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Minocycline prevents cerebral malaria, confers neuroprotection and increases survivability of mice during *Plasmodium berghei ANKA* infection

Thittayil Suresh Apoorv, Phanithi Prakash Babu *

Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad 500 046, Telangana State, India

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ABSTRACT

Cerebral malaria (CM) is a neurological complication arising due to *Plasmodium falciparum* or *Plasmodium vivax* infection. Minocycline, a semi-synthetic tetracycline, has been earlier reported to have a neuroprotective role in several neurodegenerative diseases. In this study, we investigated the effect of minocycline treatment on the survivability of mice during experimental cerebral malaria (ECM). The currently accepted mouse model, C57BL/6 mice infected with *Plasmodium berghei ANKA,* was used for the study. Infected mice were treated with an intra-peritoneal dose of minocycline hydrochloride, 45 mg/kg daily for ten days that led to parasite clearance in blood, brain, liver and spleen on 7th day post-infection; and the mice survived until experiment ended (90 days) without parasite recrudescence. Evans blue extravasation assay showed that blood-brain barrier integrity was maintained by minocycline. The tumor necrosis factor-alpha protein level and caspase activity, which is related to CM pathogenesis, was significantly reduced in the minocycline-treated group. Fluoro-Jade® C and hematoxylin-eosin staining of the brains of minocycline group revealed a decrease in degenerating neurons and absence of hemorrhages respectively. Minocycline treatment led to decrease in gene expressions of inflammatory mediators like interferon-gamma, CXCL10, CCL5, CCL2; receptors CXCR3 and CCR2; and hence decrease in T-cell-mediated cerebral inflammation. We also proved that this reduction in gene expressions is irrespective of the anti-parasitic property of minocycline. The distinct ability of minocycline to modulate gene expressions of *CXCL10* and *CXCR3* makes it effective than doxycycline, a tetracycline used as chemoprophylaxis. Our study shows that minocycline is highly effective in conferring neuroprotection during ECM.

1. Introduction

Cerebral malaria (CM) is the neurological complication caused by infection of protozoan *Plasmodium falciparum* or *P. vivax* [1]. Major clinical symptoms include convulsions, dyspnea, sudden bleeding, abnormal body posturing, coma, and death [2,3]. CM affects one percent of the malarial patients, and one out of four CM survivors continue to suffer from cognitive deficits later on in their lives [4–6]. Several theories have been postulated regarding the development of CM, which includes cytoadherence of infected red blood cells (iRBCs) to brain microvascular endothelium, hypoxia, cytokine storms leading to neuronal damage, nitric oxide, hemozoin, platelets and microparticles [7–9].

Even after standard antimalarial treatment, cerebral malaria survivors are affected by long-term cognitive impairment, for e.g. hearing loss, and impairment of attention and working memory. Cognitive impairment is mainly due to the inability of standard antimalarial treatments to prevent neuronal death in regions of the brain associated with cognition [5]. Hence, there is a need for an effective drug to prevent the cognitive deficits acquired even after standard antimalarial treatment.

Minocycline (MIN), a semi-synthetic tetracycline with a bacteriostatic property, was developed during 1966. Its empirical formula is $C_{23}H_{27}N_3O_7$, and the molecular weight is 457.5 [10]. Minocycline binds to the 30S subunit of the bacterial ribosome, and inhibits bacterial protein synthesis and cell replication [11]. Among the first and second generation of tetracyclines, minocycline has the highest lipophilicity (logP, 0.5) which is far higher compared to that of doxycycline (logP, −0.2) [12]. The high lipophilicity enables minocycline to easily cross bio-membranes and also increases its efficacy [13].

Abbreviations: CM, cerebral malaria; ECM, experimental cerebral malaria; CXCL10, C-X-C-motif ligand 10; CCL5, C-C motif ligand 5; CCL2, C-C motif ligand 2; CXCR3, C-X-C-motif receptor 3; CCR2, C-C chemokine receptor type 2.

* Corresponding author.
E-mail addresses: prakashbabuphanithi@gmail.com, prakash@uohyd.ac.in (P.P. Babu).

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In 1972, few years after its development, minocycline was studied in malarial cases [14,15] but was not observed further. The possibility of the parasite developing resistance to minocycline might be one of the reasons that hindered research on its efficacy in malaria [16]. The report of neuroprotective effects of minocycline in global brain ischemia was published in 1998 [17], which sparked intense research in the neuroprotective aspect of minocycline in different neurodegenerative diseases.

Minocycline has been shown to be effective in neurodegenerative disorders like spinal cord injury, ischemia, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and Huntington's disease. In general, minocycline is anti-inflammatory and anti-apoptotic. It reduces cyclooxygenase-2 activity, prostaglandin E2 production, and expression of inflammatory mediators like tumor necrosis factor-α (TNF-α), reactive oxygen species and inducible nitric oxide synthase. It also inhibits matrix metalloproteinases and microglial activation, downregulates pro-apoptotic protein caspase-3 and upregulates anti-apoptotic protein Bcl-2 (B-cell lymphoma 2). Extensive neuroprotective effects of minocycline have been reviewed elsewhere [18–20].

