AMP-activated protein kinase (AMPK) and matrix metalloproteinase-9 (MMP-9) in severe malaria and role of minocycline as a therapeutic agent

Thesis submitted to the University of Hyderabad
for the degree of

DOCTOR OF PHILOSOPHY

By

APOORV T. S.
(09LTPH03)

Supervisor
Prof. P. Prakash Babu

Department of Biotechnology and Bioinformatics
School of Life Sciences
University of Hyderabad
Hyderabad – 500 046 (T.S.)
INDIA

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(A Central University established in 1974 by an Act of Parliament)
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INDIA

DECLARATION

I, **Apoorv T. S.**, hereby declare that this thesis entitled "***AMP-activated protein kinase (AMPK) and matrix metalloproteinase-9 (MMP-9) in severe malaria and role of minocycline as a therapeutic agent***" submitted by me under the supervision and guidance of **Prof. P. Prakash Babu** is a bonafide research work which is free from plagiarism. I also declare that this thesis has not been submitted previously in part or in full to this or any other University or institution for award of any degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled "***AMP-activated protein kinase (AMPK) and matrix metalloproteinase-9 (MMP-9) in severe malaria and role of minocycline as a therapeutic agent***" submitted by **Apoorv T. S.** bearing registration number **09LTPH03** in partial fulfillment of the requirements for award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or institution for award of any degree or diploma.

Further, the student has the following publications before submission of the thesis for adjudication and has provided evidence for the same in the form of the reprint in the relevant area of his research.

1. **Apoorv, T.S.,** Babu, P.P., Meese, S., Gai, P.P., Bedu-Addo, G., Mockenhaupt, F.P., 2015. Matrix Metalloproteinase-9 Polymorphism 1562 C > T (rs3918242) Associated with Protection Against Placental Malaria. ***The American Journal of Tropical Medicine and Hygiene.*** 93(1), 186-188 (ISSN: 0002-9637; E-ISSN*:* 1476-1645). The 6% of 21% similarity index of this thesis is contributed by this publication (mentioned as 1.www.ajtmh.org in originality report). Chapter of the dissertation where this publication appears is **Chapter 4**.

2. **Apoorv, T.S.,** Babu, P.P., 2017. Minocycline prevents cerebral malaria, confers neuroprotection and increases survivability of mice during *Plasmodium berghei ANKA* infection. ***Cytokine.*** 90, 113-123 (ISSN: 1043-4666). Chapter of the dissertation where this publication appears is **Chapter 2**.

and

has made presentations in the following conferences:

1. Seminar on Pharmaceutical Approach for Malarial Targeting and Resistance at JSS College of Pharmacy, Ootacamund, India on 15 February 2014. He was awarded for **Best Poster**. (National Seminar)

2. Academia Sinica - University of Hyderabad Joint Workshop on Frontiers in Life Sciences, held at University of Hyderabad, Hyderabad, India on 16-17 September 2016. (National Seminar)

3. Bio-Quest 2016 (Wellcome Trust/DBT India Alliance) at School of Life Sciences, University of Hyderabad, Hyderabad, India on 15 February 2014. He was awarded for **Best Poster**.

Further, the student has passed the following courses towards fulfillment of coursework requirement for Ph.D:

Sl.No.	**Course Code**	**Name**	**Credits**	**Pass/Fail**
1	BT801	Seminar	1	Pass
2	BT802	Research Ethics & Management	2	Pass
3	BT803	Biostatistics	2	Pass
4	BT804	Analytical Techniques	3	Pass
5	BT805	Laboratory Work	4	Pass

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"Nothing can impede the success of a person dedicated to the benefit of mankind"
-SMS sent by my brother while I boarded train to Hyderabad (for joining PhD)

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ABBREVIATIONS

°C	Degree Celsius
µ	micro-
λ	wavelength
A	Adenosine (nucleotide)
ACC	Acetyl Co-A carboxylase
ADP	Adenosine diphosphate
AFR	African Region (WHO Health Region)
AMA-1	Apical membrane antigen-1
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
AMR	Region of the Americas (WHO Health Region)
ANOVA	Analysis of variance
aOR	adjusted odds ratio
Bax	Bcl-2-associated X
BBB	blood-brain barrier
Bcl-2	B-cell lymphoma-2
BDNF	brain-derived neurotrophic factor
C	Cytosine (nucleotide)
CAT	Catalase
CCL2	C-C motif ligand 2
CCL5	C-C motif ligand 5
CCR2	C-C chemokine receptor type 2
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CI	Confidence interval
CM	Cerebral malaria
CO_2	Carbon dioxide
Cox-2	Cyclooxygenase-2
CSA	Chondroitin sulphate A
CSF	Cerebrospinal fluid
CXCL10	C-X-C-motif ligand 10
CXCR3	C-X-C-motif receptor 3
DDT	Dichlorodiphenyltrichloroethane
DDW	Double distilled water
DNA	Deoxyribonucleic acid
DNCB	2,4-Dinitrochlorobenzene
DTT	Dithiothreitol
ECM	Experimental cerebral malaria
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMR	Eastern Mediterranean Region (WHO Health Region)
eNOS	endothelial nitric oxide synthase
E-selectin	endothelial-cell selectin
EUR	European Region (WHO Health Region)
FITC	Fluorescein isothiocyanate
FJC	Fluoro-Jade C
G	Guanine (nucleotide)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
GPx	Glutathione peroxidase
GSH	Reduced glutathione

GST	Glutathione-S-transferase
h	hours
H&E	hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HSP-70	Heat shock protein-70
ICAM-1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IL	Interleukin
INF-γ	Interferon-γ
iNOS	inducible nitric oxide synthase
i.p.	intra-peritoneal
iRBCs	infected red blood cells
kb	kilo base pairs (nucleotides)
kg	kilogram
LBW	Low birth weight
m	milli-
M	Molar
min	minute(s)
MIN	minocycline
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
MP	microparticle
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NO	nitric oxide
NOR	novel object recognition
NOS	nitric oxide synthase
n.s.	not significant
OR	odds ratio

pACC	phospho-acetyl Co-A carboxylase
pAMPK	phospho-denosine monophosphate-activated protein kinase
PbA	*Plasmodium berghei ANKA*
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PECAM-1	platelet or endothelial cell adhesion molecule-1 (CD31)
PfEMP-1	*Plasmodium falciparum* erythrocyte membrane protein 1
p.i.	post-infection
PTD	preterm delivery
RBC	Red blood cell/erythrocyte
RMCBS	Rapid murine coma and behavioral scale
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	ribosomal ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEAR	South-East Asia Region (WHO Health Region)
SEM	Standard error of the mean
SNP	single nucleotide polymorphism
SOD	Superoxide dismutase
T	Thymine (nucleotide)
TBS	Tris-buffered saline
T-cells	Thymus-derived lymphocytes
Th1 or 2	T-helper cell 1 or 2
T-lymphocytes	Thymus-derived lymphocytes
TLR	Toll-like receptor

TNF-α	Tumor necrosis factor-α
Tris	trishydroxymethylaminomethane
tRNA	transfer ribonucleic acid
U	enzyme unit
VCAM-1	Vascular cell-adhesion molecule-1
WHO	World Health Organization
WPR	Western Pacific Region (WHO Health Region)

Introduction

Malaria is the second deadliest disease in the world after tuberculosis. Around 300-500 million malaria cases are reported every year, and 1.5-2.7 million people die due to the disease. Almost 90% of all malarial cases arise from sub-Saharan Africa, and it mainly kills young children. Other countries where malaria is endemic are India, Brazil, Afghanistan, and Columbia **(WHO, 2015)**. The parasitic protozoon *Plasmodium* causes malaria, and it infects humans, non-human primates, reptiles, birds, and animals. There are more than 250 species of *Plasmodium* in the world, and five species of *Plasmodium* infect humans, which are *P.falciparum*, *P.vivax*, *P.ovale*, *P.malariae* and *P.knowlesi* of which the *P.falciparum* is the most virulent. The female mosquito of *Anopheles* genus transmits malaria. Malaria was termed by the Romans in the medieval period, which means 'bad air' ('*male,*' '*aria*' in Italian). Romans coined this current term by correlating the increase in malaria cases with proximity to stagnant water. As stagnant waters are adequate breeding places for mosquitoes and hence are related to higher incidences of malaria cases, the Romans wrongly believed that the 'bad air' emanating from the stagnant waters caused the disease **(Goldsmith, 2011)**.

Epidemiology

Incidence of malaria worldwide

Around 215 million malaria cases (estimate: 149-303 million) were reported globally in 2015. The three major WHO regions with the highest incidence of malaria cases were African Region, South-East Asia Region and Eastern Mediterranean Region with percentage prevalence 88%, 10%, and 2% respectively. Considering deaths due to malaria of all ages, 4,38,000 deaths were reported globally (estimate: 2,36,000-6,35,000) in 2015. The three major WHO regions with the highest incidence of malaria deaths were African Region, South-East Asia Region and Eastern Mediterranean Region with percentage prevalence 90%, 7%, and 2% respectively. In the case of children below five years old, about 3,06,000 deaths were reported (estimate: 2,19,000-4,21,000) **(WHO, 2015)**.

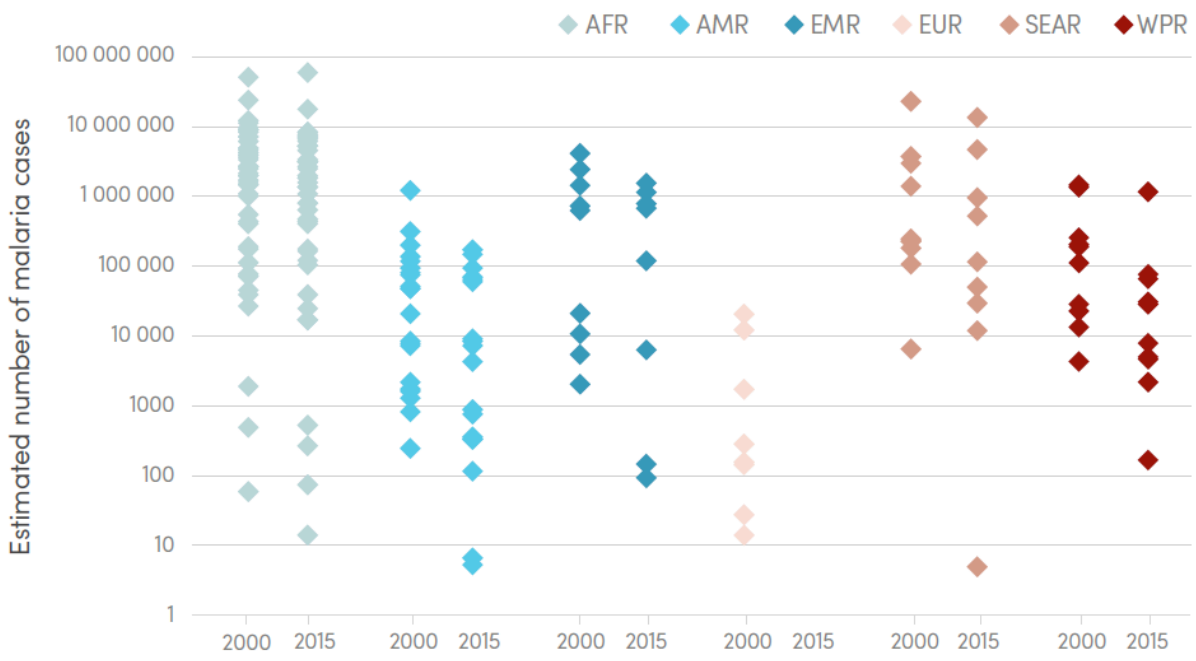


Fig.1. The number of malaria cases in the year 2000 and 2015 categorized by different WHO regions. A diamond represents each country in corresponding WHO region. Abbreviations of WHO health regions: African Region (AFR), Region of the Americas (AMR), Eastern Mediterranean Region (EMR), European Region (EUR), South-East Asia Region (SEAR) and Western Pacific Region (WPR). Image reproduced from WHO World Malaria Report 2015 **(WHO, 2015)**.

Incidence of malaria in India

In 2013, 11,02,205 malaria cases were reported but the unconfirmed estimates range from 1,00,00,000 to 2,60,00,000. Surprisingly, only 561 deaths were reported in 2013. This is because the deaths in the government hospitals are only accounted for, omitting the people who died of fever (before malaria correctly diagnosed). Hence, the deaths are adjusted for the same to 2,300-55,000. The *P.falciparum* and *P.vivax* are the prime *Plasmodium* species in India, with each species contributing 66% and 34% respectively. The *Anopheles* species of mosquitoes, which act as important malaria vectors in India, are *Anopheles culicifacies, An. fluviatilis, An. stephensi, An. minimus, An. dirus* and *An. annularis* **(WHO, 2015)**.

Plasmodium - the malaria parasite

Plasmodium is a protozoon of the phylum Apicomplexa. As the name suggests, members of the Apicomplexa have apical organelles involved in host cell invasion processes. *Plasmodium* possesses these apical organelles, namely rhoptry, dense granule,

and microneme, which secrete proteins like rhoptry neck proteins and apical membrane antigen-1 (AMA-1) for the organized and coordinated host cell invasion **(Kappe et al., 2004) (Fig.2.)**. The *Plasmodium* has 14 chromosomes, and its genome comprises of 5,300 genes, with 23 mega base pairs **(Gardner et al., 2002)**. The *Plasmodium* mitochondria's 6 kb genome is the smallest mitochondrial genome known to man, and functionally, mitochondria produce energy and pyrimidines **(Jackson et al., 2011; Vaidya and Mather, 2009)**. The *Plasmodium* also has an endosymbiotic plastid called apicoplast, which is involved in the synthesis of isoprenoid, heme and fatty acids, essential during parasite multiplication **(Ralph et al., 2001)**.

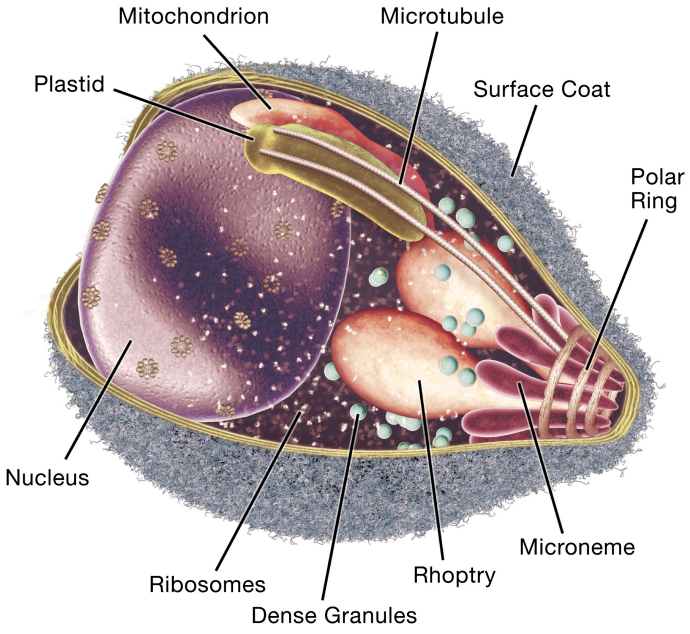


Fig.2. The *Plasmodium* parasite (merozoite).
Image reproduced from Cowman & Crabb **(Cowman and Crabb, 2006)**.

The life cycle of *Plasmodium falciparum*

The *Plasmodium* completes its life cycle in a vertebrate host and an insect host (Diptera), of which latter acts as a vector (helps in disease transmission). Here we will discuss the life cycle of *Plasmodium falciparum* in humans and female *Anopheles* mosquito **(Fig.3.)**:

Exoerythrocytic Phase (Human Liver Stage – Asexual Stage I)

When an infected female *Anopheles* mosquito bites a human, it injects saliva mixed with anticoagulant into the human blood to prevent blood clotting and facilitate efficient feeding. The *Plasmodium* sporozoites harbored in the mosquito's salivary glands gets into the human bloodstream along with the saliva. Even one sporozoite released into the bloodstream can lead to malaria infection. After its release, the sporozoite migrates to the liver via the bloodstream and penetrates a Kupffer cell (liver-resident macrophage) to gain access into the liver. The sporozoite then enters a hepatocyte and matures into a merozoite. After multiple asexual divisions of merozoite (schizogony), the infected hepatocyte bursts open releasing of thousands of merozoites into the bloodstream.

Erythrocytic Phase (Human Blood Stage – Asexual Stage II)

Each of these released merozoites infects a red blood cell (RBC)/erythrocyte which helps in evading the host immune system. Once inside the RBC, the merozoite develops into the ring-stage, the trophozoite stage and undergoes schizogony to divide into 10-30 merozoites. Around 10,000-30,000 merozoites can be produced in the liver within 5.5 to 8 days by a single sporozoite **(White et al., 2013)**. The rupture of the RBCs releases the merozoites, which infect other RBCs leading to exponential growth of *Plasmodium*. The bursting of infected RBCs and infection of new RBCs are synchronous and occur within a particular period. The period is species-specific, 48h for *P.falciparum, P.vivax, and P.ovale;* 72h for *P.malariae* and 24h for *P.knowlesi*). The repeated bursting of RBCs leads to increase in the levels of endogenous pyrogens (inflammatory cytokines) that in turn result in cyclic fevers, a characteristic feature of malaria. Interestingly, in the case of *P.vivax* and *P.ovale*, some sporozoites can remain strategically dormant in the liver to initiate malaria weeks or months later **(Kyes et al., 2001)**. Some of the merozoites also develop into dormant male and female gametocytes (gametocyte: asexual forms = 1:10 to 1:156) in preparation for the mosquito stage of *Plasmodium*.

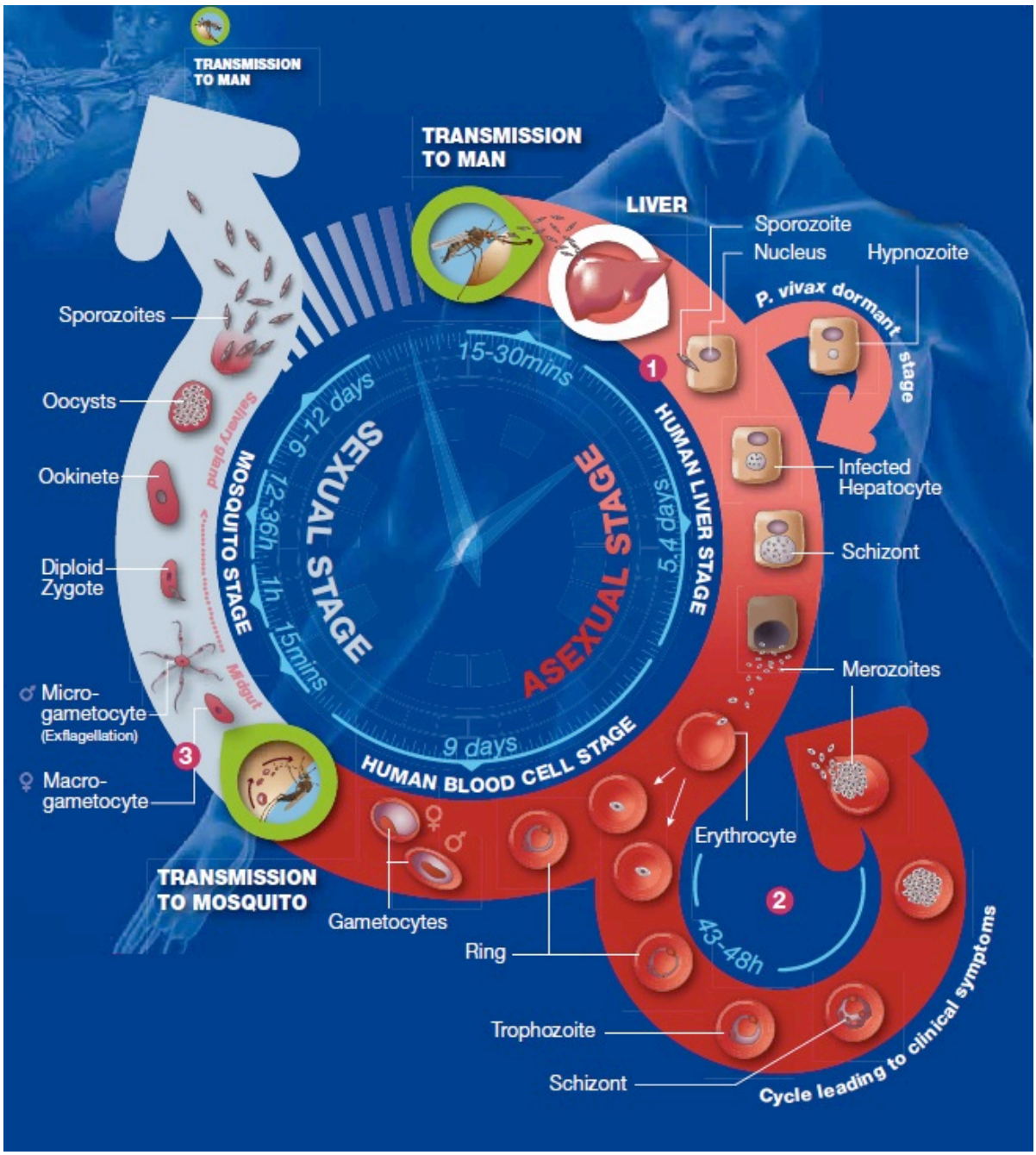


Fig.3. The life cycle of *Plasmodium falciparum*.
Image reproduced from Medicines for Malaria Venture website **(MMV)**.

Mosquito Stage (Sexual Stage)

When another mosquito bites the mammalian host infected with malaria, parasites get ingested into the mosquito's gut along with the blood **(de Souza and Riley, 2002)**. All the parasite stages perish in the mosquito gut except for the dormant gametocytes. The dormant gametocytes become active in the mosquito gut attributed to its different pH microenvironment and lower temperature compared to the mammalian host. A male

gametocyte releases eight male gametes of which one fertilizes the female gametocyte leading to the formation of a zygote (ookinete). The motile ookinete migrates to the outer wall of the gut, where it develops into an oocyst and releases thousands of sporozoites. Around 19% of the sporozoites escaping mosquito hemocyte phagocytosis traverse their way to the salivary glands of the mosquito **(Hillyer et al., 2007)**. Hence, when this mosquito bites another human, the sporozoites are released into the host bloodstream and complete the life cycle of the *Plasmodium* **(Talman et al., 2004)**.

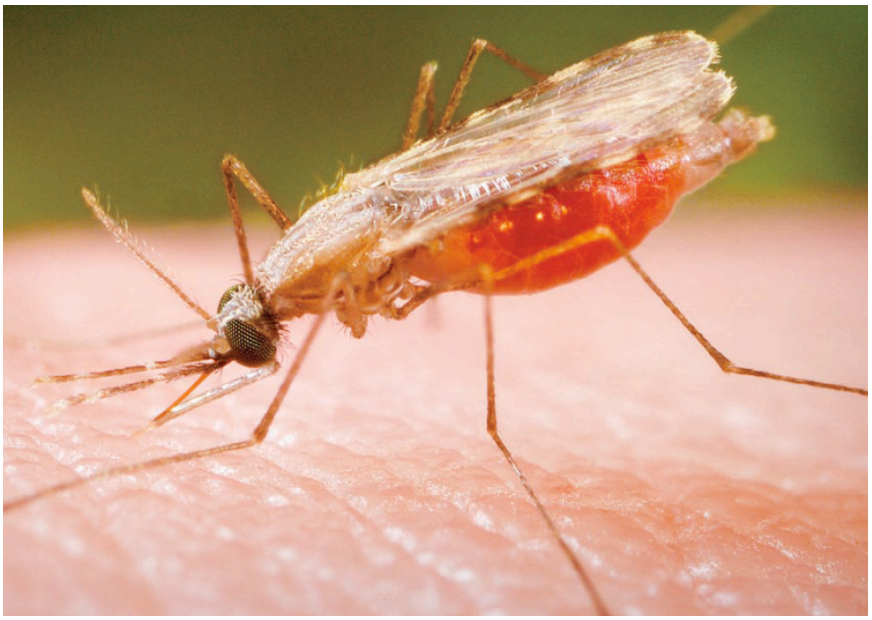


Fig.4. A female *Anopheles* mosquito during bloodmeal.
Image reproduced from the book *Mosquitoes* **(Walker, 2009)**

Mosquitoes as vectors (carriers) and role in malaria transmission

Mosquitoes ('*mosca,*' '*ito*' in Spanish means 'little fly') have an important role to play as only female *Anopheles* mosquitoes transmit human malaria **(Fig.4.)**. Only about 40 species of the > 400 species of *Anopheles* genus are involved in human malaria transmission. The important vectors of human malaria are *Anopheles gambiae* and *Anopheles arabiensis*. The male and female mosquitoes feed on plant juices like for carbohydrates and energy whereas the female mosquitoes require human blood proteins for egg production. In contrast to male mosquitoes, female mosquitoes have a specially developed proboscis to penetrate human skin and to feed on human blood.

