycline at the time of mixed cytokine injection was able to inhibit glial activation and cytoskeleton-related changes. Although these results may suggest that minocycline can be effective in tackling glial activation and the neuronal cytoskeletal changes induced by cytokine storm, one should take into account several points before jumping to this conclusion. Firstly, minocycline was administered at the same time as mixed cytokines. In a clinical setting, a patient's cytokine storm will have been present for 1-2 days before the medical consultation. Hence, the toxicity of cytokine storm on the brain would have proceeded such that minocycline is no longer of any benefit. Secondly, rats were sacrified 1 day post-

injection, but cytokine storm during influenza can last for up to 7 days. Hence, the experiment could be extended and include multiple injections of mixed cytokines and multiple oral doses of minocycline throughout the study period. This is particularly important because a longer cytokine storm could likely lead to more severe toxicity to the brain, and drug resistance to minocycline could develop after several doses and limit its effectiveness.

Based on these results, we believe that minocycline is probably protective against the neuropathological impact of cytokine storm. Future studies should verify whether minocycline relieves the neurological complications caused by influenza infection.

# Acknowledgements

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