Immune effector cells like activated T cells, macrophages, natural killer cells, and dendritic cells migrate towards increasing concentrations of chemokines produced from infection site [21]. T-cell-mediated cerebral inflammation is known to play a major role in CM pathogenesis [22]. Studies on *CXCL10*$^{-/-}$ knockout mice and neutralization of chemokine CXCL10 showed a decrease in cerebral inflammation due to the absence of CXC10-mediated T-cell recruitment in the brain [23,24]. We hypothesize that minocycline might be neuroprotective in CM, owing to its ability to modulate the expression of chemokine receptor CXCR3 (receptor for CXCL10) [25,26]. However, there have been contrasting reports of minocycline aggravating the disease, for example with different outcomes in different hosts itself [27]. Hence, it is crucial to study the effect of minocycline treatment in animal models before proceeding to human clinical trials. In this present work, we studied the effect of minocycline treatment in a mouse model of CM: C57BL/6 mice infected with *Plasmodium berghei ANKA* (*PbA*). We found that minocycline at a dose of 45 mg/kg confers neuroprotection and is highly effective in preventing cerebral malaria.

2. Materials and methods

2.1. Infection of mice and drug delivery

Female C57BL/6 mice weighing 20–25 g were procured from National Centre for Laboratory Animal Sciences (NCLAS), Hyderabad, India and housed at University of Hyderabad Animal House Facility. Filtered water and animal feed were provided *ad libitum*. Frozen *PbA* vials were collected from Malaria Parasite Bank, National Institute of Malarial Research (NIMR), New Delhi, India. All experiments were done in agreement with Institutional Animal Ethical Committee (IAEC) and National Ethical Committee (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines. Mice were divided into three groups: uninfected control, infected, and infected + minocycline-treated (n = 15). A full vial of *PbA* infected blood was mixed with pre-cooled parasite buffer [5 mM phosphate-buffered saline (PBS) pH 7.4, 0.9% NaCl] and each 200 μl of the mixture was injected to three 'source' mice via the intra-peritoneal (i.p) route. When any of the 'source mice' showed cerebral symptoms during the 5th–10th day post-infection (p.i), its blood was passaged i.p to mice of infected and minocycline groups, so that each mouse received 1×10^6 iRBCs. Mice of the control group and the infected group were given PBS i.p daily. Minocycline hydrochloride (Sigma-Aldrich, US) dissolved in PBS (pH 7.4) was given to the minocycline group daily at a dose of 45 mg/kg for ten days p.i. Another group of mice was also given a half dose of minocycline (22.5 mg/kg) with the same regimen. For CM brain samples, infected mice were sacrificed when they developed CM symptoms (strictly between 6th and 10th day p.i.) whereas the minocycline-treated mice were sacrificed on 10th day p.i. In another experiment, infected mice were separately sacrificed on 4th day p.i. after two doses of minocycline, 5th day p.i. after two doses of minocycline, and 6th day p.i. after three doses of minocycline (45 mg/kg daily).

2.2. Survivability test and parasitemia

Mice were monitored daily and the day of death was recorded. Parasitemia was recorded daily by staining caudal blood smears with Giemsa (Sigma-Aldrich, US). Parasitemia was calculated as a percentage of iRBCs to normal RBCs and plotted to parasitemia curve.

2.3. Estimation of parasite load using semi-quantitative PCR

Amplification of parasite-specific *18S rRNA* was done to confirm parasite clearance. Mice from all experimental groups were anesthetized with 10% pentobarbital i.p and perfused with saline. Brain, liver, and spleen were excised from mice and snap-frozen in liquid nitrogen. RNA from all organs was isolated using TRIZOL™ (Invitrogen, US) as per product instructions. Blood was isolated via retro-orbital sinus puncture using 6% ethylenediaminetetraaceticacid-dipped Pasteur pipette (blood: EDTA = 19:1). RNA from blood was isolated using QIAamp RNA Blood Mini Kit (Qiagen, Netherlands) as per product instructions. RNA isolated from organs as well as blood were estimated with a NanoDrop™ spectrophotometer (Thermo Scientific, US). RNA (1 μg) was converted to cDNA using BluePrint™ 1st strand cDNA synthesis kit 6115A (Takara, Japan) as per product instructions. Semi-quantitative PCR for the genes *PbA 18S rRNA* and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) were done using DreamTaq™ Green PCR Master Mix K1081 (Thermo Scientific, US) as per manufacturer's protocol using 1 μl of cDNA from each experimental group. Primers (Eurofins, India) were used at a concentration of 0.5 pmol. Housekeeping gene *GAPDH* was used as internal control. Nucleotide sequences of primers used: *PbA 18S rRNA* (5′-CGG TAA TTC CAG CTC CAA TAG CGT-3′, 5′-ATG AAG ATA TCG AGG CGG AGC CAA-3′); *GAPDH* (5′-GTG TGA ACG GAT TTG GCC GTA TTG-3′, 5′-TTT GCC GTG AGT GGA GTC ATA CTG-3′).

2.4. Evans blue extravasation assay

Evans blue dye was used to check the effect of minocycline on blood-brain barrier (BBB) integrity. Evans blue binds to the serum albumin in blood. The presence of dye in the brain implies that BBB integrity is compromised. Each mouse was injected intravenously with 100 μl of 2% Evans blue/PBS (SRL, India). After 1 h, mice were anesthetized with 10% pentobarbital i.p and perfused with saline. Brains were resected and photographed for qualitative assessment of BBB disruption. Brains were weighed and incubated in 2 ml formamide for 48 h (37 °C, in the dark). The Evans blue extracted by formamide was measured at 620 nm in an ELISA plate reader (Infinite M200, Tecan). The readings were compared to Evans blue/formamide standards to calculate 'μg of Evans blue per gm of brain tissue' [28].

2.5. Immunoblotting

Brains of each group were homogenized in isolation buffer [10 mM Tris pH 7.4, 0.32 M sucrose, 0.25 mM Na_2EDTA, 1 mM