If the female mosquito is infected with *Plasmodium*, it transmits malaria to a healthy human during the feeding process **(Becker et al., 2010)**. Several features make mosquito an efficient carrier of malaria. One of the main features is that mosquitoes can fly almost 10-12 km per night and hence increase the range of malaria transmission **(Kaufmann and Briegel, 2004)**. Mosquitoes can detect even 0.01% difference in carbon dioxide levels of host breath **(Kellogg, 1970)** and difference of 0.2^{o}C in the host body temperature **(Lehane, 1991)** and which makes its host-seeking behavior highly efficient. The host skin temperature is dependent on the number of blood vessels underneath, and the mosquito can assess the thickness and temperature of the skin to choose a suitable area for feeding **(Davis and Sokolove, 1975)**. A mosquito can ingest blood up to three times its body weight **(Nayar and Sauerman, 1975)** thereby increases the chance of *Plasmodium* gametocytes to be ingested along with blood meal. A recent study showed that *P.falciparum* releases (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate that induces a higher release of terpenes and CO_2 by the RBCs leading to increased attraction of female mosquitoes to malaria patients **(Emami et al., 2017)**. A female mosquito can lay 50-500 eggs during an oviposition **(Clements, 1992)** contributing to the exponential increase in mosquito population and makes mosquito-control programs more challenging.

Factors that increase malaria incidence

Several factors contribute to the increase in malaria incidences. Firstly, global warming that arises from the burning of fossil fuels increase the carbon dioxide levels in the atmosphere. Global warming, therefore, leads to increase in the temperature and affects weather. It has been estimated that an increase in temperature of just 1.3^{o}C doubles the mosquito population. Deforestation also contributes to the global warming and also leads to animal migration to other places. This, in turn, compels the mosquitoes to seek other hosts like the humans. A person who gets bitten by an infected mosquito in a malaria-endemic region carries malaria to unaffected regions during traveling. Also, a malaria-infected mosquito in flight cargos gets released involuntarily into unaffected countries. Civil wars and war-stricken regions compel citizens to migrate, which also leads to a

wider distribution of malaria **(Goldsmith, 2011)**. Evolution is the major role player that makes the fight against malaria tougher. The *Plasmodium* harbors *var* gene family, which creates genetic and antigenic variation among parasite progenies. This helps in evading the host immune system and has led to the development of resistance against the standard antimalarial drugs. Mosquitoes are becoming increasingly resistant to insecticides. Increased use of dichlorodiphenyltrichloroethane (DDT) against mosquitoes was successful in the 1950s. The discoverer of DDT, Paul Hermann Müller, was awarded the Nobel Prize in Physiology or Medicine in 1948 **(NobelPrize, 1948)**. However, later, evolution favored insecticide-resistant mosquitoes, and DDT increasingly became less effective.

Effect of malaria on human health and economic growth of developing countries

Poverty can be a favorable factor for malaria, as accessibility to health centers is very less in rural areas. The relation of malaria with poverty has led malaria being accounted as one of the diseases of poverty **(Gardner et al., 2002; Lou et al., 2001)**. Poor people are devoid of mosquito nets or mosquito screens at their doors or windows. Malaria patients are unable to work and cannot care for their children. The life expectancies in African countries like Zambia and Mozambique are 57.2-60.3 and 52.9-56.2 respectively, whereas in the United States it is 76.5-81.3 (2010-2015) **(UnitedNations, 2016)**. Education of children is affected due to inability to attend classes regularly, and future job prospects are dim. The tourism is affected, and foreigners fear to invest in opening new companies thinking about malaria affecting employee attendance and productivity **(Goldsmith, 2011)**. Hence, poverty is a cause of malaria and malaria is a cause for poverty. Malaria is a tropical disease as it mainly affects tropical countries (where the temperature remains above 16^{o}C throughout the year). Mosquitoes, the vector/transmitter of malaria, grow and multiply fast during temperate conditions [optimal temperature for mosquito egg hatching is 22-27^{o}C **(Impoinvil et al., 2007)**]. Malaria affects the economic growth of the countries where it is endemic, as they have to invest large amounts of money for the prevention and

eradication programs. In conclusion, malaria affects the human health and research on malaria is crucial to discover newer methods to prevent malaria.

Severe malaria

The undiagnosed or untreated malaria can worsen and develop into severe malaria, which includes cerebral malaria and placental malaria (or malaria during pregnancy). The patients suffering from severe malaria, if untreated, will die due to severe anemia (increased RBC destruction), hyperparasitemia (increase in parasite levels in the blood) or other complications.

Cerebral Malaria (CM)

Cerebral malaria (CM) is a severe form of malaria caused by humans by *P.falciparum* or *P.vivax* infection. According to World Health Organization, CM is the neurological complication arising during severe forms of *P.falciparum* malaria accompanied with coma (persistence of coma more than 30 min after a seizure). The exclusion of other forms of encephalopathy is important for confirmation of CM **(Idro et al., 2005a)**. CM is the major cause of death in malaria cases, and it has been estimated that 7% of all cases progress to CM **(Waknine-Grinberg et al., 2010).** Due to CM, children can become comatose very rapidly, and its accurate prognosis is very poor. It is estimated that 25% of the CM survivors continue to suffer from neurological and cognitive deficiencies **(John et al., 2008)**. Neurological impairment of individuals affects the economic growth of a developing country due to the loss of productivity, the number of absent days from work and school. Considering its mortality and morbidity, the prevention of CM is very crucial. 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 **(Hunt and Grau, 2003; John et al., 2008; Martins et al., 2010)**.

Clinical symptoms of CM

Major clinical symptoms of CM include convulsions, dyspnea, sudden bleeding, abnormal body posturing, coma and death **(Idro et al., 2005a; Idro et al., 2005c)**.

Like all malarial cases, CM accompanies fever, headache, and vomiting **(Mishra et al., 2007; Mishra and Newton, 2009)**. Coma is the main feature of CM. For the CM confirmation, coma scales have been devised with Glasgow coma scale < 11 in adults and a Blantyre coma scale < 3 in children **(Newton et al., 2000)**. In children, failure of corneal reflex, decorticate and decerebrate posturing is associated with poor prognosis **(Olumese et al., 1999)**. High intracranial pressure due to raised blood volume can be the reason for these abnormal posturing, papilloedema, and retinopathy. Metabolic acidosis leads to opisthotonic posturing **(Idro et al., 2005a; Idro et al., 2005b; Newton et al., 1991)**. Neuropathological analyses have shown human CM brains with petechial hemorrhages and Dürck's granulomas **(Turner, 1997)**.

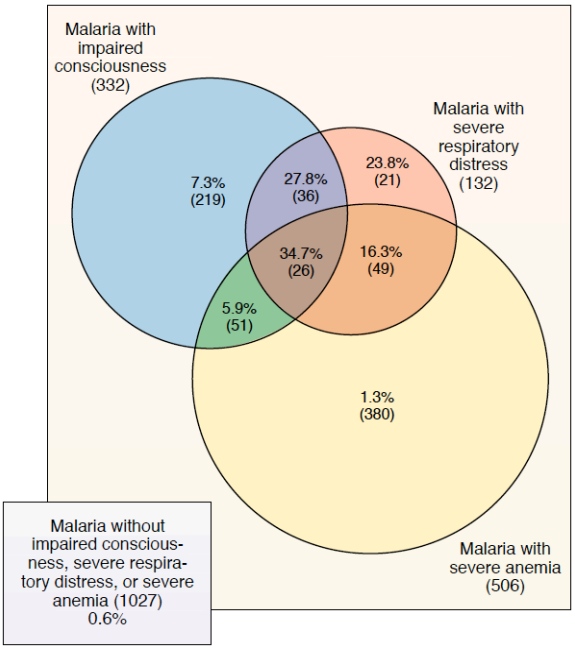


Fig.5. Pie diagram shows overlapping clinical symptoms found in cerebral malaria patients leading to poor prognosis and challenge for physicians. Mortality showed in percentages and number of patients in parentheses. Image reproduced from Marsh et al. **(Marsh et al., 1995)**.

Seizures (febrile or afebrile) are a major problem during CM as it mirrors status epilepticus. Prolonged seizures can be can be reasons for the neurological and cognitive impairments that patients suffer in later stages **(Kihara et al., 2009; Turner, 1997)**. Patients might also experience convulsions before going into a coma **(Sokrab et al., 2005)**. For example, a child playing on the ground may suddenly go into a coma.

Critical conditions like this make it a challenge for physicians to diagnose CM correctly. Also, the clinical symptoms of CM are not consistent with each patient having different overlapping symptoms **(Marsh et al., 1995) (Fig.5.)**.

Due to the less prior malaria infections and less developed immune system of children, the children and adults exhibit different symptoms during CM. Coma occurs very fast in children compared to adults and usually follows a seizure **(Molyneux et al., 1989)**. Seizures are present in 80% of the CM-affected children whereas only 20% of the CM-affected adults have seizures. Children have higher incidences of severe anemia, hyponatremia (low sodium), increased intra-cranial pressure and hypoglycemia **(Idro et al., 2005a)**. Hypoglycemia at blood plasma level has been reported in several human CM cases **(Atabani et al., 1990; Kawo et al., 1990a; Kawo et al., 1990b; Kiire, 1986; Ramos Filho et al., 1987)**. Speech, visual, hearing, and other cognitive impairments are more in the case of children **(Boivin et al., 2007; Boivin et al., 2008; Carter et al., 2005; John et al., 2008)**. In adults, jaundice is prevalent more compared to children. Also, adults take more time to regain consciousness (48 h) compared to children (24-48 h) **(Idro et al., 2005a)**.

Animal models of CM

Most of the knowledge regarding CM has been from autopsy analyses, which cannot help in understanding the sequence of events leading to death **(de Souza et al., 2010)**. The human brain is not accessible for intervention and due to ethical reasons; a 'direct' pathological study in humans is not feasible. Animal models are useful in understanding the biochemical and molecular mechanisms of CM pathogenesis due to desirable interventions.

The CM is induced in rodents only by the *Plasmodium berghei* species **(Table.1.),** and its natural host is the tree rat, *Thamnomys surdaster* **(Engwerda et al., 2005)**. In 1948, Vincke and Lips **(Vincke and Lips, 1948)** isolated the *P.berghei* species from an

Anopheles dureni mosquito. The currently accepted model for CM is the C57BL/6 black mice infected with *P.berghei ANKA* strain **(de Souza et al., 2010)**.

Table.1. The table shows the different *Plasmodia* used for CM research.
Table reproduced from Engwerda et al. **(Engwerda et al., 2005)**

Species	Subspecies	Strain	Clones	Origin	Erythrocytic cycle	Rodent species used	Neurological signs
P. berghei		ANKA	1.49L, 1.94L, 4	Katanga, Congo	21 h	Mouse	Yes
			5, BdS, cl5, 15cy1	Katanga, Congo	21 h	Rat	Yes
						Golden Hamster	Yes
		NK65		Katanga, Congo	21 h	WM rat	Yes
		SP-11		Katanga, Congo	21 h	Mouse	Yes
		Keyberg 173		Katanga, Congo	21 h	Mouse	No
						Rat	Yes
P. yoelii	yoelii	17XL	1.1, YM	Centrafrican Republic	18 h	Mouse	No
	nigeriensis	N67		Nigeria	18 h	Mouse	No
P. chabaudi	chabaudi		AS	Centrafrican Republic	24 h	Mouse	No

Recently, the use of animal models of CM has been a subject of debate, as earlier research on mouse models showed sequestration of leukocytes in brain microvessels instead of iRBCs **(Stevenson et al., 2010; White et al., 2010)**. But a recent study has shown that iRBCs sequester in the brain capillaries during murine CM similar to human CM **(Strangward et al., 2017)**. Hence, animal models yield invaluable information regarding CM pathogenesis. The introduction of new models that can fully reproduce most features of human CM will help in elucidating the unknown pathways of CM pathogenesis.

CM Pathogenesis

Even though research on CM is increasing, it is still a poorly understood disease. Researchers, especially immunologists and molecular neurobiologists have different perspectives and have a particular liking to their subject fields due to which they describe 'biased importance' of their findings. CM pathology might be the summation of cross-talks between molecules and parallel events leading to death. Several theories have

been postulated regarding the development of CM **(Fig.6.)**, which includes cytoadherence of infected red blood cells to brain microvascular endothelium, hypoxia, cytokine storms leading to neuronal damage, nitric oxide, hemozoin, platelets and microparticles **(Apoorv and Babu, 2017; Combes et al., 2006; de Souza et al., 2010; Sobolewski et al., 2005)**.

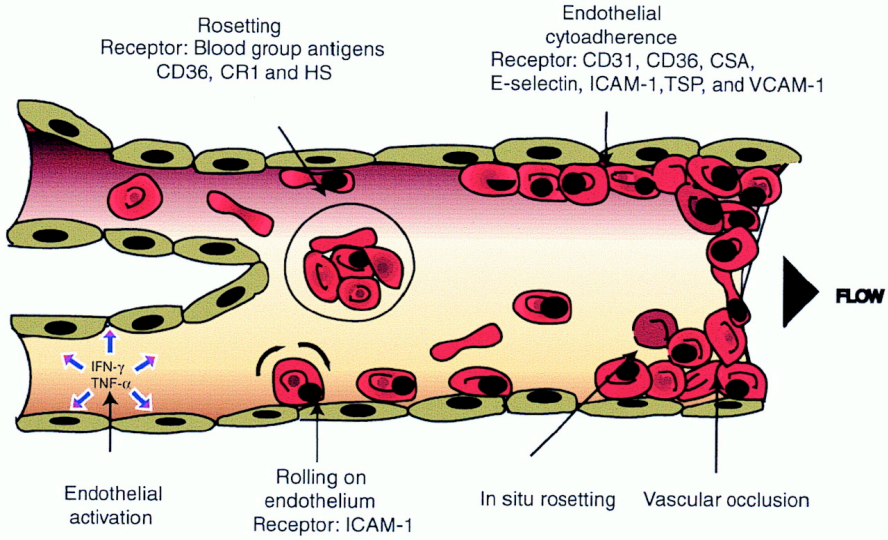


Fig.6. Schematic diagram summarizing the different theories of CM pathogenesis.
Image reproduced from Chen et al. **(Chen et al., 2000b)**

Major theories of CM pathogenesis

1. Sequestration

Sequestration is the process of infected RBCs (iRBCs) sequestering in blood microvessels, described first in 1894 **(Marchiafava and Bignami, 1894)**. Within 15 h after a normal RBC is infected, the *var* gene of the *Plasmodium* encodes a protein known as *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). These PfEMP1 proteins are transported to the iRBC outer membrane and have the ability to bind to intercellular adhesion molecule-1 (ICAM-1) expressed on the blood microvessel endothelial cells. The presence of PfEMP1 proteins makes the iRBC look 'knobby' under an electron microscope **(Trager et al., 1981; Trager et al., 1982)** and also makes the

iRBCs 'sticky' by helping the iRBC to adhere to the blood capillaries, a process known as *cytoadherence*. Cytoadherence can also occur due to the ligand-receptor interactions of the following molecules: vascular cell-adhesion molecule-1 (VCAM-1), thrombospondin, CD31 (platelet or endothelial cell adhesion molecule-1 or PECAM-1), endothelial-cell selectin (E-selectin) and CD36 **(Barnwell et al., 1989; Ockenhouse et al., 1992; Roberts et al., 1985; Treutiger et al., 1997)**.

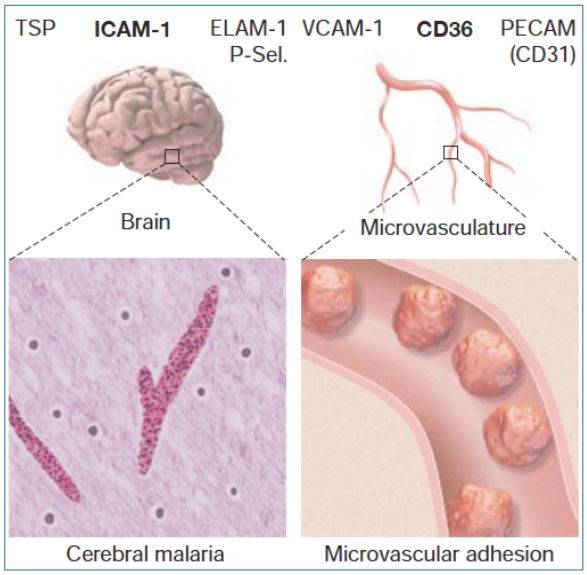


Fig.7. The receptors involved in sequestration and cytoadherence in the brain.
Image reproduced from Miller et al. **(Miller et al., 2002)**

It has been proposed that parasites adhere to microvessels so as to avoid their clearance by the spleen **(Beeson et al., 2001; de Souza et al., 2010)**. The iRBCs with its PfEMP1 knobs can adhere to uninfected RBCs forming clumps known as *rosettes*. Also, the iRBCs can adhere to other iRBCs known as *agglutination*. Hence, all the processes mentioned above can lead to the sequestration of RBCs and iRBCs to the blood capillaries thus blocking the normal blood flow **(Davis et al., 1990; Dondorp, 2008; Longo, 2012; MacPherson et al., 1985)**. This leads to lowered supply of nutrients to the brain, resulting in hypoxia and coma **(Ponsford et al., 2011)**.

However, the sequestration hypothesis is a matter of debate **(Clark and Alleva, 2009)** due to the absence of sequestration in some patients **(White et al., 2010)**.

2. Cytokines

The immune system protects the individual against infection; for example, spleen and T-lymphocytes help in parasite clearance **(Nie et al., 2009)**. However, hyperactivation of the immune system is detrimental. For example, high fevers can lead to chills, seizures and brain damage in patients **(Longo, 2012)**. Among all the theories, the cytokine theory is the most extensively studied due to the involvement of multiple immune factors in host-defense against malaria.

Cytokines are the cell-signaling proteins produced by various immune cells. The major pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is involved in various pathological conditions. TNF-α is also a pyrogen; because during infection, it stimulates the hypothalamus to increase the body temperature leading to fever. In 1987, Grau et al. **(Grau et al., 1987)** reported that antibody treatment against TNF-α, protected the mice against CM thus proving the major involvement of TNF-α in CM pathogenesis. However, contrasting results were found by Hermsen et al. **(Hermsen et al., 1997b)** who showed that TNF-α were immunologically but not biologically active, which was the reason why anti-TNF-α treatments fail. A significant *in vitro* study showed that parasitic proteins of the blood microvessel sequestered-iRBCs undergo trogocytosis to get transferred from iRBC membrane to endothelial plasma membrane. This causes the T cells to consider the host cells with parasitic proteins to be 'foreign', leading to the opening of the intercellular junctions and thereby compromising blood-brain barrier. The transfer of *Plasmodium* proteins to the host cells were accelerated in the presence of TNF-α, thus strengthening the TNF-α theory **(Jambou et al., 2010)**. This seminal paper is almost like a paradigm shift in the field of malaria and clearly explains the basis of CM pathogenesis.

T lymphocytes are a major role player in CM. The *PbA*-infected mice were protected from CM when T cells cultured *in vitro* against *Plasmodium* were transferred to the mice **(Finley et al., 1983)**. However, T cell recruitment in the brain leads to neuroinflammation, and $CD8^+$ T cells contribute to iRBC sequestration and CM pathogenesis **(McQuillan et al., 2010)**. The depletion of T cells (both $CD4^+$ or $CD8^+$) has been reported to protect mice from CM **(Chang et al., 2001; Grau et al., 1986; Hermsen et al., 1997a)**. A recent study on murine CM showed that brain stem region undergoes neuronal apoptosis induced by $CD8^+$ T cells. The treatment of mice with lymphocyte function-associated antigen-1 prevented adhesion of $CD8^+$ T cells in the brain microvessels **(Swanson et al., 2016)**. Most of the $CD8^+$ T cells infiltrated into the brain expressed granzyme-B and the knockout mice for granzyme-B were protected from CM **(Haque et al., 2011)**. The CD8+ T cells are also involved in the breakdown of the blood-brain barrier **(Suidan et al., 2008)**. Haque et al. **(Haque et al., 2001)** reported that gamma delta T cells infiltrated into the mouse brain upon treatment with interleukin-2 and even made the resistance mice (DBA/2) susceptible to CM. This was confirmed by antibody treatment against the gamma delta T cells which led to the protection of mice against CM.

Microglia (resident macrophages of the brain) help in microbe clearance, nervous system repair and, preservation of axonal pathways and synaptic transmission. Microglia can act as antigen-presenting cells and also produce inflammatory cytokines to coordinate the immune defense **(Colonna and Butovsky, 2017)**. Infection of the brain like CM can activate microglia (amoeboid-shaped) leading to release of proinflammatory cytokines and chemokines. A major chemokine reported to be associated with CM is the CXCL10 and its receptor CXCR3 **(Armah et al., 2007; Griffith et al., 2007; Jain et al., 2008; Nie et al., 2008; Nie et al., 2009)**. The T cells express the receptor CXCR3 and the $CXCR3^+$ T cells resident in the spleen are attracted to the CXCL10 chemokine released by the activated microglia present in the brain and infiltrated natural killer cells, leading to the migration of T cells towards the infection site and inflammation **(Bakmiwewa et al., 2016; Campanella et al., 2008a; Hansen et al.,**

2007; Nie et al., 2009; Wilson et al., 2013). Recent work shows that the CXCL10 derived from neutrophils and monocytes hinders the effective control of malaria infection **(Ioannidis et al., 2016)**. Astrocytes present in the brain also contribute to CM pathogenesis as the microglial phagocytosis of iRBCs and increased deposition of parasite vesicles in the astrocytes lead to increase in CXCL10 levels in mice blood plasma and brain **(Shrivastava et al., 2017)**. Also, astrocyte apoptosis mediated by Fas and Fas-ligand binding is known cause for CM pathology **(Potter et al., 2006)**.

The immune cells and factors like neutrophils **(Chen et al., 2000a)**, natural killer cells **(Hansen et al., 2007)**, dendritic cells **(deWalick et al., 2007)**, CCR5 **(Belnoue et al., 2003)**, suppressor of cytokine signalling-1 (SOCS1) **(Bullen et al., 2003)**, lymphotoxin-beta receptor **(Randall et al., 2008)**, C1q, C5 **(Lackner et al., 2008; Patel et al., 2006)**, CCL2, CXCL9 **(Belnoue et al., 2008; Campanella et al., 2008a)**, protein kinase C theta **(Ohayon et al., 2010a)**, interleukin-6 and interleukin-17 **(Wu et al., 2010)** have been associated with CM pathogenesis. On the contrary, other immune factors like interleukin-10 **(Couper et al., 2008; Kossodo et al., 1997)** and cytolytic T lymphocyte-associated Ag-4 (CD152) **(Jacobs et al., 2002)** have been reported to be protective during CM.

3. Nitric oxide (NO)

Nitric oxide is a gaseous free radical involved in normal physiological as well as pathological conditions. It is produced physiologically by the three nitric oxide synthases (NOS): inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS). The iNOS present in dendritic cells, macrophages and neutrophils can produce NO from L-arginine and L-citrulline. The NO, in turn, combines with superoxide anion ($\bullet O_2^-$) to form peroxynitrite ($ONOO^-$). NO also leads to the production of nitric dioxide (NO_2) and S-nitrosothiols. The NO, $ONOO^-$ and NO_2 together form the reactive nitrogen species (RNS) which induce oxidative stress to kill microbes and are hence important in immunity **(Owen et al., 2013)**. NO does not

need any cellular receptors for action and hence, can easily cross plasma membranes and reach brain leading to localized effects.

The first argument is that increase in NO levels can lead to CM. This was supported by the fact that brains of African children affected by CM showed an increase in iNOS staining **(Clark and Cowden, 2003)** and higher NO levels in the cerebrospinal fluid **(Weiss et al., 1998)**. Also, the peripheral blood mononuclear cells from malaria patients showed higher NOS activity *ex vivo* and correlated to lowered levels hemoglobin levels **(Keller et al., 2004)**. However, contrastingly, the levels of arginine required for NO production, were found to be lower in children affected by CM **(Lopansri et al., 2003)**. Also, the disease severity was inversely proportional to the NOx levels in urine and plasma **(Anstey et al., 1996; Boutlis et al., 2003)**. These points suggest that NO might not be the cause for CM pathogenesis, but might be beneficial during CM. However, the increase in NO bioavailability using treatments like tetrahydrobiopterin, arginine and sodium nitrate failed to prevent CM in mice **(Martins et al., 2012)**. The short half-life of NO in plasma (less than 5 sec) makes it difficult for correct estimation of NO levels **(Wennmalm et al., 1993)** and might be the reason for contrasting results of different studies. However, the most recent report about adjunctive therapy with inhaled NO did not significantly ameliorate the CM outcome in patients **(Mwanga-Amumpaire et al., 2015)**.

4. Hemozoin and toxins

About 80% of the host RBC hemoglobin is used up by the *Plasmodium* for its growth **(Rosenthal and Meshnick, 1996)**. The digestion of the heme produces α-hematin, which is toxic to *Plasmodium*. Hence, the *Plasmodium* crystallizes the α-hematin into the non-toxic β-hematin, also known as hemozoin. The presence of hemozoin in the iRBCs is the basis of action of the artemisinin, the potent antimalarial. The unstable peroxide bridge of the artemisinin is broken down in the presence of hemozoin leading to the production of free radicals and specific killing of parasites present in iRBCs.

In vitro studies showed that the monocytes phagocytize the hemozoin leading to a reduction of CD11c and CD54 expression, and leads to the release of TNF-α and matrix metalloproteinase-9 **(Prato et al., 2005; Schwarzer et al., 1998)**. However, another study showed that hemozoin acts as an adjuvant for *Plasmodium* DNA and immunologically stimulates TLR9 **(Parroche et al., 2007)**. The treatment of *PbA*-infected mice with heme (hemin; 30 mg/kg) two times per day between days three and six p.i. led to decreased neuronal damage. Heme treatment led to increased heme oxygenase-1 gene expression, prevented microglial activation and decreased TNF-α, IFN-γ and CXCL10 cytokine gene expressions **(Dalko et al., 2016)**.

The *Plasmodium* plasma membrane is anchored with merozoite surface proteins linked by the glycosylphosphatidylinositol (GPI). The macrophages produce various cytokines during exposure to the GPI toxin **(Gazzinelli and Denkers, 2006; Gazzinelli et al., 2014)**. GPI leads to activation of the TLR4 dimer and NOS in macrophages. Also, the GPI with two chains of fatty acids stimulates the TLR2-TLR6 dimer complex whereas the GPI with three chains of fatty acids stimulates the TLR1-TLR2 dimer complex. The ICAM-1, VCAM-1, and E-selectin proteins are upregulated on endothelial cells upon exposure to GPI, thereby facilitating sequestration **(Durai et al., 2013; Krishnegowda et al., 2005; Schofield and Hackett, 1993; Schofield et al., 1996; Tachado et al., 1996)**.

5. Platelets

Platelets are non-nucleated megakaryocyte fragments that are involved in blood clotting. The interaction between platelets and endothelial cells is one of the events in CM pathogenesis **(Lou et al., 1997)**. An *in vitro* study showed that platelets affect permeability and are cytotoxic to endothelial cells of the brain during *Plasmodium* infection **(Wassmer et al., 2006a)**. Another *in vitro* study by the same group supported this view by showing that platelets release transforming growth factor β1 that leads to the apoptosis of brain endothelial cells **(Wassmer et al., 2006b)**. Sequestration of platelets is elevated in the mouse brain during experimental CM. The

platelets bound to brain endothelium and expressing P-selectin and CD40, binds to the leukocytes expressing P-selectin glycoprotein ligand 1 and CD40L respectively. The platelet can act as a bridge in cytoadherence of iRBCs to endothelium, through binding of PfEMP1 of iRBCs and CD36 expressed by platelets **(Schofield and Grau, 2005)**. The antibody treatment against lymphocyte function-associated antigen 1, a protein expressed by platelets, prevented CM **(Grau et al., 1993; Grau et al., 2003)**.

Platelets also help in the agglutination of iRBCs with the involvement of CD36 receptor and the agglutination is associated with severe malaria **(Pain et al., 2001)**. The children affected by severe malaria had an increase in mean platelet volume. The increase in thrombocytosis might be the reaction of bone marrow to compensate for the increased destruction of peripheral platelets **(Ladhani et al., 2002)**. In contrast, another study showed that early treatment of the mice (day one p.i.) with antibodies against CD41 led to increased survivability of *PbA*-infected mice. However, similar treatments on days 4, 5 or 6 had no effect and mice had poor survivability. As platelet sequestration in the brain microvessels occur on day six p.i., and the study suggested that platelets might be involved in early stages of CM pathogenesis and act as initiators of the disease. As the early, as well as late antibody treatments, agree and disagree with the platelet sequestration hypothesis respectively, the study suggests parallel events in CM pathogenesis leading to mortality **(van der Heyde et al., 2005)**. The platelet factor 4 (PF4)/CXCL4, a chemokine derived from platelets, is also involved in CM pathogenesis **(Srivastava et al., 2010)**.

6. Microparticles

Microparticles (MP) are small membrane-bound particles (0.1 – 1 μm diameter) released from the cell surface when its phospholipid bilayer asymmetry is lost **(Couper et al., 2010a; Enjeti et al., 2007)**. Platelets normally release them in healthy animals, but leucocytes, endothelial cells, and erythrocytes can also release MPs under certain conditions like stress, complement activation, cell death or damage **(Rubin et al., 2010)**. They are different from exosomes (0.03 - 0.1 μm) as exosomes are formed from

the exocytosis of intracellular vesicular bodies and involved in the presentation of antigens **(Enjeti et al., 2007)**. MPs play a major role in coagulation, vascular homeostasis, inflammation and disease pathogenesis **(Rubin et al., 2010)**.

The endothelial microparticles were found to be present in malaria-affected and comatose children **(Combes et al., 2004)**. In human CM, coma scale and thrombocytopenia were associated with platelet-derived microparticles **(Pankoui Mfonkeu et al., 2010)**. Also, elevated levels of cell-derived microparticles and TNF-α were linked to human CM **(Sahu et al., 2013)**. The ATP-binding cassette transporter A1 is involved in the transfer and exposure of phosphatidylserine on the outer side of the plasma membrane hence highlighting cell apoptosis. In the case of murine CM, it was shown that knockout mice for gene of ATP-binding cassette transporter A1 ($ABCA1^{-/-}$) were protected from experimental cerebral malaria with lowered production of TNF-α **(Combes et al., 2005)**. In another study, the microparticles present in plasma of infected mice were shown to stimulate macrophage activity *in vitro* leading to the production of TNF-α and CD40 **(Couper et al., 2010b)**.

Placental malaria or Malaria during pregnancy

Every year, approximately 50 million pregnant women are at risk due to malaria. If a pregnant woman is infected with malaria, the iRBCs get sequestered in the placenta (a uterine organ that provides oxygen and nutrients to the fetus via umbilical cord). This affects the normal blood flow and nutrient supply to the fetus leading to low-birth weight during delivery or stillbirth. The loss of iRBCs during malaria leads to maternal anemia, which is a factor for placental malaria **(Brabin et al., 2004)**. The density of iRBCs sequestered in the placenta is higher compared to that of iRBC densities in the peripheral circulation **(Duffy, 2001)**.

Similar to cerebral malaria, the parasite PfEMP1 protein promotes the sequestration of iRBCs in the intervillous spaces **(Rogerson et al., 2003b; Rogerson et al., 2007)**.

The PfEMP1 of the iRBCs bind to the hyaluronic acid **(Beeson et al., 2000)**, chondroitin sulphate A (CSA) **(Rogerson and Brown, 1997)** present in the trophoblast of the placenta **(Fig.8.)**. However, there is no binding of iRBCs to ICAM-1 or CD36, and there is an absence of rosetting **(Maubert et al., 1998; Rogerson et al., 2000; Rogerson et al., 2007)**. The intervillous spaces of the placenta get infiltrated with monocytes and hemozoin deposition in prominent **(Fig.8.)**.

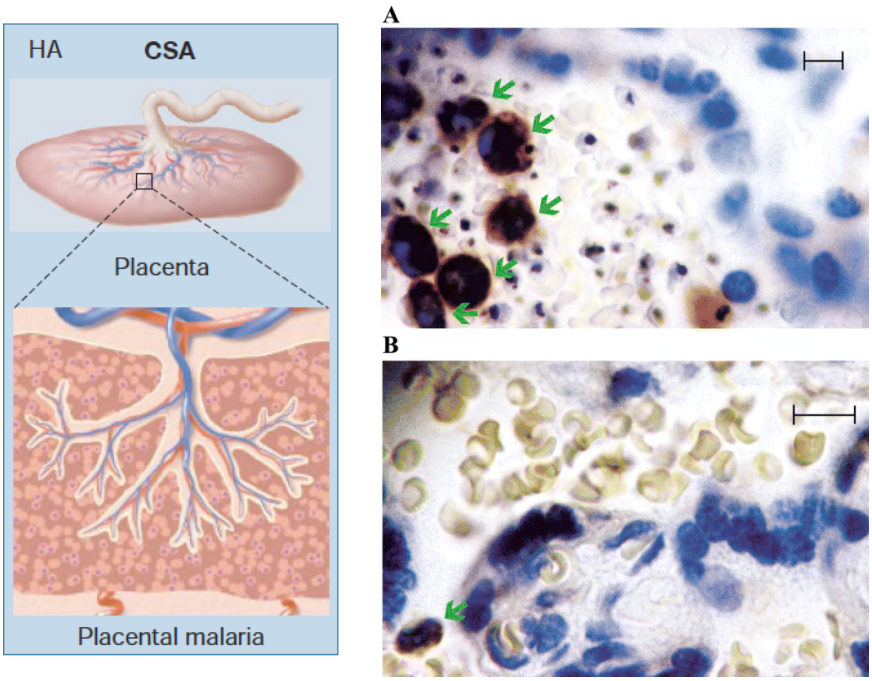


Fig.8. The illustration showing the receptors involved in sequestration and cytoadherence in the placenta; and immunohistochemical staining of the placenta (A) infected and (B) non-infected, with CD68, a marker for monocytes and macrophages (green arrows shows CD68 staining, bar = 10μm). Illustration reproduced from Miller et al. **(Miller et al., 2002)** and immunohistochemical images reproduced from **(Suguitan et al., 2003)**.

The immune system of the mother plays a major role in the placental malaria outcome. Placental malaria affects primigravid (first-time pregnant) women more as multigravid (more than one pregnancy) women have immunologically exposed to the parasite in malaria endemic areas. Fried et al. **(Fried et al., 1998)** showed that the IgG antibodies of multigravidae serum but not primigravidae serum were able to block the binding of iRBCs and CSA thus providing evidence for the previous statement. The exposure of iRBCs lead placental cells to release chemokines like CXCL10 **(Chaisavaneeyakorn et al., 2002; Suguitan et al., 2003)** and monocyte chemoattractant protein 1 (MCP1) **(Abrams et al., 2003; Suguitan et al., 2003)**

which attract monocytes and macrophages. Release of TNF-α from placental-resident monocytes inhibits erythropoiesis, lead to anemia and is also associated with placental malaria pathogenesis **(Becker et al., 2004; Rogerson et al., 2003a)**. Healthy pregnancies require Th2-type of cytokine response. However, during placental malaria, Th2-type cytokine response is shifted to Th1-type response at the placenta that leads to pre-term deliveries **(Fievet et al., 2001; Kwak-Kim et al., 2005; Wegmann et al., 1993)**.

It was shown that a polymorphism of the calcium pump ATP2B4 (rs10900585) was protective during placental malaria **(Bedu-Addo et al., 2013)**. Low-birth weight deliveries during placental malaria were found to be associated with polymorphisms of *TLR4* (Asp299Gly) and *TLR9* (T-1486C) **(Mockenhaupt et al., 2006b)**. Hence, the studies on genetic polymorphisms in precious human samples provide novel insights into the placental malaria pathogenesis.

Objectives of the present study

1. To study the status of AMP-activated protein kinase (AMPK) in mouse brain during experimental cerebral malaria

2. To study the effect of minocycline treatment on murine cerebral malaria outcome

3. To elucidate the molecular basis of neuroprotection imparted by minocycline during murine cerebral malaria

4. To study the association of matrix metalloproteinase-9 polymorphism 1562 C>T (rs3918242) with placental malaria

CHAPTER 1

To study the status of AMP-activated protein kinase (AMPK) in mouse brain during experimental cerebral malaria

1. Introduction

Although brain consumes 50% of the whole body glucose, neurons have a low storage capacity of glucose and have limited production of glycogen. Instead, energy demands of the neurons are met by astrocytes, which stores and provides the glycogen necessary for neurons. Limited energy stores and high nutrient demands by neurons make brain prone to metabolic stress during pathological conditions like infection **(Poels et al., 2009)**. Hypoglycemia is a major symptom of malaria infection. In contrast, several studies on glucose consumption in malaria patients reported an increase in plasma glucose levels **(Agbenyega et al., 2000; Davis et al., 1993; Dekker et al., 1997; van Thien et al., 2001)**. However, Jakka et al. **(Jakka et al., 2006)** reported that CSF glucose concentrations in the brain are decreased to 2.7 mmol/L in CM patients compared to 3.3 mmol/L in controls which point the difference in glucose levels of brain and plasma. The parasite *Plasmodium* consumes host glucose for its energy demands, and the glucose consumption of parasitized RBCs are increased 75 times compared to normal RBCs **(Sherman, 1979)**. This takes a heavy toll on the energy supply of malaria patients making the scenario worse. Metabolic sensing and energy balance is hence required for proper functioning of the brain during pathological conditions like malaria infection.

Adenosine 5'-monophosphate-activated protein kinase (AMPK), as the name suggests, is a protein kinase activated during increased levels of AMP. It is an enzyme complex comprising of α catalytic subunit (isoforms α1 or α2), β and γ regulatory subunits (isoforms β1 or β2, and isoforms γ1 or γ2 or γ3). The breakdown of high-energy phosphates like ATP releases energy for the cell. Utilization of ATP leads to increase in levels of ADP and AMP that indicates a decline in cellular energy. AMPK gets activated during this energy depletion, and it replenishes the ATP levels by inhibiting synthesis of

proteins and lipids (anabolic pathways) and activating oxidation of fatty acids (catabolic pathways). Hence, AMPK is an important metabolic sensor and helps in maintaining energy homeostasis. Also, AMPK is related to autophagy (cell survival mechanism), which is important during metabolic stress and pathologic conditions **(Weisova et al., 2011; Williams et al., 2011)**.

The mTOR is well known to inhibit autophagy and AMPK inhibits mTOR to promote autophagy **(Kim et al., 2011)**. Gordon et al. **(Gordon et al., 2015)** showed that rapamycin treatment directed against the mammalian target of rapamycin (mTOR) protects mice from CM. It was suggested that AMPK might be playing a role in mTOR regulation in T cells and affecting the CM outcome **(Gordon et al., 2015)**. Also, Ruivo et al. **(Ruivo et al., 2016)** reported that *Plasmodium* modulates host AMPK in the liver during infection, and activation of AMPK decreases the parasite size. However, AMPK is like a 'double-edged sword,' as its activation in neurons has been shown to neuroprotective as well as inhibitory depending on the conditions and cell types used **(Steinberg and Kemp, 2009)**. Understanding the activation status of AMPK in cerebral malaria might promote of future drug discoveries for AMPK activation. The present study investigated the status of AMPK in the mouse brain affected by CM at mRNA and protein level. The C57BL/6 mice infected by rodent-specific *Plasmodium berghei ANKA* were used for present work. We found a decrease in the levels of activated AMPK in the mouse brain during CM.

2. Materials and Methods

2.1 Infection of mice

C57BL/6 mice (male or female, 20-25 g) were bought from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Tarnaka, Hyderabad and maintained at Animal Housing Facility, University of Hyderabad. Mice were provided with animal feed and filtered water *ad libitum*. Animal experiments were commenced after 4-day acclimatization. The Institutional Animal Ethics Committee (IAEC) [as per the guidelines of Committee for the Purpose of Control and Supervision

of Experiments on Animals (CPCSEA)] approved the experiment plan; and the animal experimentation followed all ethical guidelines. *PbA* vials were collected from Malaria Parasite Bank, National Institute of Malarial Research (NIMR), New Delhi, India. After thawing on ice, the *PbA* infected blood was suspended in 5mM PBS. Three mice were injected intra-peritoneally with 200 µl of the *PbA* suspension. The 'source' mice were monitored daily, and when any mouse exhibited CM symptoms after day five p.i. (post-infection), its blood was collected by retro-orbital sinus puncture. This blood was used to infect other mice as previously described except each mouse received 1 X 10^6 iRBCs. Uninfected mice were used as control group. Parasitemia (% of iRBCs/uninfected RBCs) was calculated daily by staining caudal blood smears with 20% Giemsa (Sigma-Aldrich, US). Mice in the infected group that exhibited CM symptoms, like seizures and coma, (day 6-10 p.i.) were anesthetized using an intraperitoneal injection of sodium pentobarbital (100mg/kg) and killed for brain samples. Control mice were killed for brain samples on the same day. Except for histology, all brains were excised, snap-frozen in liquid nitrogen and stored in -80 ^{o}C for further analyses.

2.2 Real-time PCR

RNA was isolated from mouse brains using TRI Reagent® (Sigma-Aldrich, US) as per the standard protocol **(Chomczynski, 1993)** and was quantified using NanoDrop™ spectrophotometer. RNA (1 µg) from each experimental group were then converted to cDNA using BluePrint™ 1st strand cDNA synthesis kit 6115A (Takara) following manufacturer instructions. The cDNA (1 µl) of both experimental groups were subjected to real-time PCR using SYBR® *Premix Ex Taq*™ [Tli RNase H Plus] (Takara Bio Inc., Japan) for the quantifying the expression of the genes *PRKAA1, PRKAA2, PRKAB1, PRKAB2, PRKAG1,* and *PRKAG2.* Genes were amplified using temperature conditions: 40 cycles of 95^{o}C (15 s), 58^{o}C (30 s) and 72^{o}C (30 s) with a final melting stage of 95^{o}C (15 s), 60^{o}C (60 s), 95^{o}C (30 s) and 60^{o}C (15 s). The $\Delta\Delta Ct$ values were used to quantify the gene expressions of *PRKAA1* and *PRKAA2.* Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH)* was used as a reference gene for loading control.

Table.2. Primer nucleotide sequences

PRKAA1	Forward *5'-GGA AGG TTG GAC GAA AAG GAA AGC-3'*
	Reverse *5'-ATG TGT GCA TCA AGC AGG ACG TT-3'*
PRKAA2	Forward *5'-TGC AAA CAT GGG CGG GTT GAA GA-3'*
	Reverse *5'-TTT GGC GAT CCA CAG CTA GTT CGT-3'*
PRKAB1	Forward *5'-AAG GAC ACG GGC ATC TCT TGT GA-3'*
	Reverse *5'-TCT TGT ACC GGT GTG TTG CAC TGA-3'*
PRKAB2	Forward *5'-ATG ATC CGT CAG AGC CTG TGG TTA-3'*
	Reverse *5'-ACG TCT CCG AGC TTT CCA TAG AGT-3'*
PRKAG1	Forward *5'-GCC ACT TGT CTG CAT CTC TCC AAA-3'*
	Reverse *5'-TTT GGC GAT CCA CAG CTA GTT CGT-3'*
PRKAG2	Forward *5'-CTC GCA GTA TTT TGA GGG TGT GGT-3'*
	Reverse *5'-TCT GTC TCC TTC TGT TTG GCA CCT-3'*
GAPDH	Forward *5'-GTG TGA ACG GAT TTG GCC GTA TTG-3'*
	Reverse *5'-TTT GCC GTG AGT GGA GTC ATA CTG-3'*

2.3 Immunoblotting

The protocol described by Timmons et al. was used for extracting cytosolic fractions from mouse brains **(Timmons et al., 2011)**. Mice brains were homogenized in isolation buffer (brain: isolation buffer = 100 mg: 1ml; isolation buffer - 10mM Tris pH 7.4, 0.32M sucrose, 0.25mM Na_2EDTA, 1mM Na_3VO_4, 1mM PMSF, 20mM beta-glycerophosphate & 20mM NaF) using a Polytron® tissue homogenizer. Homogenate was centrifuged at 2000 x g for 3min (4°C) to obtain supernatant '1'. Pellet was resuspended in isolation buffer and centrifuged at 2000 x g for 3min (4°C) to obtain supernatant '2'. Supernatants '1' and '2' were combined and centrifuged at 20,000 x g for 10min (4°C) to obtain the supernatant, i.e., total cytosolic fraction. Protein concentrations were estimated by Bradford method **(Bradford, 1976)**, and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 100 μg of protein used to load each well of 10% SDS-PAGE gel **(Laemmli, 1970)**. After separation of proteins,

gel was transferred overnight to nitrocellulose membrane **(Towbin et al., 1979)**. Nitrocellulose membrane was blocked for 1h (5% skimmed milk in TBS), probed with corresponding primary antibodies (1:1000 in TBS) overnight at 4°C. Primary antibodies anti-phospho-AMPK, anti-AMPK, anti-phospho-ACC (acetyl Co-A carboxylase), anti-ACC, and anti-β-tubulin (Cell Signaling, USA) were used for the study. The membrane was washed three times (TBS, TBST, and TBS) for 5 min each and probed with horseradish peroxidase-tagged secondary antibody (1:30,000) (Sigma-Aldrich, US) for 1h at room temperature. The membrane was washed three times (TBS, TBST, and TBS) for 5 min each. Protein bands were visualized using luminol (Thermo Fisher Scientific, US) and imaged in VersaDoc™ MP 4000 System (Bio-Rad Laboratories, Inc., US). The protein bands were densitometrically quantified using ImageJ software (NIH, US).

2.4 Immunofluorescence

Mice were first anesthetized using an intraperitoneal injection of sodium pentobarbital (100 mg/kg). After perfusion with normal saline (0.9% NaCl) and 4% paraformaldehyde (PFA)/PBS, brains were excised from the mice. Brains were then stored for 48h in 4% PFA/PBS and infiltrated with paraffin wax before microtomy. For immunofluorescence, brain sections (20 μm) were first deparaffinized with xylene and then hydrated with 100%, 95%, and 70% alcohol. Sections were then washed in DDW, PBS and incubated with primary antibody anti-phospho-AMPK (Cell Signaling, US)(1:100) overnight at 4°C. After PBS washes, sections were incubated with Alexa® Fluor-tagged secondary antibody (Life Technologies, US) for 1h at room temperature. Sections were again washed with PBS and subjected to confocal microscopy for fluorescence analysis (Carl-Zeiss, Germany).

2.5. Minocycline treatment

Minocycline, a semi-synthetic tetracycline, was used to treat mice daily (day 1 p.i. for 10 days) to check the effect on brain pAMPK levels. Minocycline was administered intra-peritoneally at a dose of 45 mg/kg.

2.5 Statistical Analysis

The paired t-test was used to analyze all data and graphs were made using Prism 5.0 (GraphPad Software Inc., La Jolla, US). All data were represented as the mean ± standard error of the mean. Results with a P value less than 0.05 were considered significant.

3. Results

3.1 *Parasitemia*

As expected, mice of the CM group had a parasitemia 10-15% before succumbing to cerebral symptoms on days 6-10 p.i. **(Fig.9.)**.

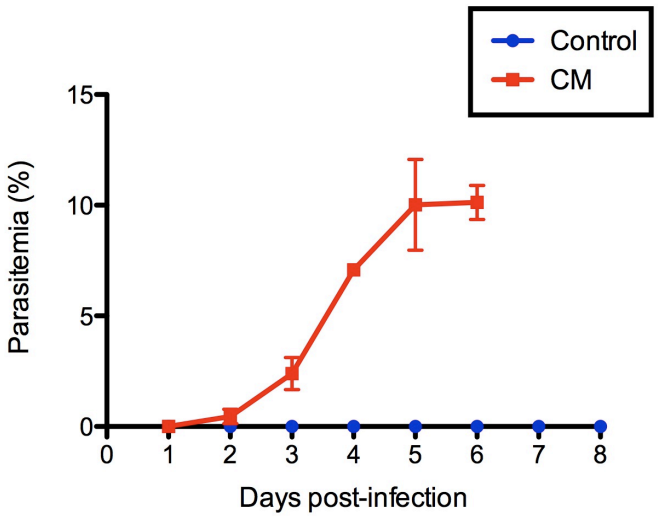


Fig.9. Graph showing the day-wise parasitemia levels in the control and CM group.

3.2 Gene expression of AMPK catalytic subunit isoforms α1 and α2 are decreased in the brain during cerebral malaria

We found statistically significant reduction in the gene expressions of *Prkaa1* (α1 subunit, $P < 0.001$, 95% CI = 0.911 to 1.055) and *Prkaa2* (α2 subunit, $P = 0.025$; 95% CI = 0.139 to 1.216) in the brains of CM mice compared to uninfected control **(Fig.10.)**.

In contrast, gene expression of *Prkab2* (β2 subunit) significantly ($P < 0.05$) increased in CM group compared to control. There was no statistically significant difference ($P > 0.05$) in the mRNA levels of *Prkab1* (β1 subunit), *Prkag1* (γ1 subunit) and *Prkag2* (γ2 subunit) between both groups **(Fig.10.)**.

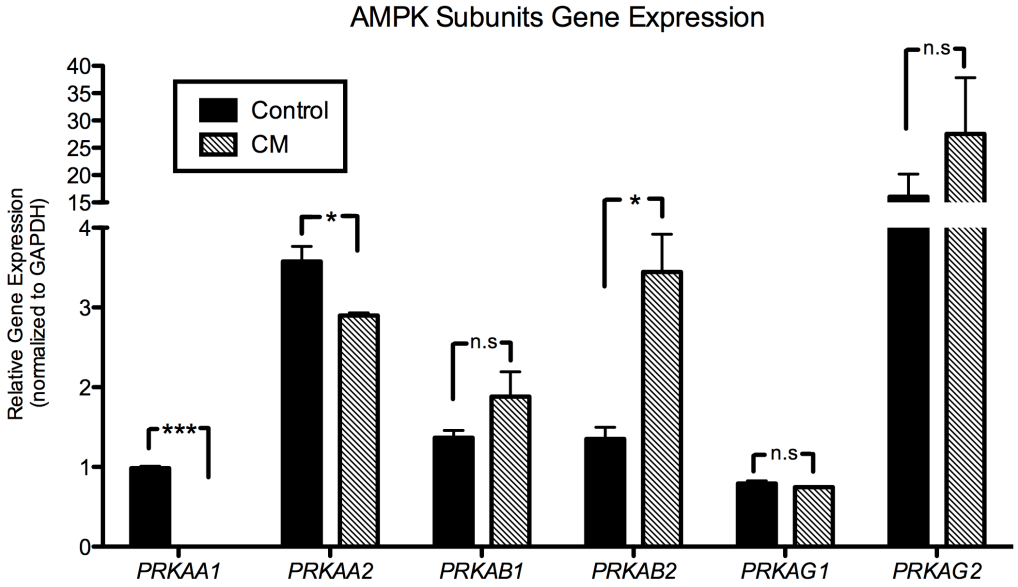


Fig.10. Gene expression of AMPK subunit isoforms α1, α2 and β2, are decreased brain during cerebral malaria. The graph shows the gene expressions of *Prkaa1* (α1 subunit), *Prkaa2* (α2 subunit), *Prkab1* (β1 subunit), *Prkab2* (β2 subunit), *Prkag1* (γ1 subunit) and *Prkag2* (γ2 subunit) in the brains of control and CM mice. The experiment was repeated three times and statistical significances are indicated by: ***, $P < 0.001$; *, $P < 0.05$ and n.s, not significant ($P > 0.05$).

3.3 Total phospho-AMPK (activated AMPK) levels are decreased in the brain during experimental cerebral malaria and was increased after minocycline treatment

There was a statistically significant reduction in the protein levels of phospho-AMPK/AMPK ratio in CM compared to uninfected control ($P = 0.0383$; 95% CI = 0.02985 to 0.06535) **(Fig.11A & B.)**. There was no statistically significant decrease in phospho-ACC/ACC ratio in the brain compared to control ($P = 0.1763$; 95% CI = -3.709 to 1.312) **(Fig.11A & C.)**. Decrease of pAMPK levels in the brain was also confirmed by immunofluorescene studies, which showed a decrease in the number of $pAMPK^{+}$ cells in cortex and striatum of CM brain compared to control **(Fig.12A-E.)**. Total phospho-

AMPK (activated AMPK) levels in mouse brain during CM were increased after minocycline treatment showing the AMPK activating effect of minocycline **(Fig.13A & B)**.

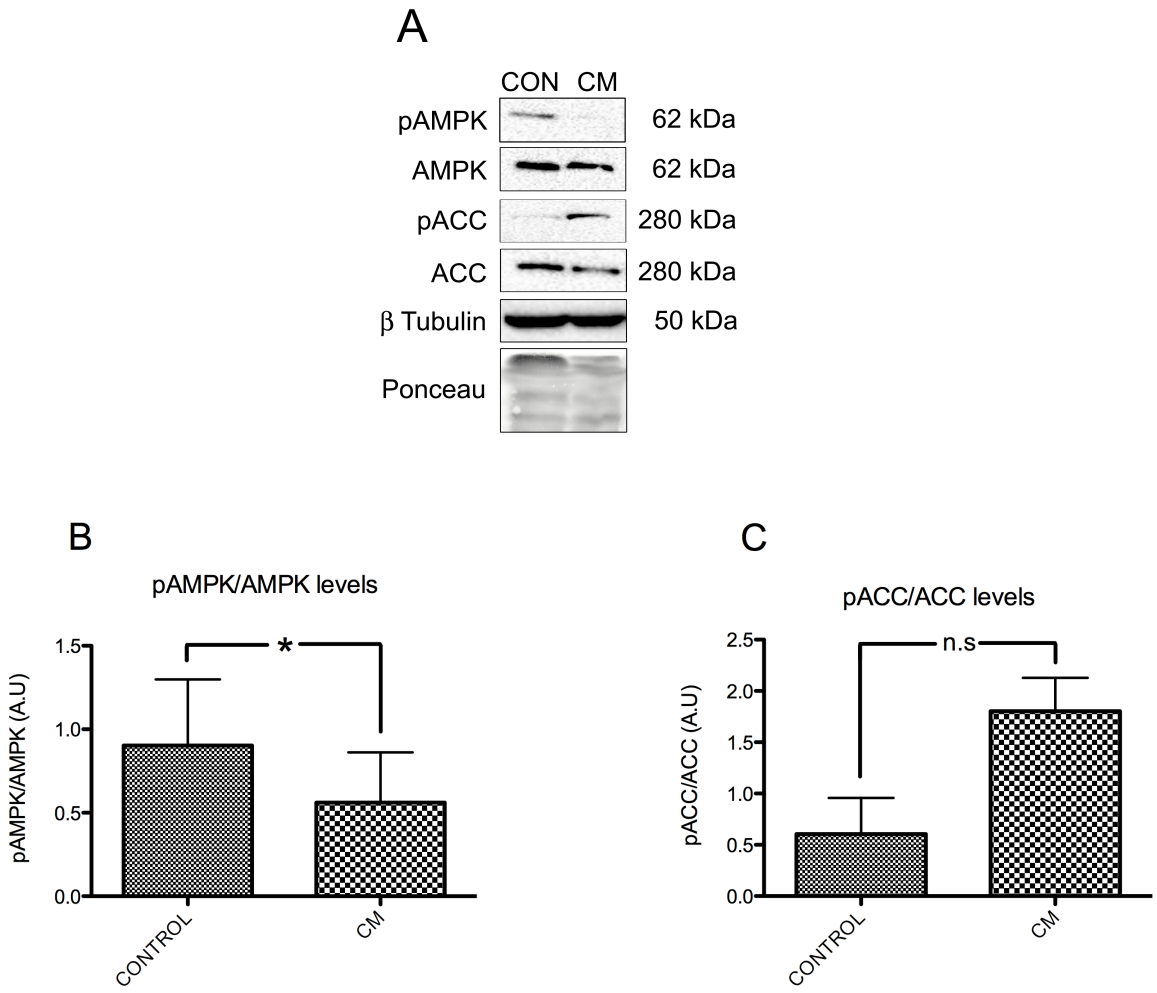


Fig.11. Total phospho-AMPK (activated AMPK) levels are decreased in the brain during experimental cerebral malaria. (A) Representative immunoblots of proteins, p-AMPK, AMPK, p-ACC, ACC in experimental groups: control (CON) and cerebral malaria (CM). Immunoblot of β-tubulin confirms equal loading and absence of protein degradation. (B) Graph showing the pAMPK/AMPK levels in control and CM groups; (n=5). (C) Graph showing the pACC/ACC levels in control and CM groups; (n=3). Statistical significance is indicated by * ($P < 0.05$); and 'n.s' indicate 'not significant' ($P > 0.05$).

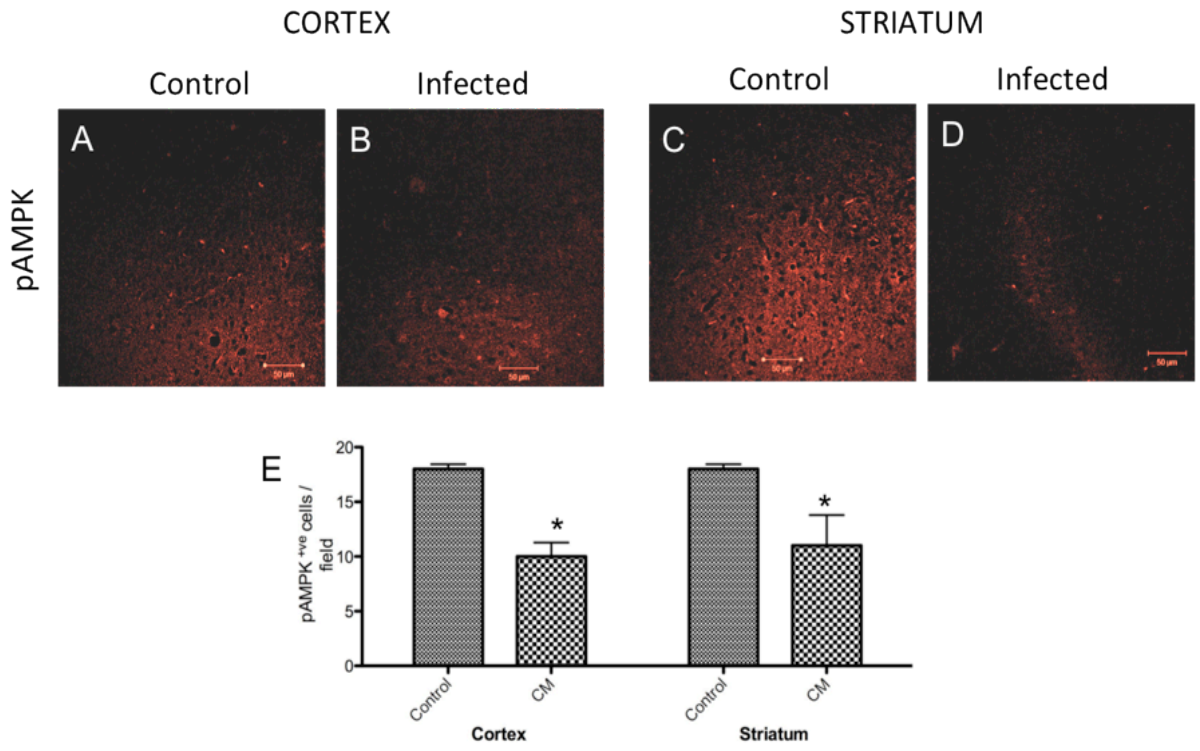


Fig.12. Total phospho-AMPK (activated AMPK) levels are decreased in the brain during experimental cerebral malaria. (A-D) Immunofluorescene results showing cortex and striatal regions of experimental groups, control & infected, stained with p-AMPK (red). Bar = 50μm. (E) Graph showing the pAMPK^{+} cells in control and CM groups; (n=3). Statistical significance is indicated by * ($P < 0.05$).

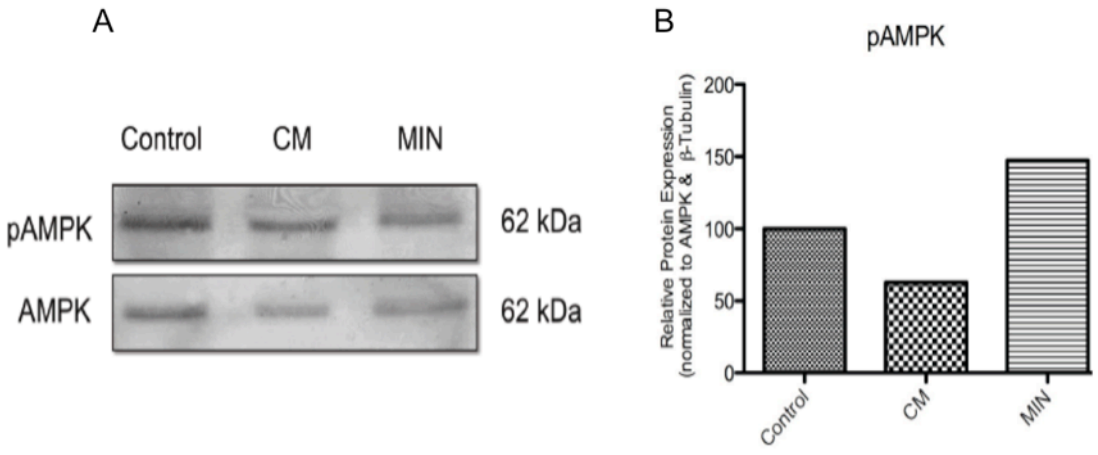


Fig.13. Total phospho-AMPK (activated AMPK) levels in mouse brain during CM are increased after minocycline treatment. (A) Representative immunoblots of proteins, p-AMPK and AMPK in experimental groups: control (CON) and cerebral malaria (CM) and minocycline-treated (MIN). (B) Graph showing the pAMPK/AMPK levels in experimental groups, control, CM and MIN; (n=3).

4. Discussion

Kinases phosphorylate several carbohydrates, proteins or lipids leading to change in activity or binding capacity of the substrates. Hence kinases directly affect cellular metabolism and molecular pathways during normal physiological as well as pathological conditions. Previous research on CM has shown that kinases like glycogen synthase kinase 3 (GSK3), Janus kinase 3 (JAK3), c-Jun N-terminal kinase (JNK), protein kinase C-theta and tyrosine kinases are associated with CM pathogenesis **(Anand and Babu, 2011; Bongfen et al., 2012; Dai et al., 2012; Fauconnier et al., 2011; Kumar et al., 2003; Lacerda-Queiroz et al., 2015; Lu et al., 2006; Ohayon et al., 2010b)**.

AMPK is very crucial for energy homeostasis in the brain, and we show that p-AMPK levels are decreased in the brain during cerebral malaria. Maintenance of optimal levels of AMPK during CM is necessary mainly due to following two reasons. Firstly, brain-derived neurotrophic factor (BDNF) is decreased during ECM **(de Miranda et al., 2015)**. Chen et al. **(Chen et al., 2005)** reported in a mouse model of stroke that AMPK facilitate neurogenesis by the production of BDNF. Secondly, it has been reported that statin treatment confers neuroprotection and reduced neuroinflammation during ECM **(Reis et al., 2012)**. Statins are known to activate AMPK, which in turn activates endothelial nitric oxide synthase (eNOS), and facilitate production of nitric oxide (NO) **(Chen et al., 2005; Li and McCullough, 2010; Sun et al., 2006)**. Endothelial NOS and NO are known to promote neurogenesis and angiogenesis. Also, nitric oxide (NO) dysfunction has been reported in ECM **(Ong et al., 2013)**, and NO treatment led to amelioration of ECM **(Jeney et al., 2014; Zanini et al., 2011)**. Hence, AMPK might be involved directly or indirectly in CM pathogenesis.

The genes of AMPK, *PRKAA1*, *PRKAA2, PRKAB1, PRKAB2, PRKAG1, PRKAG2* and *PRKAG3* code for subunits α1, α2, β1, β2, γ1, γ2, and γ3 respectively. These subunits generate twelve combinations of AMPK enzyme complex and are differentially distributed in the brain **(Ross et al., 2016)**. Among the AMPK α catalytic subunits, α2

is considered to be more important than α1 for brain function as there is a wider distribution of α2 in the mouse brain and there were no change in glucose homeostasis in AMPK $\alpha 1^{-/-}$ knockout mice **(Li and McCullough, 2010; Turnley et al., 1999; Viollet et al., 2003)**. The mRNA levels of AMPK subunit isoforms α1 and α2 were found to be highly downregulated during CM compared to control. The β subunit of AMPK is associated with sensing of glycogen levels **(Polekhina et al., 2003)**. We found an increase in the mRNA levels of β2 isoform in CM brain but no difference in β1 isoform. Studies on knockout mice **(Dasgupta et al., 2012)** revealed that β2 subunit is important during metabolic stress for energy homeostasis. As there are no reports on the levels of glycogen in CM brain, we can interpret the increase in mRNA levels of β2 subunit as a compensatory mechanism of the mouse brain during metabolic stress. The function of γ subunits is not well understood except that AMP binding leads to change in conformation of γ subunit and exposure of catalytic domain of α subunit. We found no change in the gene expressions of γ subunits, γ1 and γ2.

We found a decrease in the protein levels of pAMPK in the CM brain correlating to α subunit gene expression. Our work is in agreement with the work of Ruivo et al. **(Ruivo et al., 2016)** who reported a decrease in pAMPK levels during malaria and the modulation of AMPK by the *Plasmodium* parasite. As there have been reports of anti-inflammatory functions of AMPK **(Zhang et al., 2014; Zhao et al., 2008)** and induction of AMPK leads to poor growth and decrease in the parasite size **(Ruivo et al., 2016)**, there might be a possibility of *Plasmodium* modulating AMPK in the brain too. However, as the earlier work **(Ruivo et al., 2016)** was reported in the liver where the parasite load is more compared to brain, we attribute the decreased AMPK levels in the CM brain due to metabolic fluctuations.

Conventionally, ACC phosphorylation is used as a marker of AMPK activity, as AMPK inhibits fatty acid synthesis by inactivation of this rate-limiting enzyme **(Laderoute et al., 2006)**. Although pAMPK levels were decreased in CM, phosphorylation of ACC increased in CM, which was not statistically significant. The

negative correlation of pAMPK and pACC might be due to phosphorylation status of ACC is not entirely dependent on AMPK activity. A recent study **(Dkhil et al., 2016)** showed that increase in epinephrine in the mouse brain during malaria. The hormone epinephrine is known to activate protein kinase A that phosphorylate and inhibit phosphatase 2A leading inactivation of ACC **(Berg et al., 2015; Lee and Kim, 1978)**. Perhaps the increase in epinephrine in the CM brain might have resulted in inhibition of phosphatase 2A and thereby not affecting the phospho-forms of ACC. Nevertheless, further research is essentially required to unravel the exact mechanism of AMPK signaling after ECM.

5. Conclusion

As AMPK is downregulated in cerebral malaria, there is an increasing need for the discovery of novel drugs that activate and maintain 'optimal levels' of AMPK in the brain. Such novel drugs will confer neuroprotection and hence, might be promising candidates to be used as adjunctive therapy with standard antimalarials.

CHAPTER 2

To study the effect of minocycline treatment on murine cerebral malaria outcome

1. Introduction

Even after standard antimalarial treatment, cerebral malaria survivors are affected by long-term cognition impairment like attention and working memory **(John et al., 2008)**. Cognition impairment is mainly due to the inability of standard antimalarial treatments to prevent neuronal death in regions of the brain associated with cognition **(John et al., 2008)**. Hence, there is a need for an effective drug to prevent the cognitive deficits acquired after standard antimalarial treatment.

In the previous objective, we had additionally checked the effect of minocycline on the AMPK levels. Immunoblotting of pAMPK in experimental groups control, CM and minocycline-treated, which showed that minocycline was able to increase the pAMPK levels compared to CM group **(Fig.13.)**. 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 **(Redin, 1966)**. Minocycline binds to the 30S subunit of the bacterial ribosome, and inhibits bacterial protein synthesis and cell replication **(Jordan et al., 2007)**. Among the first and second generation of tetracyclines, minocycline has the highest lipophilicity (log P, 0.5) which is far higher compared to that of doxycycline (log P, -0.2) **(Fuoco, 2012)**. High lipophilicity enables minocycline to easily cross bio-membranes and also increases its efficacy **(Elewa et al., 2006)**.

In 1972, few years after its development, minocycline was studied in malarial cases **(Colwell et al., 1972; Willerson et al., 1972)** but was not looked further upon. The possibility of the parasite developing resistance to minocycline might be one of the reasons that hindered research on its efficacy in malaria **(Jacobs and Koontz, 1976)**. A report of neuroprotective effects of minocycline in global brain ischemia was published

in 1998 **(Yrjanheikki et al., 1998)**, 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 inhibits matrix metalloproteinases and microbial 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 **(Blum et al., 2004; Kim and Suh, 2009; Yong et al., 2004)**.

Immune effector cells like activated T cells, macrophages, natural killer cells, and dendritic cells migrate towards increasing concentrations of chemokines produced from infection site **(Ioannidis et al., 2014)**. T-cell-mediated cerebral inflammation plays a major role in CM pathogenesis **(Nitcheu et al., 2003)**. 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 **(Miu et al., 2008; Nie et al., 2009)**. We hypothesized that minocycline might be neuroprotective in CM, owing to its ability to modulate the expression of chemokine receptor CXCR3 (receptor for CXCL10) **(Kast, 2008; Kremlev et al., 2007)**. But there have been contrasting reports of minocycline aggravating the disease, for example with different outcomes in different hosts itself **(Diguet et al., 2004)**. Hence, it is very important to study the effect of minocycline treatment in animal models before proceeding to human clinical trials. In this study, we checked the effect of minocycline treatment in a mouse model of CM: C57BL/6 mice infected with *Plasmodium berghei ANKA*.

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), National Institute of Nutrition, Tarnaka, Hyderabad 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 [5mM phosphate-buffered saline (PBS) pH 7.4, 0.9% NaCl] and each 200 µl of the mixture was injected to three 'source' mice via intra-peritoneal (i.p) route. When any of the source mice showed cerebral symptoms during the days 5-10 p.i, its blood was passaged i.p to mice of infected and minocycline groups, so that each mouse received 1 x 10^6 iRBCs. Mice of control group and 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 (22.5mg/kg) with the same regimen. For CM brain samples, infected mice were sacrificed when they developed CM symptoms (strictly between 6^{th} and 10^{th} day p.i.) whereas the minocycline-treated mice were sacrificed on 10^{th} day p.i. In another experiment, infected mice were separately sacrificed on 4^{th} day p.i. after two doses of minocycline, 5^{th} day p.i. after two doses of minocycline, and 6^{th} day p.i. after three doses of minocycline (45 mg/kg daily).

2.2. Survivability test and parasitemia

Mice were monitored on daily basis 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 parasitic 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 (1µg) 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 1µl of cDNA from each experimental group. Primers (Eurofins, India) were used at a concentration of 0.5 picomoles **(Table.3.)**. House keeping gene *GAPDH* was used as internal control.

Table.3. Nucleotide sequences of primers used

PbA 18S rRNA	Forward *5'-CGG TAA TTC CAG CTC CAA TAG CGT-3'*
	Reverse *5'-ATG AAG ATA TCG AGG CGG AGC CAA-3'*
GAPDH	Forward *5'-GTG TGA ACG GAT TTG GCC GTA TTG-3'*
	Reverse *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^{o}C, in 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' **(Promeneur et al., 2012)**.

2.5. Immunoblotting

Brains from each group were homogenized in isolation buffer [10 mM Tris pH 7.4, 0.32 M sucrose, 0.25 mM Na_2EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, 20 mM β-glycerophosphate and 20 mM NaF] using Omni Tip™ tissue homogenizer (Omni International, US). The cytosolic fractions from mice brain homogenates were acquired using the protocol as described by Timmons et al. **(Timmons et al., 2011)**. The homogenate was centrifuged (Kubota, Japan) at 2,000 X g 3 min 4^{o}C to get supernatant 'A' and nuclear pellet 'N'. The pellet N was then re-suspended in isolation buffer and centrifuged again at 2,000 X g, 3 min, 4^{o}C. This supernatant 'B' was combined with supernatant 'A' and centrifuged at 20,000 X g, 10 min, 4^{o}C to obtain supernatant: the complete cytosolic fraction. The protein concentration of complete cytosolic fraction was estimated using Bradford method **(Bradford, 1976)**. Cytosolic fractions from each experimental group (100 µg protein) were separated using 10% and 15% sodium-dodecyl-sulphate polyacrylamide gels, and transferred to nitrocellulose membrane. The nitrocellulose membranes were blocked with 5% skimmed milk in tris-buffered saline (TBS) and incubated with anti-TNF-α and anti-β-tubulin (Cell Signaling Technologies, US) primary rabbit antibodies (dilution 1:1000) overnight at 4^{o}C. Membranes after washing were incubated with alkaline phosphatase-labeled anti-rabbit secondary antibodies (1:30,000) (Sigma-Aldrich, US). Protein bands were detected using 5-bromo-4-chloro-3-indolyl-phosphate - nitro blue tetrazolium (BCIP-NBT) method. Protein levels were quantified by densitometry using ImageJ software (NIH, US).

2.6. Caspase activity assay

The fluorescence emitted during cleavage of false caspase substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin relates to the aggregate enzymatic activity of pro-apoptotic effector caspases 3, 6 and 7. The cytosolic fractions from mice brain homogenates were acquired as in immunoblotting (sub-section 2.4). Cytosolic fractions from each experimental group (100 μg protein) were made up to 100 μL using caspase assay buffer (20 mM HEPES pH 7.4, 10% sucrose, 100 mM NaCl, 10 mM DTT, 0.1% CHAPS, 1 mM EDTA) and incubated at 37°C for 1 h. Caspase substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Sigma-Aldrich, US) was added to all reaction tubes to a final concentration of 5 μM, and caspase assay buffer added to make final reaction volume 1ml. The tubes were incubated again at 37°C for 1 h. The solutions were subjected to spectrofluorometry (FluoroMax®). Fluorometric detection was done at excitation λ = 400 nm and emission λ = 450-500 nm.

2.7 Oxidative Stress Assays

For cytosolic fraction from mice brains, protocol as described by Timmons et al. was followed **(Timmons et al., 2011)**.

2.7.1. Superoxide Dismutase (SOD)

The enzyme activity of SOD was determined following the protocols of Das et al (2001), and Misra and Fridovich (1972) **(Das et al., 2001; Misra and Fridovich, 1972)**. Carbonate/bicarbonate buffer (50 mM $Na_2CO_3.10H_2O/NaHCO_3$, pH 10.2, 700 μM epinephrine) was used for the assay. Tissue lysates were added to the carbonate/bicarbonate buffer (lysate : buffer = 1 : 9) to start the reaction and absorbance was checked at 480 nm. The SOD activity inhibited epinephrine oxidation and the amount of inhibition was used calculate the enzymatic activity; expressed as U/mg protein.

2.7.2. Catalase (CAT)

The enzyme activity of CAT was determined following the protocols of Aebi (1984) and Das et al. (2001) **(Aebi, 1984; Das et al., 2001)**. The tissue lysate (90 µl) was kept on ice for 30min, after which 10µl of Triton X-100 was added. In another tube, 400 µl of phosphate buffer (50mM $Na_2HPO_4.2H_2O/NaH_2PO_4.H_2O$, pH 7, 1mM EDTA) was mixed with 500 µl of H_20_2. The tissue lysate was added to this mixture to start the reaction and absorbance was checked at 240 nm for 30 sec. The molar extinction coefficient 43.6 mM^{-1} cm^{-1} was used to determine the enzyme activity. The enzyme activity was correlated to degradation of H_20_2 and was expressed as U/mg protein.

2.7.3. Glutathione Peroxidase (GPx)

The enzyme activity of GPx was determined following the protocols of Das et al (2001), and Flohé and Günzler (1972) **(Das et al., 2001; Flohe and Gunzler, 1984)**. A hundred microlitres each of phosphate buffer (50mM $Na_2HPO_4.2H_2O/NaH_2PO_4.H_2O$, pH 7), NAPDH (1.5 mM) and GSH (100mM) were mixed together in a tube. The tissue lysate (100 µl) and 500 µl of phosphate buffer (50mM $Na_2HPO_4.2H_2O/NaH_2PO_4.H_2O$, pH 7, 0.34 U glutathione reductase, 1mM EDTA) were added to this mixture; and incubated at 37°C for 10 min. A hundred microlitres of t-butyl hydroperoxide (12mM) were added to the mixture to bring the volume to 1 ml. Absorbance was checked at 340 nm for 3 min. The molar extinction coefficient 6.22 mM^{-1} cm^{-1} was used to determine the enzyme activity. The enzyme activity was correlated to oxidation of NADPH and was expressed as U/mg protein.

2.7.4. Glutathione-S-Transferase (GST)

The enzyme activity of GST was determined following the protocol of Habig et al (1974) **(Habig et al., 1974; Ilavenil, 2012)**. A hundred microlitres of tissue lysate, 100 µl 2,4-Dinitrochlorobenzene (DNCB), 1.7 ml DDW and 1ml of phosphate buffer (similar to CAT activity assay except pH was 6.5 and without EDTA) were mixed in a tube and incubated at 37°C for 5min. A hundred microlitres of GSH was added to this tube to start the reaction. Absorbance was checked at 340 nm to measure the rise in

enzyme optical density. The enzyme activity was correlated to the formation of DNCB-conjugate and was expressed as U/mg protein.

2.7.5. Reduced Glutathione (GSH)

The GSH levels were estimated following the protocols of Ellman et al. (1959) and Moron et al. (1979) **(Ellman, 1959; Moron et al., 1979)**. Two hundred microlitres of tissue lysate were mixed with 2.8 ml of phosphate buffer [0.2M $Na_2HPO_4.2H_2O/NaH_2PO_4.H_2O$, pH 8, 0.6 mM 5, 5' dithio (2-nitrobenzoic acid) (DTNB)] to start the reaction. Absorbance at 412 nm quantified the levels of the compound formed by reaction of GSH and DTNB, which correlated to the GSH levels. Results were compared with phosphate buffer containing 200µl of 5% TCA substitute to tissue lysate. The GSH levels were expressed as µmol/g tissue.

2.8. Real-Time PCR

The cDNA for real-time PCR was synthesized using same method aforementioned in semi-quantitative PCR section. Quantitative PCR for the genes *CXCL10* and *GAPDH* was done using SYBR® *Premix Ex Taq*™ [Tli RNase H Plus] (Takara, Japan) as per manufacturer's protocol in 7500 Fast Real-time PCR system (Applied Biosystems, US). Primers (Eurofins, India) were used at a concentration of 0.1 picomoles **(Table.4.)** and 1 µl of cDNA from each experimental group was used for PCR. *GAPDH* primers, described in semi-quantitative PCR section, were used for quantitative PCR for use as an internal control. Relative changes in gene expression were calculated using $\Delta\Delta C_t$ values.

2.9. Statistical Analysis

All results are shown as the mean ± standard error of the mean. All data were analyzed using one-way ANOVA with Holm-Sidak post-test except for Kaplan-Meir survival curve and graphs were plotted using GraphPad Prism 5.0 software (GraphPad Software, Inc., US). Significance was represented by asterisks: *** indicate $P < 0.001$, ** indicate $P = 0.001$ - 0.01, * indicate $P < 0.05$, and n.s indicate not significant, $P > 0.05$.

Table.4. Nucleotide sequences of primers used

TNF-α	Forward *5'-CCA ACG GCA TGG ATC TCA AAG ACA-3'*
	Reverse *5'-ATG AGA TAG CAA ATC GGC TGA CGG-3'*
IFN-γ	Forward *5'-TCC TCA TGG CTG TTT CTG GCT GTT-3'*
	Reverse *5'-TGT CAC CAT CCT TTT GCC AGT TCC-3'*
CXCL10	Forward *5'-CAA GGG ATC CCT CTC GCA AGG AC-3'*
	Reverse *5'-GGC AAT GAT CTC AAC ACG TGG GCA-3'*
CXCR3	Forward *5'-CAA GGG ATC CCT CTC GCA AGG AC-3'*
	Reverse *5'-GGC AAT GAT CTC AAC ACG TGG GCA-3'*
CCL5	Forward *5'-ATA TGG CTC GGA CAC CAC TC-3'*
	Reverse *5'-GTG ACA AAC ACG ACT GCA AG-3'*
CCL2	Forward *5'-CCA GAT GCA GTT AAC GCC CCA-3'*
	Reverse *5'-CCT CTC TCT TGA GCT TGG TGA CAA A-3'*
CCR2	Forward *5'-CAC ACC CTG TTT CGC TGT AGG AAT-3'*
	Reverse *5'-CTG CAT GGC CTG GTC TAA GTG CT-3'*
MMP-2	Forward *5'- ATC AAC TTT GGA CGC TGG GAG CAT-3'*
	Forward *5'- TTC CCA TAC TTT ACG CGG ACC ACT-3'*
MMP-9	Forward *5'- CAA GGA CGG TTG GTA CTG GAA GTT-3'*
	Forward *5'- ACA CCC ACA TTT GAC GTC CAG AGA-3'*

3. Results

3.1. Minocycline treatment prevents CM, leads to parasite clearance and increases survivability of mice

Sixty percent of mice of infected group succumbed to CM on day 6-8 whereas the rest 40% died of severe anemia on day 14. Mice that received both doses of minocycline (45 mg/kg and 22.5 mg/kg) survived for 90 days corresponding to end of the experiment **(Fig.14A)**. The parasitemia in CM mice was 10-15% during the day of death, i.e. day 7 post-infection (p.i); whereas the parasites were cleared in both minocycline groups on day

7 p.i **(Fig.14B)**. To rule out chances of recrudescence, parasitemia was checked in both minocycline groups after 90 days. Interestingly during days 1 and 2 p.i, parasitemia was found to be lower in minocycline group that received 22.5 mg/kg compared to the group that received 45 mg/kg. But minocycline group that received 22.5 mg/kg showed increased reticulocytosis compared to 45 mg/kg group. As reticulocytosis is linked to anemia **(Jarra and Brown, 1989)**, the dose 45 mg/kg was considered to be optimal and continued for rest of the study. Semi-quantitative PCR, a more sensitive method than microscopy, showed the absence of parasite-specific *18S rRNA* in blood, brain, liver and spleen of minocycline treated group confirming parasite clearance **(Fig.14C)**.

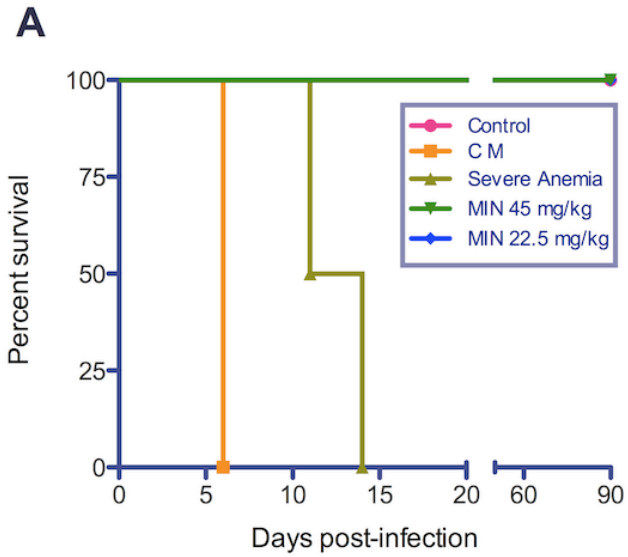
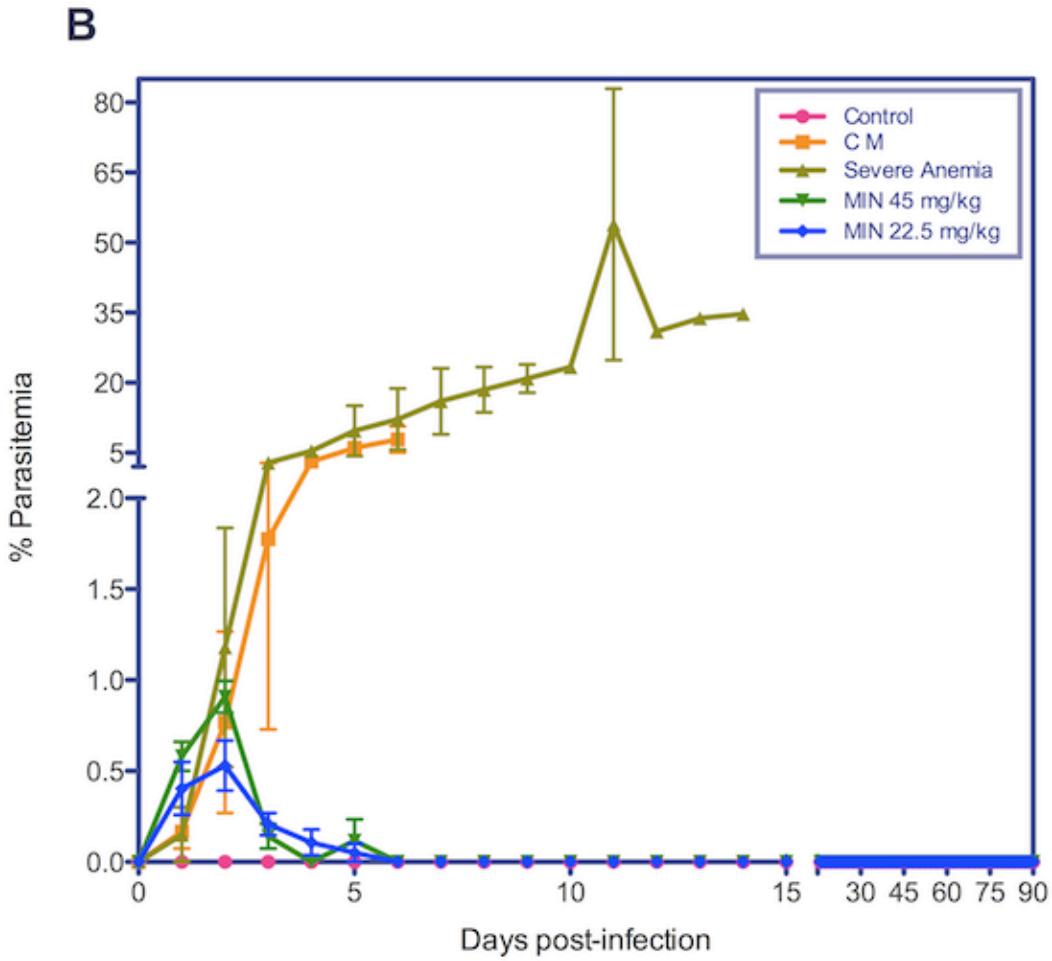
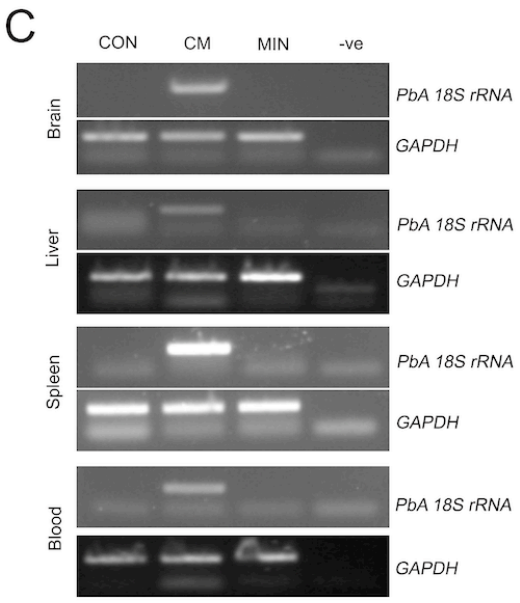


Fig.14. Minocycline treatment prevents CM, leads to parasite clearance and increases the survivability of mice. (A) Kaplan-Meier survival curve showing day-wise percent survival of experimental groups upon minocycline treatment: control (pink line), CM (orange line), severe anemia (brown line), minocycline dose 45 mg/kg (MIN 45 mg/kg, green line) and minocycline dose 22.5 mg/kg (MIN 22.5 mg/kg, blue line). There was 100 % survivability in both minocycline-treated groups (45 mg/kg and 22.5 mg/kg). Due to 100 % survivability, the representative lines of the experimental groups control, minocycline 45 mg/kg and minocycline 22.5 mg/kg in the graph are merged. The experiment was repeated three times (n = 15) and representative data expressed as mean ± SEM is shown. **(B)** Representative parasitemia curve showing day-wise parasitemia of experimental groups: control (pink line), CM (orange line), severe anemia (brown line), minocycline dose 45 mg/kg (MIN 45 mg/kg, green line) and minocycline dose 22.5 mg/kg (MIN 22.5 mg/kg, blue line). Both minocycline doses cleared the parasites on day 6 p.i. but dose 45 mg/kg was found to be favorable and continued throughout rest of the study. The experiment was repeated three times (n = 15) and representative data expressed as mean ± SEM is shown. **(C)** Gene expression of parasite-specific *18S rRNA* in brain, liver, spleen and blood, that correspond to parasitic load. Lanes CON, CM and MIN correspond to control, cerebral malaria and minocycline treated respectively. Lane '–ve' corresponds to 'no template negative control'. House keeping gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as loading control. No amplification was found in minocycline treated group confirming parasite clearance.


B
% Parasitemia
80
65
50
35
20
5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
30 45 60 75 90
Days post-infection
Control
C M
Severe Anemia
MIN 45 mg/kg
MIN 22.5 mg/kg


C
CON
CM
MIN
-ve
Brain
Liver
Spleen
Blood
PbA 18S rRNA
GAPDH

3.2. Minocycline maintains blood-brain barrier (BBB) integrity

The brains of CM mice after Evans blue injection were blue in color indicating that BBB is compromised **(Fig.15B)**. In the case of control and minocycline group, brains were normal pinkish in color **(Fig.15A and C)**. The formamide extraction revealed that the amount of Evans blue extravasated into brain was significantly decreased ($P < 0.001$) in minocycline treated group compared to CM mice **(Fig.15D)**. Low Evans blue extravasation implies that minocycline maintains BBB integrity during infection.

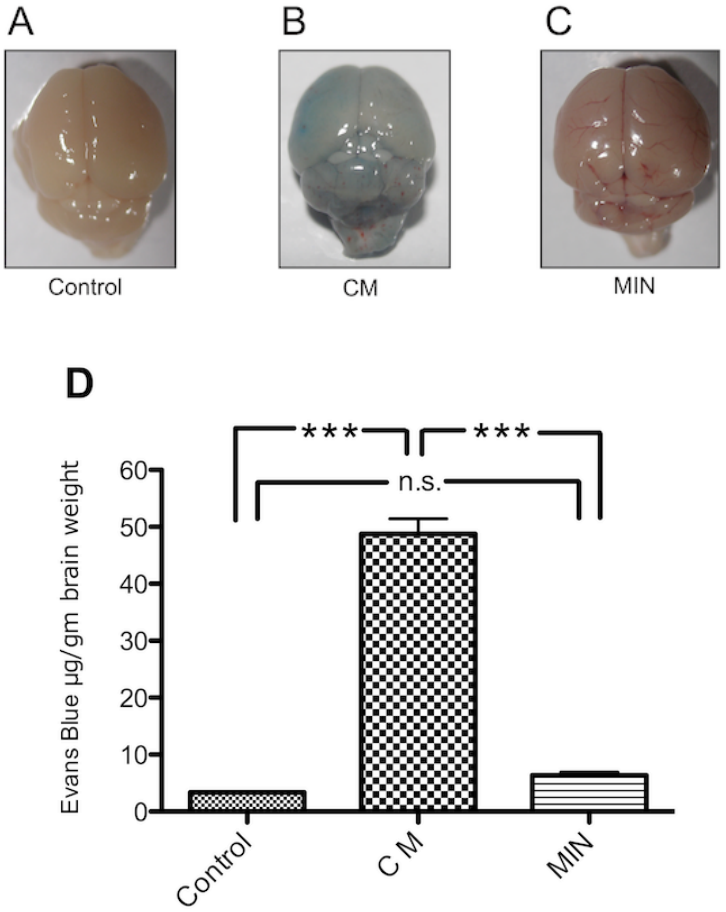


Fig.15. Minocycline maintains BBB integrity. (A-C) Representative images of the brain of different experimental groups, control, CM and minocycline-treated (MIN), after Evans blue injection. The brains of CM group (B) were blue in color showing the breakdown of blood-brain barrier whereas brains of minocycline treated group [C] were normal pinkish in color similar to control (A) indicating maintenance of blood-brain barrier. The experiment was repeated three times and representative data is shown. **(D)** Graph showing the amount of Evans blue extravasated into brains of different experimental groups. There was a significant reduction in Evans blue leakage into the brain in the minocycline-treated group compared to CM group. The experiment was repeated three times; results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM. (*** indicate $P < 0.001$ and n.s indicate not significant, $P > 0.05$).

3.3. Minocycline decreases the protein expression of pro-inflammatory cytokine tumor necrosis factor- α (TNF-α)

The brain cytosolic fractions of CM mice showed high protein expression of TNF-α compared to control group ($P < 0.001$). There was highly significant reduction in the protein levels of TNF-α in minocycline-treated group comparison to CM group ($P < 0.001$; **Fig.16A and B**).

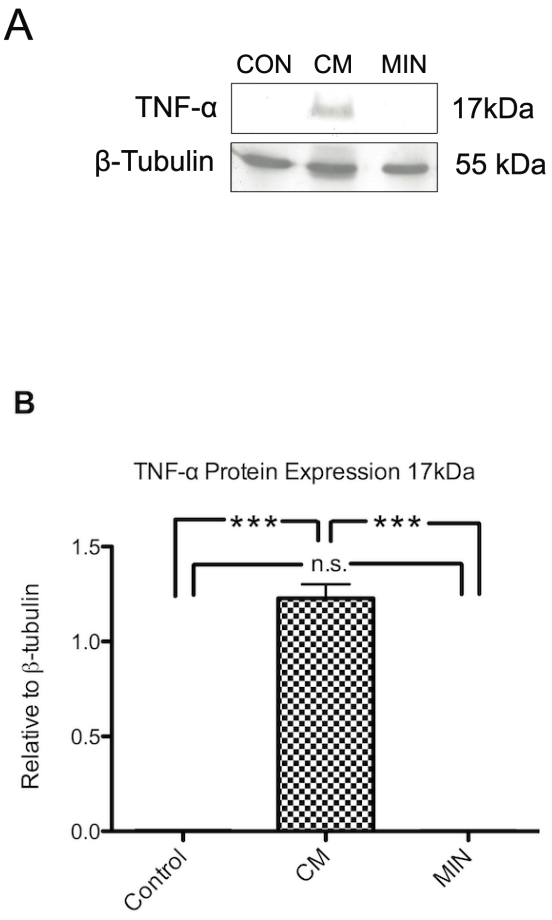


Fig.16. Minocycline decreases the protein expression of pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-alpha). (A) Immunoblot showing the TNF-alpha protein levels in brain samples of different experimental groups: control (CON), cerebral malaria (CM) and minocycline (MIN). The experiment was repeated three times. **(B)** Graph showing the TNF-alpha protein expression in relation to β-tubulin. There was a significant reduction in the TNF-alpha protein expression in the minocycline-treated group compared to CM group. The results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM. (*** indicate $P < 0.001$, ** indicate $P = 0.001$ - 0.01 and n.s indicate not significant, $P > 0.05$).

3.4. Minocycline decreases effector caspase activity

Brain cytosolic fractions of CM mice showed high caspase (effector caspases 3, 6 and 7) activity compared to control group. Minocycline treatment led to inhibition of caspase activity ($P < 0.001$; **Fig.17**). Interestingly, the caspase activity level in minocycline group was even less than in control group, but this was not statistically significant ($P > 0.05$).

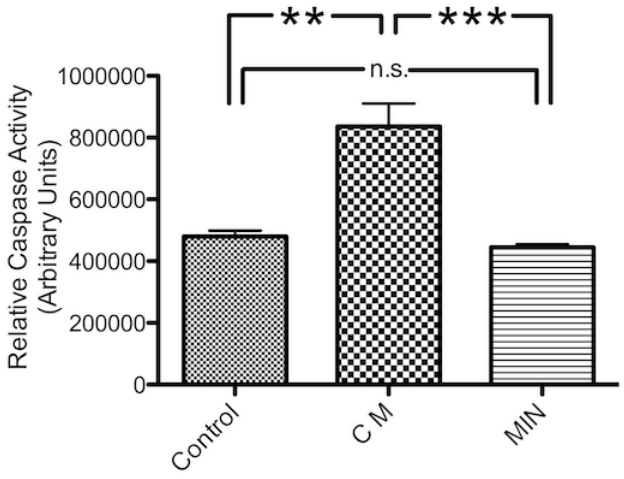


Fig.17. Minocycline decreases effector caspase activity. The graph shows caspase activity levels in brain samples of different experimental groups: control, CM and minocycline-treated (MIN). There was a significant reduction in the activity of effector caspases (-3, -6 and -7) in minocycline-treated group compared to CM group. The experiment was repeated three times; results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM. (*** indicate $P < 0.001$, ** indicate $P = 0.001$ - 0.01 and n.s indicate not significant, $P > 0.05$).

3.5. Minocycline increases ROS scavenging enzyme activities

There was a decrease in the activities of ROS scavenging enzymes and decrease in GSH levels in the CM group **(Fig.18A-E)**. But minocycline treatment led to statistically significant increase in the enzyme activities of SOD ($P < 0.05$; **Fig.18A**), catalase ($P < 0.001$; **Fig.18B**), glutathione peroxidase ($P < 0.05$; **Fig.18C**) and glutathione-S-transferase ($P < 0.01$; **Fig.18D**). There was no statistically significant difference of GSH levels between the CM group and minocycline group ($P > 0.05$; **Fig.18E**).

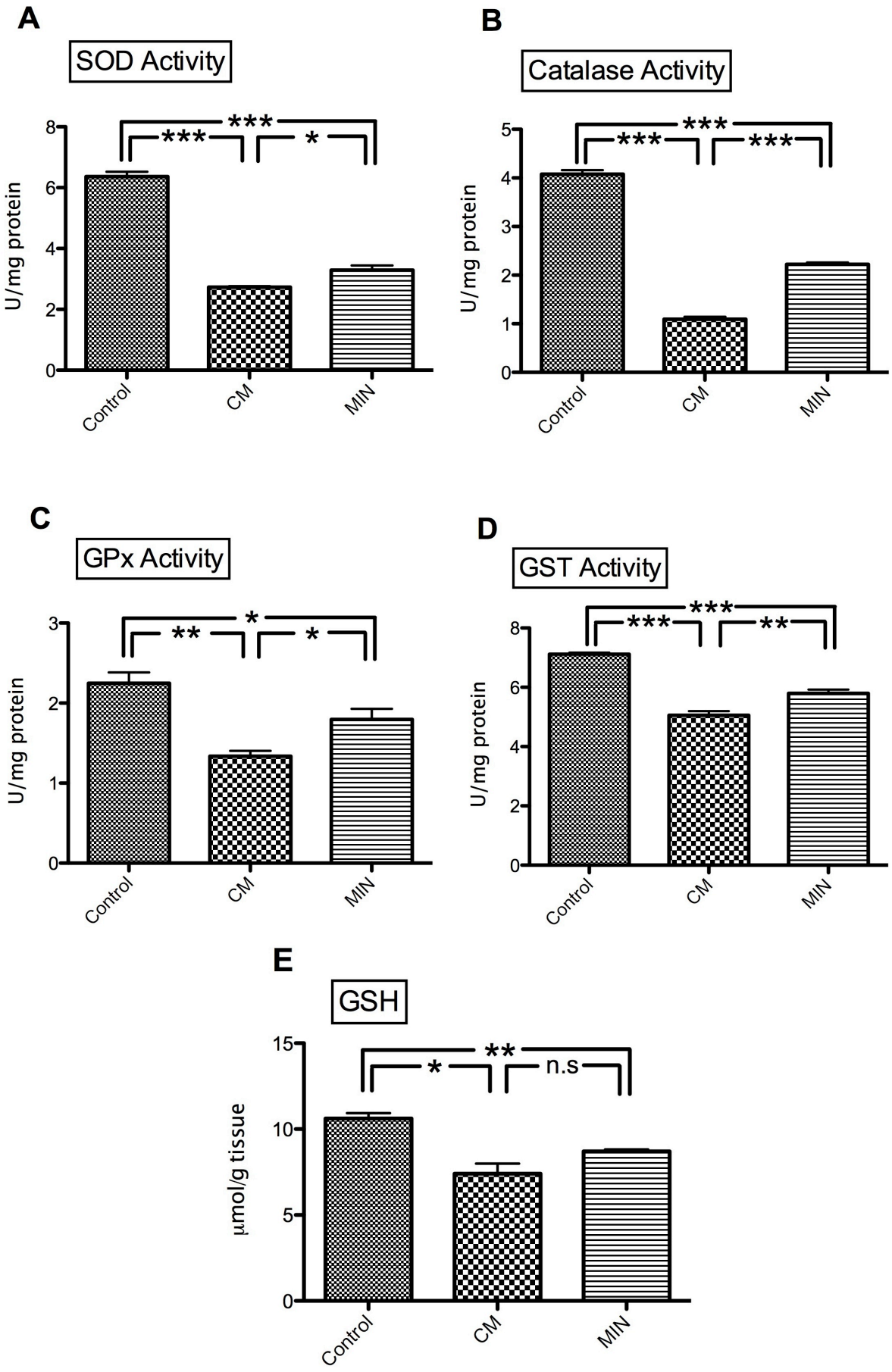
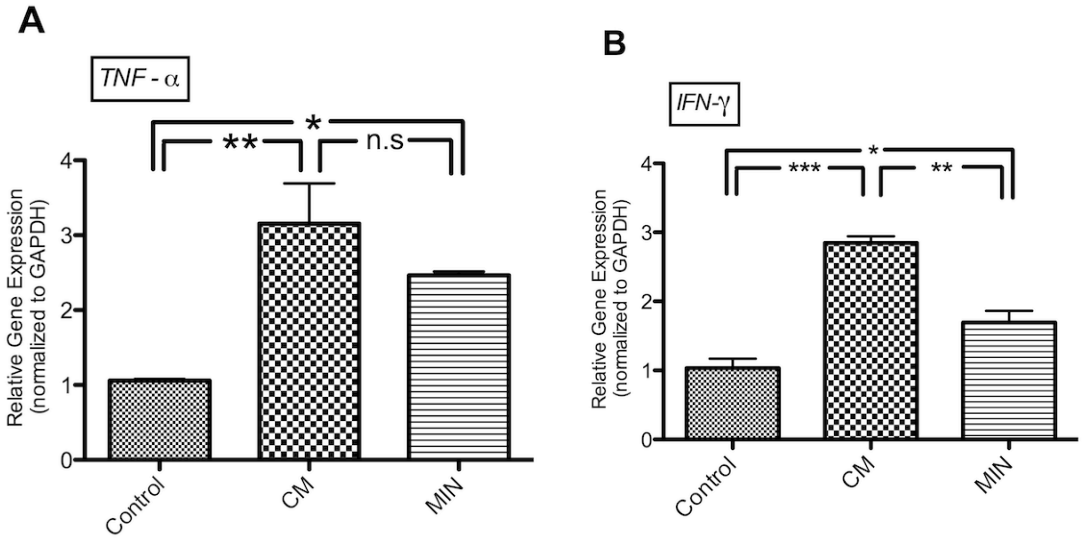
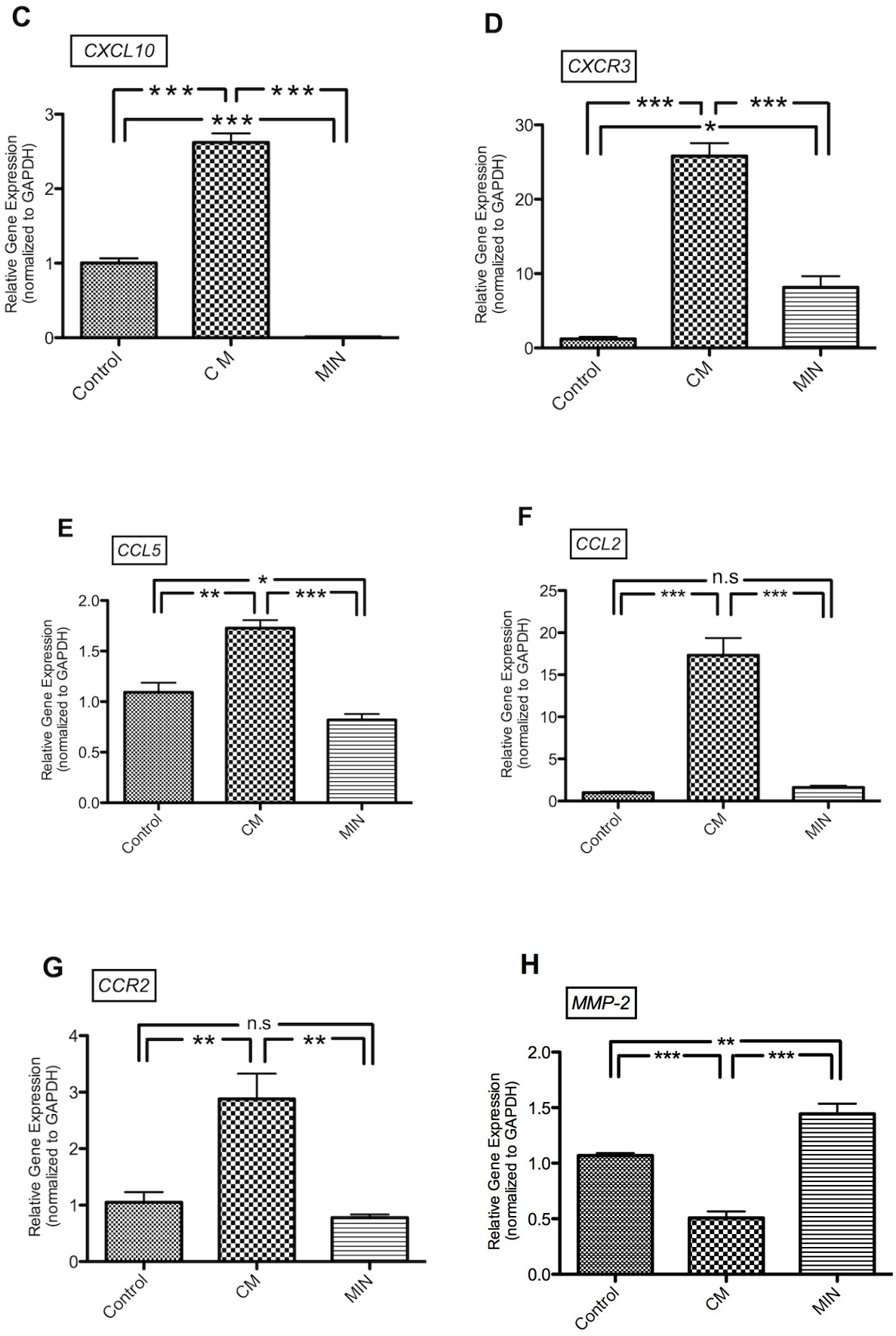

A
SOD Activity
U/mg protein
8
6
4
2
0
***
***
*
Control
CM
MIN
B
Catalase Activity
U/mg protein
5
4
3
2
1
0
***
***
***
Control
CM
MIN
C
GPx Activity
U/mg protein
3
2
1
0
*
**
*
Control
CM
MIN
D
GST Activity
U/mg protein
8
6
4
2
0
***
***
**
Control
CM
MIN
E
GSH
µmol/g tissue
15
10
5
0
**
*
n.s
Control
CM
MIN

Fig.18.(A-E). Minocycline increases activities of ROS scavenging enzymes. The graphs showing the enzyme activities of SOD (A), CAT (B), GPx (C) and GST (D) and levels of GSH (E). Minocycline treatment significantly increased the enzyme activities of SOD ($P < 0.05$; Fig..A), CAT ($P < 0.001$; Fig..B), GPx ($P < 0.05$; Fig..C) and GST ($P < 0.001$-0.01; Fig..D). There was no significant change in the levels of GSH after minocycline treatment ($P = 0.057$; Fig..E). Each experiment was repeated thrice; results were analyzed by one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM. P value < 0.001 represented as ***, P value 0.001 - 0.01 represented as **, P value < 0.05 represented as ** and P value > 0.05 represented as n.s (not significant).

3.6. Minocycline decreases gene expressions of inflammatory mediators but increases gene expressions of gelatinases

Consistent with previous reports, gene expressions of *IFN-gamma*, *CXCL10, CXCR3, CCL5*, *CCL2* and *CCR2* were elevated in the brains of CM mice. The gene expressions of gelatinases, *MMP-2* and *MMP-9* were decreased in CM group. With exception of *TNF-alpha,* minocycline treatment led to significant decrease in the gene expressions of *IFN-γ* ($P < 0.01$; **Fig.19B**), *CXCL10* ($P < 0.001$; **Fig.19C**), *CXCR3* ($P < 0.001$; **Fig.19D**), *CCL5* ($P < 0.001$; **Fig.19E**), *CCL2* ($P < 0.001$; **Fig.19F**) and *CCR2* ($P < 0.01$; **Fig.19G**). Interestingly, *CXCL10* gene expression was almost nullified in minocycline group (relative gene expression = 0.0105). Minocycline treatment led to increase in gene expressions of *MMP-2* ($P < 0.001$; **Fig.19H**) and *MMP-9* ($P < 0.001$; **Fig.19I**).




C
CXCL10
Relative Gene Expression (normalized to GAPDH)
Control
C M
MIN
D
CXCR3
CM
E
CCL5
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CCL2
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MMP-2

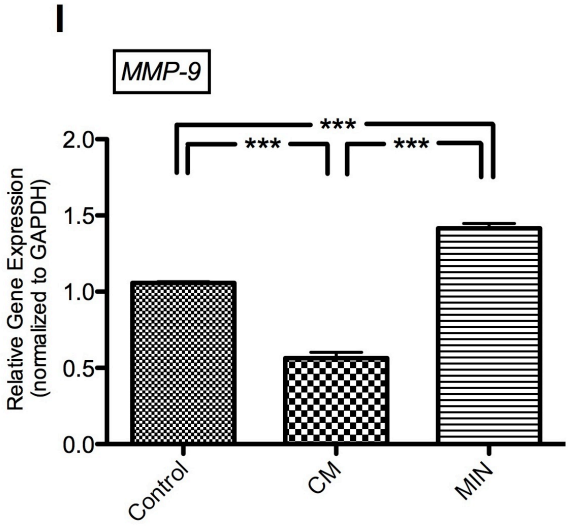

Fig.19.(A-I). Minocycline decreases inflammatory cytokine and chemokine gene expression but increases gelatinase gene expression. Graphs showing gene expressions of *TNF-alpha* (A), *IFN-gamma* (B), *CXCL10* (C), *CXCR3* (D), *CCL5* (E), *CCL2* (F), *CCR2* (G), *MMP2* (H) and *MMP9* (I) in brains of different experimental groups control, CM and minocycline-treated (MIN). There was a significant reduction in the mRNA levels of *IFN-gamma*, *CXCL10, CXCR3, CCL5*, *CCL2* and *CCR2* in minocycline-treated group compare to CM group. There was no significant decrease in the *TNF-alpha* mRNA levels upon minocycline treatment. In the case of gelatinases, MMP2 and MMP9, there was a statistically significant decrease in gene expressions but the gene expressions were increased after minocycline treatment. The experiment was repeated three times; results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM. (*** indicate $P < 0.001$, ** indicate $P = 0.001$ - 0.01, * indicate $P < 0.05$, and n.s indicate not significant, $P > 0.05$).

3.7. Minocycline decreases inflammatory cytokine and chemokine gene expression irrespective of its anti-parasitic activity

In another batch of infected mice treated with minocycline, the mice were sacrificed before parasite clearance. There was no significant change among the minocycline-treated groups compared to CM group ($P > 0.05$; **Fig.20A**). This indicates that the parasite loads in minocycline-treated groups were identical to that of CM group and incomplete parasite clearance by minocycline upon tissue sampling. Gene expression of *IFN-γ* was significantly decreased ($P < 0.01$) in the case of all minocycline-treated groups compared to CM group (**Fig.20B**). There was a highly significant reduction in the *CXCL10* gene expression in experimental groups, 4^{th} day p.i. after two minocycline doses ($P < 0.001$), 5^{th} day p.i. after two minocycline doses ($P < 0.01$), and 6^{th} day p.i. after three minocycline doses ($P < 0.001$, **Fig.20C**). In the case of *CXCR3* gene expression, there was a highly significant reduction in experimental group 6^{th} day p.i. after three minocycline doses compared to CM ($P < 0.001$, **Fig.20D**). There was no significant

reduction of *CXCR3* gene expression in 4th day p.i. after two minocycline doses, and 5th day p.i. after two minocycline doses ($P > 0.05$).

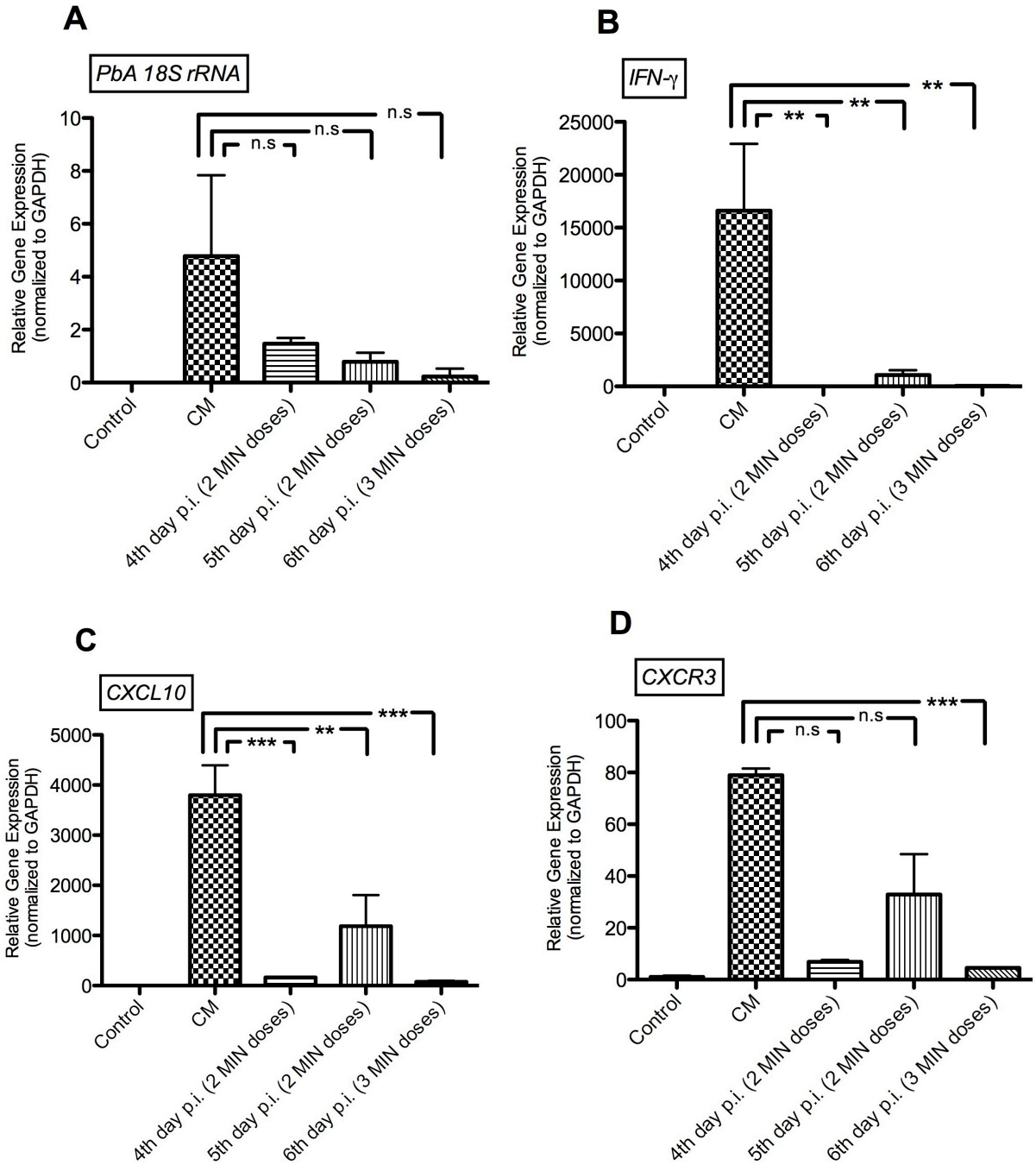

Fig.20.(A-D). Minocycline decreases inflammatory cytokine and chemokine gene expression independent of anti-parasitic activity. Graphs showing gene expressions of *PbA I8S rRNA* (A), *IFN-γ* (B), *CXCL10* (C) and *CXCR3* (D), in brains of different experimental groups control, CM, 4th day p.i. after two minocycline doses, 5th day p.i. after two minocycline doses, and 6th day p.i. after three minocycline doses. The experiment was repeated three times; results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM. (*** indicate $P < 0.001$, ** indicate $P = 0.001$ - 0.01, * indicate $P < 0.05$, and n.s indicate not significant, $P > 0.05$). MIN = minocycline dose, 45 mg/kg daily.

3. Discussion

Most of the knowledge regarding human CM has been from autopsy reports that represent the terminal stages and hence, cannot reveal much about the events that lead to CM. As it is unethical to experiment on humans, animal models have been developed for suitable interventions before the terminal stages. For our study, we used the currently accepted model of CM: C57BL/6 mice infected with *PbA* **(de Souza et al., 2010)**.

In the case of rodents, the dose of minocycline which gives neuroprotective effect is reported to be in the range 10-90 mg/kg **(Elewa et al., 2006)**. A study on the mouse model of Japanese Encephalitis Virus infection was done using two dose regimens, 45 mg/kg and 22.5 mg/kg **(Mishra and Basu, 2008)**. In our study, the effective dose of minocycline was decided on a trial-and-error method. Both doses of 45 mg/kg and 22.5 mg/kg were found to increase the survivability of *PbA* infected mice treated with minocycline. But we found increased reticulocytosis in the mice group that received 22.5 mg/kg. As reticulocytosis is linked to anemia **(Jarra and Brown, 1989)**, we considered the dose of 45 mg/kg to be favorable and was continued through rest of the study.

Minocycline, like any other tetracycline, is anti-parasitic **(Colwell et al., 1972; Dahl et al., 2006; Willerson et al., 1972)**. Tetracyclines bind to the 30S subunit of bacterial ribosomes and prevent binding of aminoacyl-tRNA, leading to inhibition of protein synthesis **(Jordan et al., 2007)**. *Plasmodium* contains apicoplasts (apicomplexan plastid), a cyanobacteria-derived endosymbiotic plastid. The absence of apicoplasts does not lead to the death of parasites, but leads to an error in the development of parasite progenies and thereby an impairment of successful invasion of uninfected blood cells **(Ralph et al., 2001)**. As apicoplasts are of bacterial origin, tetracyclines affect apicoplast of the *Plasmodium* **(Dahl et al., 2006; Jordan et al., 2007)**. In our study, the parasites exposed to minocycline showed morphological irregularities, probably due to impaired protein synthesis, which decreased its virulence. In addition, minocycline has been shown to decrease the activities of *Plasmodium*

mitochondria and plastids **(Lin et al., 2002)**. These might be the reasons for parasite clearance in minocycline-treated mice and mice continued to survive for 90 days (mice monitored for 90 days and experiment ended).

Autopsy studies have shown that TNF-α and interleukin-1β levels are elevated in the human CM brains **(Brown et al., 1999)**. Activation of microglia (resident macrophages in the brain) during infection, leads to the release of inflammatory mediators like pro-inflammatory cytokine TNF-α, reactive oxygen intermediates, reactive nitrogen intermediates; and other products like matrix metalloproteinases, glutamate and quinolinic acid **(Rock and Peterson, 2006)**. Jambou et al. **(Jambou et al., 2010)** showed that TNF-α enhances the transfer of *P.falciparum* antigens from iRBC to human brain endothelial cells, leading to an opening of intercellular junctions and compromising BBB integrity. BBB breakdown occurs via soluble guanylyl cyclase and protein tyrosine kinase activation **(Jambou et al., 2010; Mayhan, 2002)** contributing in CM pathogenesis. In our study, we found that minocycline was able to effectively decrease TNF-α protein levels in brain and maintain BBB integrity. Minocycline, with its ability to inhibit T-cells from producing inflammatory cytokines like TNF-α and interferon-γ **(Kloppenburg et al., 1996)**, might have prevented BBB disruption in minocycline-treated mice.

The release of cytochrome-c, Smac/Diablo (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI) and AIF (apoptosis-inducing factor) from the mitochondria are the molecular events ultimately leading to apoptosis. Minocycline has earlier been shown to inhibit these steps **(Kim and Suh, 2009)**. In addition, minocycline has also been reported to downregulate caspase-1 and caspase-3 expression; and upregulate the anti-apoptotic protein Bcl-2 levels **(Blum et al., 2004; Castanares et al., 2005)**. Minocycline has been shown to inhibit both caspase-dependent and caspase-independent apoptosis **(Heo et al., 2006; Wang et al., 2003)**. Similarly, we found a decrease in apoptosis and inhibition of effector caspase activity in the minocycline-treated group showed compared to the CM group. The

inhibition of apoptosis implies that minocycline effectively inhibits neuronal cell death and provides neuroprotection during CM.

Earlier reports showed that activities of ROS scavenging enzymes like SOD, catalase, glutathione peroxidase and GSH levels were decreased during murine CM **(Dkhil et al., 2016; Linares et al., 2013; Zanini et al., 2012)**. Minocycline has been reported to have anti-oxidant properties, which can be attributed to the chemical structure of minocycline, making it an effective anti-oxidant similar to tocopherol/vitamin E **(Kraus et al., 2005)**. Also, Choi et al. **(Choi et al., 2005)** reported that minocycline was able to inhibit ROS produced by the NADPH oxidase leading to neuroprotection of substantia nigra. Consistent with these reports, we found increase in the activities of ROS scavenger enzymes in the mouse brains of minocycline group proving the anti-oxidant property of minocycline.

Murine cerebral malaria pathology has been correlated to T cell homing in the brain and chemokine receptor CXCR3 **(Campanella et al., 2008b; Miu et al., 2008; Nie et al., 2009)**. Several studies have demonstrated the ability of minocycline to decrease the production of *CXCR3* mRNA **(Kast, 2008; Kremlev et al., 2007)**. Bakmiwewa et al. (2016) showed that IFN-γ induces production of CXCL10 by astrocytes during murine cerebral malaria **(Bakmiwewa et al., 2016)**. Consistent with this study, our gene expression studies in mouse brain showed that minocycline decreased the mRNA levels of *IFN-γ* that accompanied the decrease in expressions of *CXCL10* and *CXCR3.* It is probable that lowered production of CXCL10 in the brain might have contributed to high splenic retention of $CD4^+$ T cells, reduced chemoattraction towards the brain and decreased cerebral inflammation explaining the neuroprotection conferred to infected mice upon minocycline treatment. Also, it is well known that $CD4^+$ T cells play an important role in immunity against malaria via production of cytokines that improve phagocytosis by macrophages **(Nie et al., 2009)**. The rise in splenic $CD4^+$ T cell population might have also contributed to parasite clearance, in addition to the anti-parasitic property of minocycline. Moreover, the leukocyte migration to the brain is also

dependent on the increased gene expressions of CCL5 and CCR2 **(Belnoue et al., 2008)**. Our study shows that minocycline treatment also decreases the gene expressions of *CCL5* and *CCR2* in the mouse brain; consequently preventing cerebral inflammation. The gene expressions gelatinases, *MMP-2* and *MMP-9* were decreased in CM group in agreement with the previous study **(Van den Steen et al., 2006)** and the gene expressions were increased after minocycline treatment. Further studies on protein expressions will reveal whether the MMP-2 and MMP-9 protein levels are maintained at optimal levels after minocycline treatment.

During pathological conditions, CXCL10 is expressed by neurons and astrocytes to attract $CXCR3^{+}$-microglia. Increased expression of CXCL10 and CXCR3 is correlated with microglial activation and dendrite loss **(Rappert et al., 2004)**. Minocycline, by decreasing the production of CXCL10 and CXCR3, might decrease dendrite loss and impart neuroprotection. Wilson et al. **(Wilson et al., 2013)** showed *in vitro* that CXCL10 induces caspase-dependent apoptosis of human brain microvascular endothelial cells and inhibition of CXCL10 using atorvastatin as an adjunctive therapy in experimental cerebral malaria increased the survivability of mice up to 30%. Lowered production of CXCL10 would also have decreased the caspase-dependent apoptosis of endothelial cells associated with BBB.

Limitation of our study is the lack of *Plasmodium* strain resistant to minocycline. Neuroprotective role of minocycline may be further confirmed using a resistant *Plasmodium* strain. The resistance of *Plasmodium* to tetracyclines has been associated to copy numbers of *P. falciparum GTPase TetQ gene* (*pfTetQ*) and *P. falciparum metabolite drug transporter gene (pfmdt)* **(Briolant et al., 2010)**. Extra passaging of *PbA* in mice with increasing doses of minocycline can result in a resistant parasite strain **(Jacobs and Koontz, 1976)**. Future experiments with resistant strain including behavioral analyses can further confirm cognitive improvement in mice. In our study, we showed that minocycline is neuroprotective in murine cerebral malaria. Although there is a chance of parasite developing resistance to minocycline in future, only the anti-parasitic

effect of the minocycline will be affected. The neuroprotective action of minocycline will be intact and will help in preventing cognitive deficits during infection or post-infection.

Another batch of infected mice was treated with daily doses of minocycline (45 mg/kg), two to four days before the onset of cerebral symptoms and mice were sacrificed before complete parasite clearance. Our gene expression studies of the mice brains revealed that minocycline reduced the mRNA levels of inflammatory mediators IFN-γ and CXCL10, and the reduction was irrespective of the parasite load in the brain. Also, minocycline was able to affect *CXCR3* gene expression when three doses of minocycline were given before 6^{th} day (p.i.). Therefore, our gene expression studies confirm that the neuroprotective activity of minocycline was not a consequence of parasite clearance. Although doxycycline, another tetracycline, has been in use as chemoprophylaxis with standard antimalarials, doxycycline lacks the ability to modulate *CXCR3* gene expression and confer neuroprotection **(Kast, 2008)**; which points out that minocycline is more effective than doxycycline for preventing CM.

When we tried rescue treatment during final stages of CM, mice succumbed to CM and rescue treatment was a failure. It has already been discussed that minocycline might be affecting the parasite apicoplasts **(Ralph et al., 2001)**. As mice during the final stage of CM survive only for 3-6 h, minocycline might not be having sufficient 'time window' to affect the parasite division. Hence, except for final stages of CM, minocycline might be a suitable candidate as an adjunctive therapy for conferring neuroprotection and preventing CM.

Considering the pharmacokinetics of minocycline, its half-life in humans is 12-18h **(Agwuh and MacGowan, 2006)** whereas 2-3h in mice **(Andes and Craig, 2002; Carty et al., 2008)**. During i.p administration, the peritoneal cavity acts as a reservoir, slowly releasing and maintaining the levels of minocycline in plasma, thus aiding parasite clearance **(Fagan et al., 2004)**. In addition, mice are given only a single 'high' minocycline dose daily. However, this 'high' dose can be considered to be low dose taking

into consideration the high liver metabolism in rodents. Hence, the mice are in reality, are under-dosed **(Yong et al., 2004)**. However, even after under-dosing, we got favorable outcomes of increased survivability in mice, and most importantly, neuroprotection.

4. Conclusions

Our study shows a promising role of minocycline in experimental cerebral malaria. As minocycline has a neuroprotective action in CM, it might have a major role to play as adjunctive therapy with other antimalarials. But it has to be validated by human clinical trials for use as a neuroprotective agent in human severe malaria cases. If successful in clinical trials, minocycline will help in decreasing the morbidity and mortality associated with this dreadful neurological complication.

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CHAPTER 3

To elucidate the molecular basis of neuroprotection imparted by minocycline during murine cerebral malaria

1. Introduction

In the previous chapter, we had checked the effect of minocycline treatment on the survivability of C57BL/6 mice infected with *PbA*. In this chapter, we conducted histological analysis of CM brain and behavioral studies on mice to check the effect of minocycline treatment.

Learning and memory, the important cognition-associated traits, are affected in CM survivors **(Boivin et al., 2007; Grote et al., 1997; Kihara et al., 2009; Richardson et al., 1997)**. Petechial hemorrhage in the brain is a well known pathology associated with human CM **(Turner, 1997)** and murine CM **(Nacer et al., 2012)**. Hemorrhages can lead to cell death **(Qureshi et al., 2003)** and hemorrhages during CM have been reported to cause memory impairment **(Gall et al., 1999)**. Hence, in the present study, we focused on the ability of minocycline treatment to mitigate hemorrhages in the brain.

Neuronal dendrites harbor spine-like protrusions known as dendritic spines that receive signal transmissions from other excitatory neurons **(Rochefort and Konnerth, 2012)**. The dendritic spines thus help in synaptic transmission along the neurons. The dendritic spine morphology is associated with long-term potentiation and synaptic plasticity. Dendritic spines of length 0.5-6 μm and are classified according to their morphology: cup-shaped, mushroom-shaped, stubby and thin **(Hering and Sheng, 2001; Peters and Kaiserman-Abramof, 1970)**. Bilousova et al. **(Bilousova et al., 2009)** showed in a murine model of fragile X syndrome that minocycline treatment helps in the maturation of dendritic spines. Hence, we checked whether the hippocampal dendritic spine densities of the brain of CM mice are improved upon minocycline treatment. Next

we wanted to correlate the effect of minocycline on CM outcome at histological level and mouse behavior. Hence, we compared the outcome to uninfected control using standard cognitive tests.

2. Materials and Methods

2.1. Infection of mice and drug delivery

C57BL/6 mice were procured from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Tarnaka, Hyderabad and housed at University of Hyderabad Animal House Facility. Filtered water and animal feed were provided *ad libitum*. *PbA* vials were collected from Malaria Parasite Bank, National Institute of Malarial Research (NIMR), New Delhi, India. All experiments in agreement with Institutional Ethical Committee and National Ethical Committee (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines.

Mice were divided into three groups: control (C), infected (I) and infected + minocycline-treated (M). A full vial of *PbA* infected blood was mixed with ice-cold parasite buffer (5mM PBS pH 7.4, 0.9% NaCl) and each 200µl of the mixture was injected intraperitoneally (i.p) to three 'source' mice. When any of the source mice showed cerebral symptoms during days 5-10 p.i, its blood was passaged i.p to mice of groups I & M so that each mouse received 1 x 10^6 pRBCs. Mice of group C & I were given PBS i.p daily. Minocycline hydrochloride (Sigma-Aldrich, M9511) dissolved in PBS pH 7.4 was given to the group M daily dose of 45 mg/kg BW for ten days p.i. The mice that developed cerebral symptoms were considered as CM group and were euthanized for brain sample.

2.2. Survivability Test and Parasitemia

Mice were monitored on daily basis and the day of death was recorded. Blood smears were taken from tail vein daily till the day mouse survived. Smears were stained with Giemsa (Sigma-Aldrich GS-500), and parasitemia was recorded. Parasitemia was calculated as the percentage of pRBCs to normal RBCs.

2.3. Hematoxylin & Eosin (H&E) staining

H&E staining helps in assessing the extent of cell death in tissue sections. Mice were anesthetized with 10% pentobarbital i.p. Brains from each group were perfused with saline and then 4% paraformaldehyde in PBS (pH 7.4). The brains were resected, fixed in 4% paraformaldehyde for 48 h and embedded in paraffin wax. Brain samples were cut into 20 μm sections using microtome (Leica RM 2145, Germany). For H&E staining, the sections were first deparaffinized in xylene. Sections were then hydrated in alcohol series, stained with Meyer's hematoxylin and eosin; dehydrated in alcohol series, cleared in xylene and mounted with DPX. Brain sections were visualized under a light microscope (Olympus BX-51, Japan) and photographed at X400 magnification.

2.4. Fluoro-Jade® C staining

Fluoro-Jade® C (Millipore, US) stains degenerating neurons and is used to assess the degree of neuronal degeneration and cell death. Staining protocol was followed as of Schmued et al. except Fluoro-Jade® C was used at a concentration of 0.001% **(Schmued et al., 2005)**. Fluoro-Jade® C-stained brain sections were imaged using FITC filter in laser scanning confocal microscope (Carl-Zeiss, Germany). Fluoro-Jade® C-positive cells were counted per field using ImageJ software (NIH, US).

2.5. Golgi-Cox Staining for dendritic spine density

For neuronal dendritic spine analyses, Golgi-Cox staining was performed using a modified version of the standard staining protocol **(IHCWorld).** Mice from all experimental groups were anesthetized using Diethyl Ether (SRL, Mumbai). Later, mice were perfused with PBS (pH 7.4) and 4% paraformaldehyde solution. Brains were carefully excised and impregnated with Golgi-Cox solution (5% K_2CrO_4, 5% $HgCl_2$, 5% $K_2Cr_2O_7$ and water at ratios of 5:5:4:10) (Fischer Scientific) for 14 days, with solution refreshed every two days. Brains were then cryoprotected in 15% sucrose and 30% sucrose at 4°C, for 24h each. They were cut into 50μm sections using cryotome (Leica CM1850) and transferred to amino-propyl-tri-ethoxy silane-coated (Sigma-Aldrich) microscopic glass slides. The sections were covered with square-shaped parafilm and

treated with 20% NH_4OH (Fischer Scientific) for 10min **(Wright et al., 2011)**. Sections were dehydrated in alcohol series, cleared in xylene and mounted in DPX. Spine analyses were conducted on Golgi-Cox solution impregnated brain sections. Neurons with only the following features were used for the study: (i) dendrites which are not truncated, (ii) dendrites stained consistently, and (iii) single and untangled dendrites **(Titus et al., 2007)**. Three to five dendrites (with minimum length of 15mm) **(Magarinos et al., 2011)** of a neuron was analyzed and ten-eleven neurons were analyzed of an experimental group **(Peters and Kaiserman-Abramof, 1970)**. Sections were photographed under a light microscope (Olympus BX-51) at 1000X magnification, and the microphotographs were analyzed for spine density difference. Spine analyses by this method do not assess spine density in 3-D but focuses on spines that are parallel to the section plane. Even though total number of dendritic spines might be underestimated, the method can be used to assess the effect of drug treatments as the analysis is consistent among the experimental groups. ImageJ software (NIH) was used to calculate linear spine density **(Spires-Jones et al., 2011)**, which was presented as the number of spines per 7 μm of the dendrite length.

2.6. Immunoblotting

The immunoblotting protocol as described in subsection 2.5 of Chapter 2 was used, except for the primary antibodies. The primary antibodies anti-cyclooxygenase-2 (Cox-2), anti-heat shock protein-70 (HSP-70), anti-Bcl-2-associated X (Bax), anti-B-cell lymphoma 2 (Bcl-2) and anti-caspase-3 were used for immunoblotting and β-tubulin was used as internal control.

2.7. Rapid Murine Coma and Behavioral Scale (RMCBS) for CM assessment

Mice were assessed for the development of CM using rapid murine coma and behavioral scale (RMCBS) as described by Carroll et al. (2010). The following table shows the different behavior and corresponding scores used for calculating the RMCBS **(Table.1.)**. Uninfected control mice show an RMCBS score of 20, but *PbA* infection

leads to a drop in score. Mice were assessed every 12h when the RMCBS score was 16-20 and every 4h if RMCBS was 11-15. Mice were used for cognitive impairment tests when the RMCBS reached <12 **(Carroll et al., 2010)**.

Table.5. Different behavioral parameters used for calculating the Rapid Murine Coma & Behavioral Scale (RMCBS). Mouse behavior parameters are scored and added to obtain the final RMCBS score. The mice with score <12 are eligible for drug treatment or cognitive test. Table adapted from Carroll et al. **(Carroll et al., 2010)**.

SCORE GIVEN	**0**	**1**	**3**
COORDINATION			
Gait	No Gait	Ataxic	Normal Gait
Balance	No extension	Keeps feet on wall	Lifts complete body
EXPLORATORY BEHAVIOR			
Motor Performance	No exploration	2-3 corners in 90 sec	4 corners in 15 sec
STRENGTH AND TONE			
Body Position	Sideways	Hunched position	Extended fully
Limb Strength	Fails to grasp	Weak grasp (weak pull)	Strong grasp (strong pull)
Reflexes and Self-Preservation			
Touch Escape (3 attempts)	No touch escape	1-sided response	2-sided response
Pinna Reflex (3 attempts)	No pinna reflex	1-sided response	2-sided response
Toe Pinch (3 attempts)	No reflex	1-sided response	2-sided response
Aggression	No aggression	Attempt to bite during tail prick	Attempt to bite before tail prick (in 5 sec)

Hygiene-Related Behavior			
Grooming	Loss of hair and no grooming	Piloerection	Clean fur (sheeny)

2.8. Spatial Learning And Memory

2.8.1. T-Maze – for checking memory retention

T-maze is used to study cognitive dysfunction in rodents. In the present study, T-maze was used to study the effect of minocycline in cognition improvement **(Deacon and Rawlins, 2006)**. The tendency to choose a different option in succession to a previously chosen option is known as alternation. If the mouse chooses a different option without the motivation of food, darkness or other mice, it is called as spontaneous alternation. The spontaneous alternation is dependent mainly on the activities of the brain regions: hippocampus, septum and cerebellum; and the neurotransmitters: dopamine, acetyl choline and gamma-amino butyric acid **(Lalonde, 2002)**.

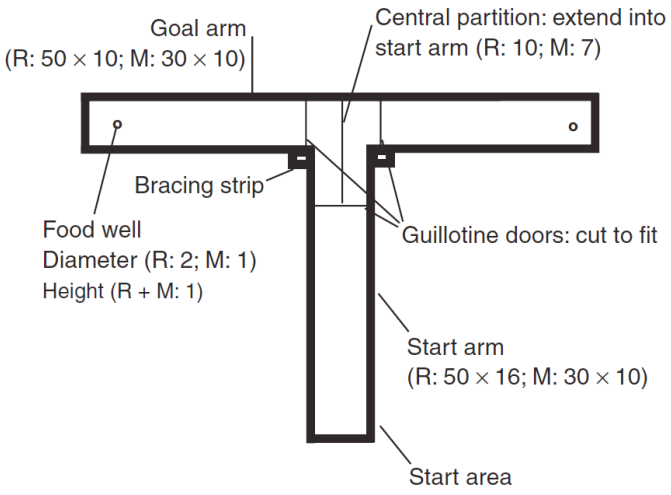


Fig.21. T-maze used for spontaneous alternation in rodents ('R' represents rats and 'M' represents mice). Dimensions are in centimeters. Image reproduced from Deacon and Rawlins **(Deacon and Rawlins, 2006)**.

As the name suggests, the T-maze shaped like a 'T' consisting of a stem (start arm) and two goal arms **(Fig.20.)**. For assessing spontaneous alternation, all guillotine doors were removed except for central partition and the mouse was released into the stem arm (mouse is not let to habituate to the maze before, as novelty is a factor for spontaneous

alternation). Mouse was allowed to choose among the left and right arms. When the arm was chosen, the guillotine door of the corresponding arm was closed. Mouse was confined in the maze for 30 sec and then removed from the maze. All guillotine doors and central partition was removed. Mouse was reintroduced into the stem arm so that mouse faced opposite direction of goal arms (180^{o} of earlier release) and was let to choose a goal arm **(Fig.21.)**. The maze was cleaned with 10% ethanol in between each trial, and each trial was completed within 2 min.

Fig.22. T-Maze

2.9. Non-Spatial Learning And Memory

2.9.1. Novel Object Recognition (NOR) test for checking retrograde amnesia

Rodents tend to explore preferentially novel objects. The ability of rodents to recognize novel objects is dependent on its working memory. In our study, we checked the status of working memory of the mice with the help of novel object recognition test and was done similarly to the work by Desruisseaux et al. **(Desruisseaux et al., 2008)**. A 3-chambered glass apparatus was used for the study **(Fig.22.)**. The apparatus had dimensions 24cm x 12cm x 12cm (length x width x height) and was divided into 3 chambers using two glass sheets of dimensions 12 cm x 8 cm (length x width). Two 3 cm x 3 cm-squared holes were made in the bottom of each glass sheet to facilitate movement of mice between the chambers. The side chambers were numbered as chambers 1 and 3, and the middle chamber was numbered as chamber 2. Before the day of infection, mice of all experimental groups were habituated to the 3-chambered apparatus and mice were allowed to explore two identical objects kept at chambers 1 and 3, for 3 min. Timers

were used to record the time taken by the mice in exploring the novel objects. Mice were then shifted to the home cage. NOR test was commenced when the mice of the infected group had an RMCBS score of < 12, five days post-infection. All mice were introduced into the chamber 2 of the 3-chambered apparatus to explore two objects (familiar object replaced by a novel object) for 3 min. Touching, sniffing or intentional proximity within 3 cm of the object was considered as exploration. The exploration times were recorded, and the preference score was calculated (percentage of time taken to explore novel object and time taken to explore familiar object).

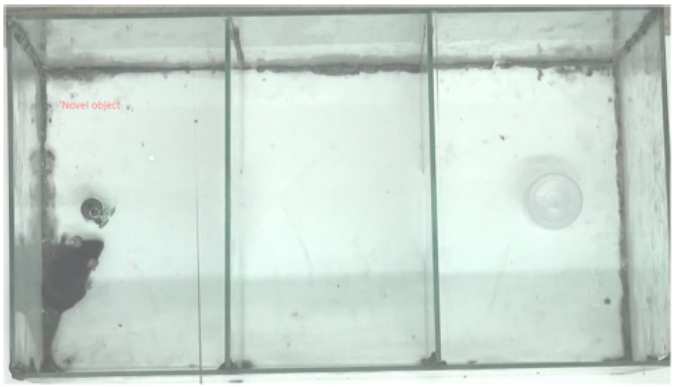
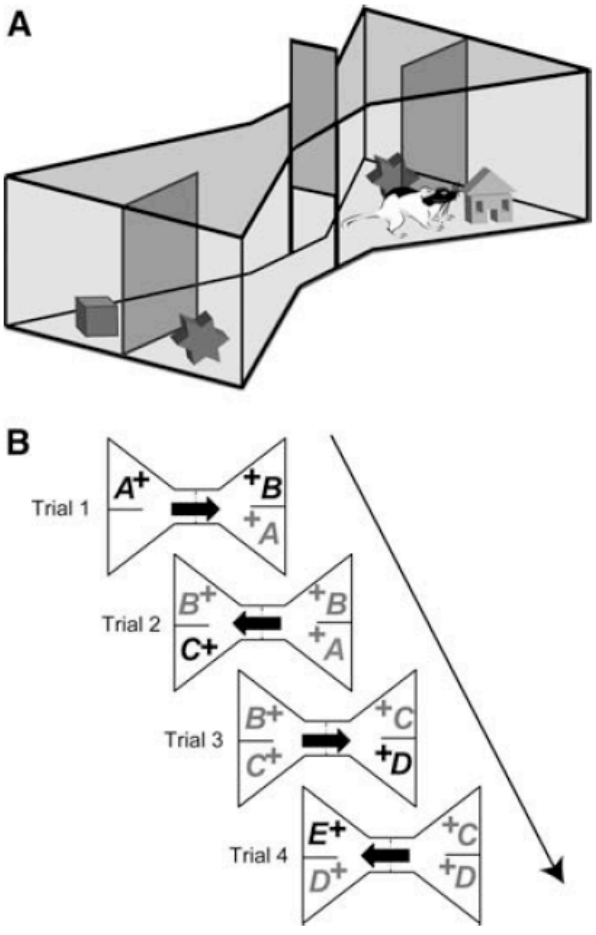


Fig.23. Novel Object Recognition Test with 3-chambered apparatus

2.9.2. Bow-Tie Maze NOR test for checking anterograde amnesia

Bow-tie maze was used to measure the spontaneous preference for novel objects and to assess the recognition memory **(Albasser et al., 2010) (Fig.23A)**. The experiment was commenced when the RMCBS score of the infected mice reached <12, five days post-infection. All mice were subjected to 4 trials in bow-tie maze. During the first trial, mouse was allowed to explore a novel object placed on one side of the bow-tie maze for 1 min. For the second trial, mouse was allowed to move into another side of the maze to explore a familiar object (previously explored novel object) and a novel object for 1 min. In the successive trials, one between the two objects to be explored was a familiar (previously explored) object as described **(Fig.23B)**. The proximity within ± 1 cm of the object, except for mounting on the object, was considered as exploration time and was recorded with a timer **(Fig.23C)**. Experiments were video recorded with a digital camcorder (Sony™ Handycam®) mounted on a tripod (Manfrotto) held above the maze

and the exploration time was also counted by analyzing the video. The following measures were used to calculate the discrimination ratios. The exploration time of novel object minus the exploration time of familiar object gave 'D1'. The sum of individual exploration times of novel objects minus the sum of individual exploration times of familiar objects gave the 'cumulative D1'. The 'D1' was divided by total exploration time of both objects to get 'D2'. This discrimination ratio ranged from +1 to − 1 correlating to preference to either novel object or familiar object respectively. The results were represented as 'updated D2', obtained by D2 recalculation after every trial **(Albasser et al., 2010)**.



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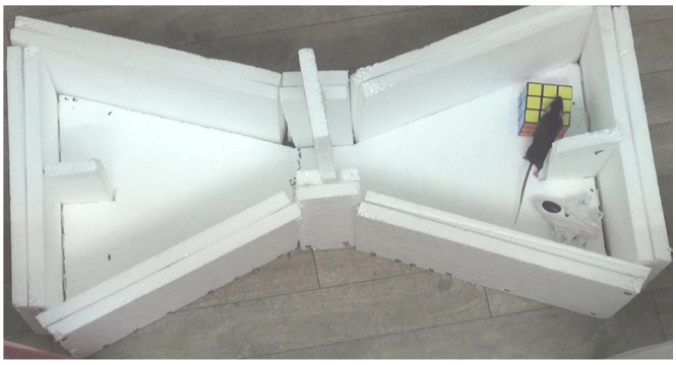

Fig.24.(A). Illustration of a Bow-Tie Maze. **(B)** The pattern of different trials used in the experiment. The mouse was allowed to choose one familiar object and a novel object. The next trial set up in opposite side of the maze had the previously novel object (now familiar object) and another novel object. **(C)** The image of original Bow-Tie Maze used for this study. Images (A) & (B) reproduced from Albasser et al. **(Albasser et al., 2010)**.

2.10. Statistical Analysis

The data were analyzed by one-way ANOVA and post-analyzed by Tukey's multiple comparison tests. Post-analysis for Fluoro-Jade C-staining was done with Holm-Sidak test. Results were depicted as mean ± S.E.M using Prism 5.0 software (GraphPad Software, Inc., US). P values less than 0.05 were considered as statistically significant. The P values <0.05, <0.01 and <0.001 were depicted as *, ** and *** respectively. Not-significant results were depicted as 'n.s' and 95% confidence intervals (95% CI) were reported.

3. Results

3.1. Minocycline contributes to decreased apoptosis and hemorrhage

Hematoxylin & Eosin (H&E) staining of the mouse brain sections showed increased apoptosis (identified by condensed cytoplasm with dark shrunken nuclei and large interstitial spaces) and hemorrhages in CM mice **(Fig. 24B, E, and H)**. Apoptosis was decreased and hemorrhages absent in minocycline group **(Fig. 24C, F & I)**.

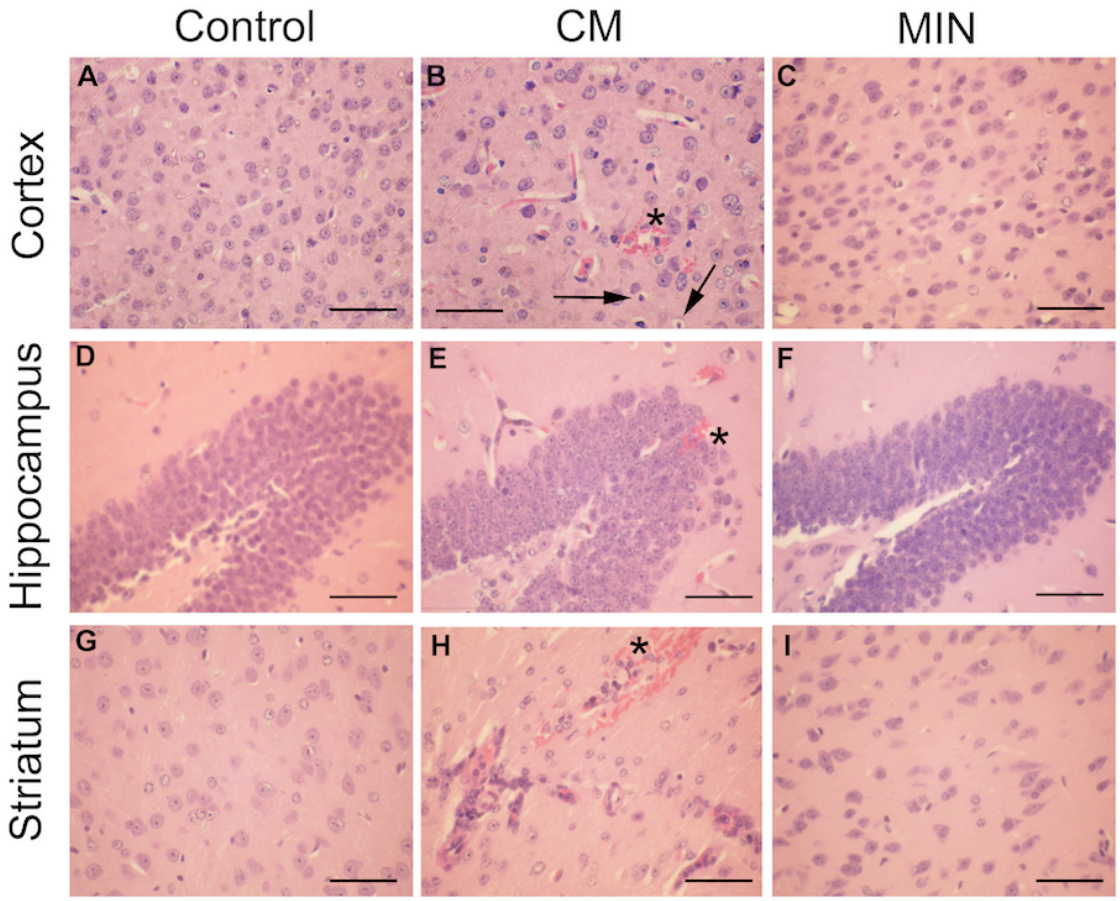

Fig.25. Minocycline treatment leads to decreased apoptosis and hemorrhage. (A-I) Representative images of brain regions (cortical, hippocampal and striatal) of experimental groups control, CM and minocycline-treated (MIN) after H&E staining (Magnification, X400). Arrows indicate the cells undergoing apoptosis and asterisks indicate hemorrhages. The CM group (B, E and H) showed the presence of hemorrhages whereas the minocycline-treated group showed the absence of hemorrhages (C, F and I). Hemorrhage was present in the dentate gyrus of the hippocampus in CM group (E) whereas dentate gyrus protected upon minocycline treatment. (Bars, 50 μm)

3.2. Minocycline prevents neurodegeneration

Fluoro-Jade C (FJC) staining revealed increased number of FJC-positive cells in CM mice brains **(Fig. 25B, E and H)**. In contrast, brains of minocycline group showed a decrease in FJC-positive cells **(Fig. 25C, F and I)** as comparable to that of uninfected control **(Fig. 25A, D and G)**. Specifically, there was a significant reduction of FJC-positive cells in the cortex and striatal regions of the minocycline group ($P < 0.01$). Most importantly, we found the absence of FJC-positive cells in memory associated-hippocampal region of the brain ($P < 0.001$; **Fig.25F**).

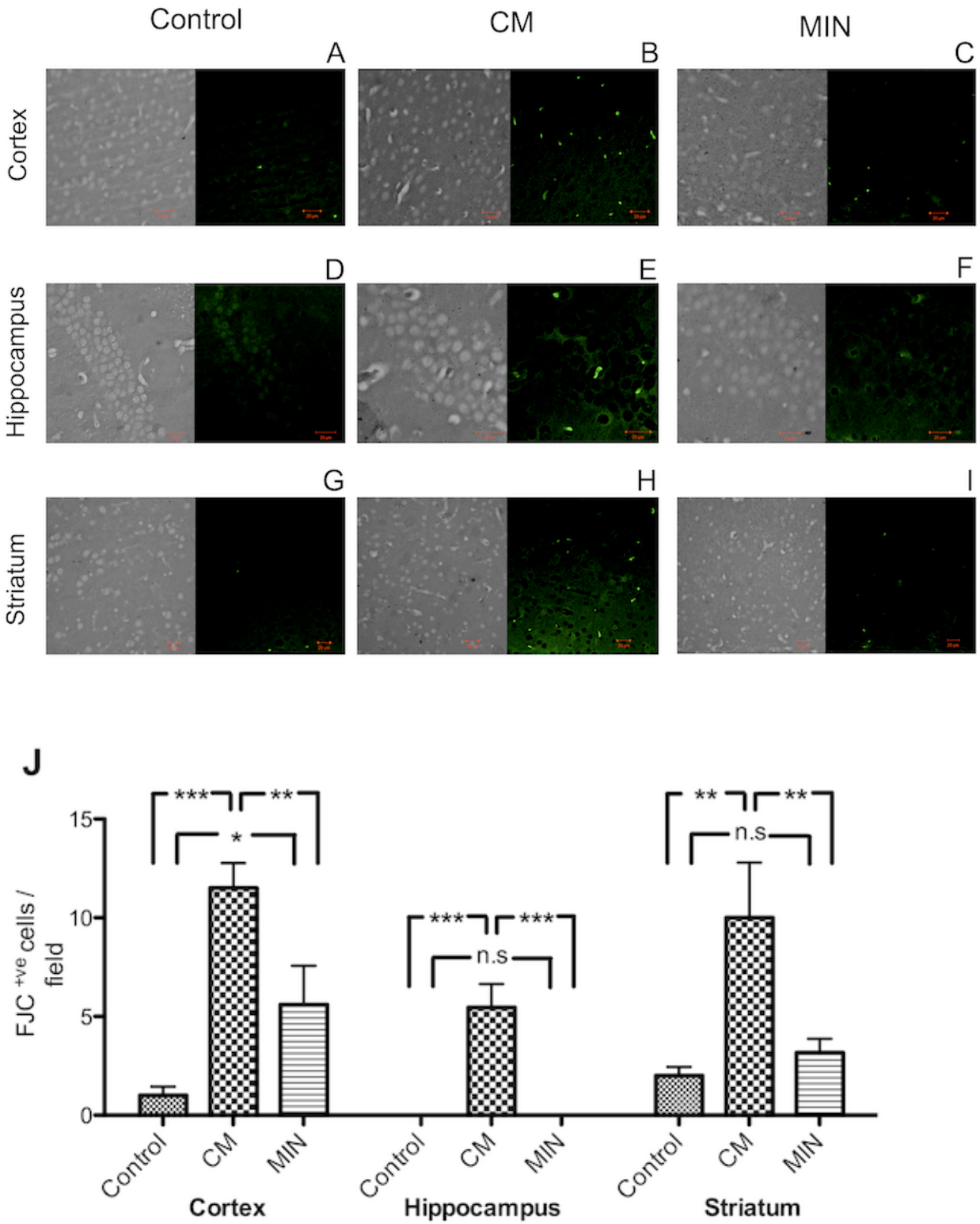

Fig.26. Minocycline prevents neurodegeneration. (A-I) Representative images of brain regions (cortical, hippocampal and striatal) of experimental groups control, CM and minocycline-treated (MIN) after Fluoro-Jade® C staining. Cells stained green indicate degenerating neurons. The CM group (B, E and H) showed increased in degenerating neurons. There was a decrease in neurodegeneration upon minocycline treatment (C, F and I). (Bars, 20 μm). **(J)** Graph showing the number of Fluoro-Jade C positive cells per field (n = 5 fields) in different experimental groups control, CM and minocycline-treated (MIN). Results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM. (*** indicate $P < 0.001$, ** indicate $P = 0.001$ - 0.01, * indicate $P < 0.05$, and n.s indicate not significant, $P > 0.05$).

3.3. Minocycline treatment has no effect on the hippocampal dendritic spine density

We found decrease in hippocampal dendritic spine densities in the case of CM brain compared to uninfected control and the dendritic spine densities were improved upon minocycline treatment **(Fig.26 and 27)**. But as P was > 0.05 we cannot rule out the null hypothesis that there is no difference between the experimental groups. The 95% CI of differences between the groups was as follows: control v/s CM (-0.1601 to 1.9600), CM v/s minocycline-treated (-1.46 to 0.6601) and control v/s minocycline-treated (-0.5601 to 1.560).

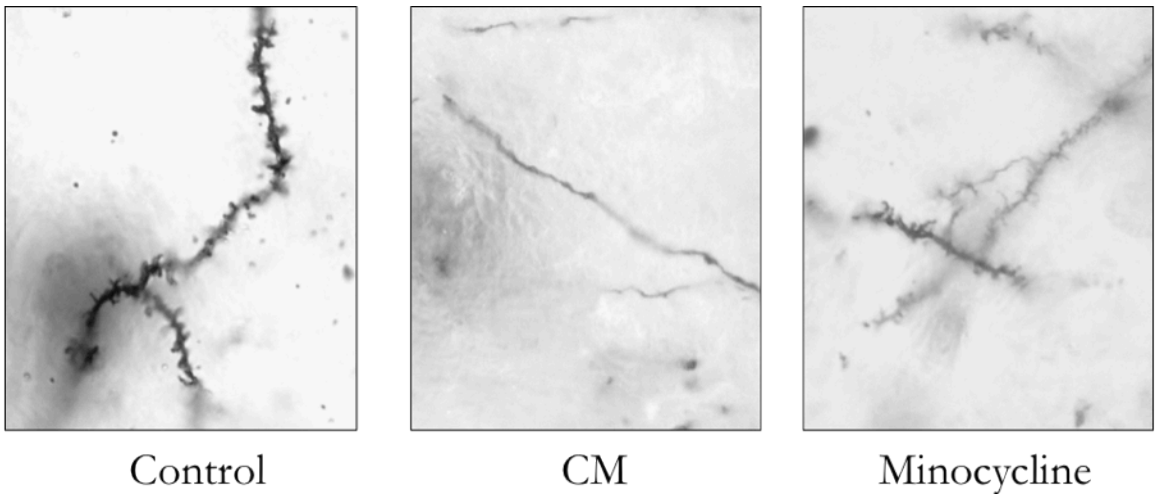

Fig.27. The representative microphotographs showing the Golgi-Cox-stained hippocampal dendritic spines of different experimental groups. The experimental groups of mice were uninfected control, cerebral malaria (CM) and minocycline-treated. Magnification = 1000X. For clarity, the images were converted to black and white using Image J (NIH, US).

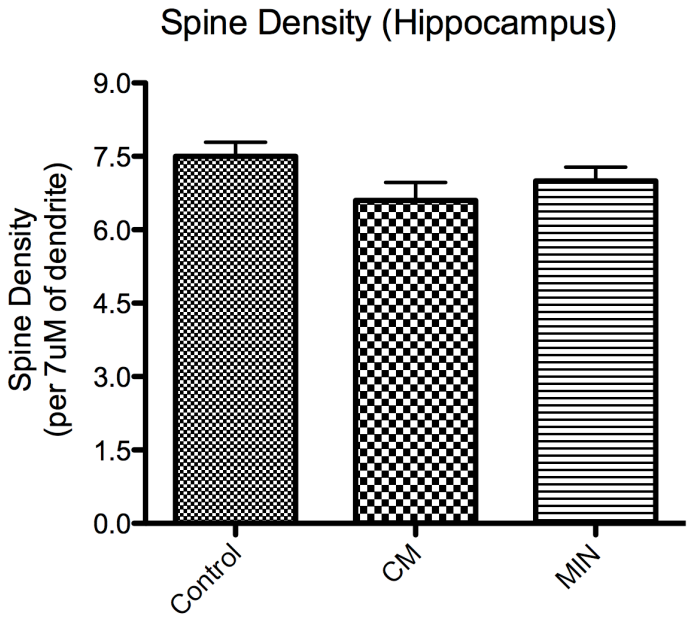

Fig.28. Minocycline treatment has no effect on hippocampal dendritic spine density. Graph showing hippocampal spine density of different experimental groups per 7 μm of dendrite. The experimental groups of mice were uninfected control, cerebral malaria (CM) and minocycline-treated. (n=50).

3.4. Minocycline decreases the protein levels of pro-apoptotic proteins

Minocycline was able to effectively decrease the protein levels of inflammation-associated Cox-2, HSP-70 and pro-apoptotic caspase-3 compared to the CM group ($P < 0.001$) **(Fig.28A and C)**. Interestingly, active caspase-3 levels in the minocycline-treated group were lower compared to that of CM group but this was not statistically significant ($P > 0.05$). The Bcl-2/Bax ratio was reduced in during CM whereas minocycline effectively improved the Bcl-2/Bax ratio thereby inhibiting apoptosis ($P < 0.05$) **(Fig.28C)**.

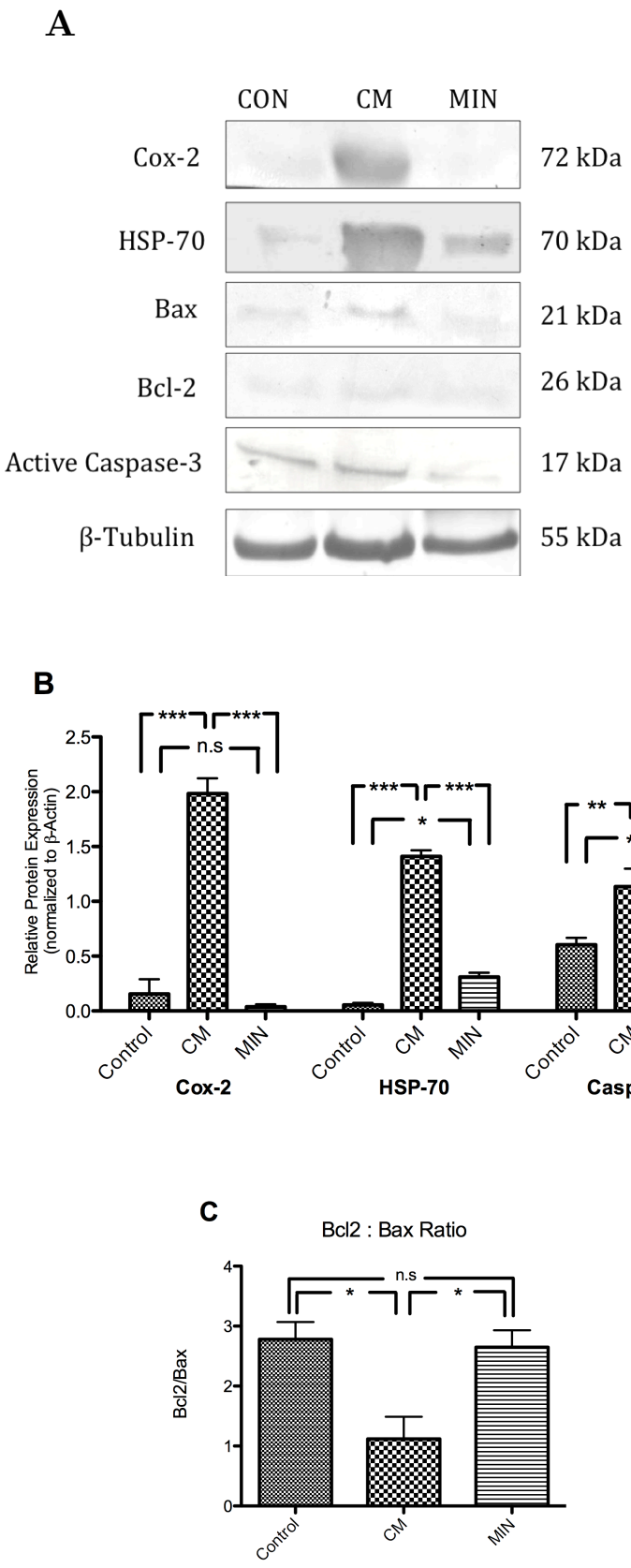

Fig.29. Minocycline decreases the protein levels of pro-apoptotic proteins. (A) Representative immunoblots of proteins Cox-2, HSP-70, Bax, Bcl-2, active caspase-3 and β-actin of different experimental groups, uninfected control, cerebral malaria (CM) and minocycline-treated (MIN). **(B)** The relative protein expression levels of Cox-2, HSP-70 and active caspase-3 after normalizing to β-actin. **(C)** The Bcl-2/Bax ratio in different experimental groups. P values less than 0.05 were considered as statistically significant. The *P* values <0.05, <0.01 and <0.001 were depicted as *, ** and *** respectively. Not-significant results were depicted as 'n.s'.

3.5. Spatial Learning and Memory

3.5.1. Minocycline treatment has no effect on the spontaneous alternation outcome in T-maze

As expected, there was a decrease in the percentage of correct alternation in the CM group compared to control **(Fig.29.)**. Also, the percentage of correct alternation in the minocycline treated group increased in comparison to CM group. As $P > 0.05$, we cannot rule out the null hypothesis that there is no difference between the experimental groups. The 95% CIs of difference between the groups were as follows: control v/s CM (-53.19 to 91.34), CM v/s minocycline-treated (-84.11 to 80.44) and control v/s minocycline-treated (-61.43 to 95.92).

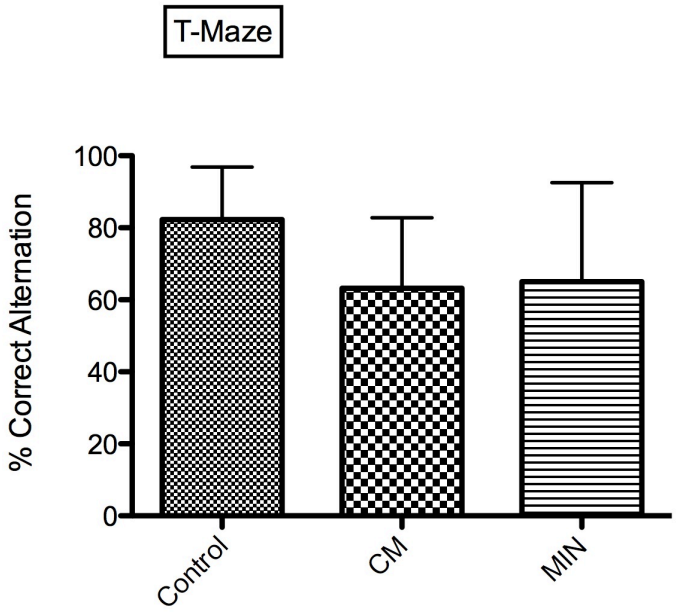

Fig.30. Graph showing the percentage of correct alternation of different experimental groups during T-maze test. The experimental groups of mice were uninfected control, cerebral malaria (CM) and minocycline-treated (MIN). The experiment was repeated two times (n=5).

3.6. Non-Spatial Learning and Memory

3.6.1. Minocycline treatment has no effect on the novel object preference score in T-maze

There was a decrease in the novel object preference score in the CM group compared to control **(Fig.30.)**. Also, the novel object preference score in the minocycline treated

group increased in comparison to CM group and control group. As $P > 0.05$, we cannot rule out the null hypothesis that there is no difference between the experimental groups. The 95% CIs of difference between the groups were as follows: control v/s CM (-2.133 to 4.384), CM v/s minocycline-treated (-5.299 to 1.219) and control v/s minocycline-treated (-4.173 to 2.344).

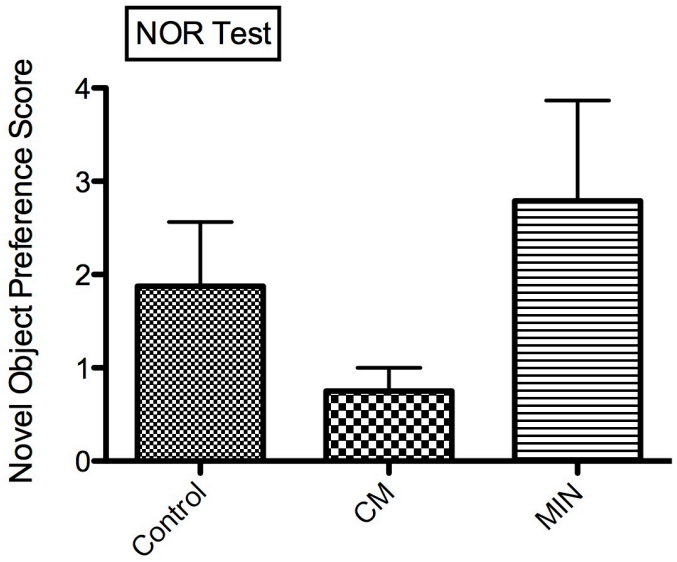

Fig.31. Graph showing the novel object preference of different experimental groups during T-maze test. The experimental groups of mice were uninfected control, cerebral malaria (CM) and minocycline-treated (MIN). The experiment was repeated two times (n=5).

3.6.2. Minocycline treatment has no effect on the novel object preference score but improved total exploration time in bow-tie maze test

As expected, the total exploration time of the CM mice were lower compared to that of uninfected control mice ($P < 0.01$) **(Fig.31.)**. The minocycline treatment was able to effectively increase the total exploration time of mice compared to the CM group ($P < 0.05$). Interestingly, there was no statistically significant ($P > 0.05$) difference between uninfected control and minocycline-treated mice. There was a decrease in the novel object preference score in the CM group compared to control **(Fig.34.)**. Also, the novel object preference score in the minocycline treated group increased in comparison to CM

group. As $P > 0.05$, we cannot rule out the null hypothesis that there is no difference between the experimental groups. The 95% CIs of difference between the groups were as follows: control v/s CM (-1.328 to 3.211), CM v/s minocycline-treated (-2.759 to 1.781) and control v/s minocycline-treated (-2.168 to 3.073).

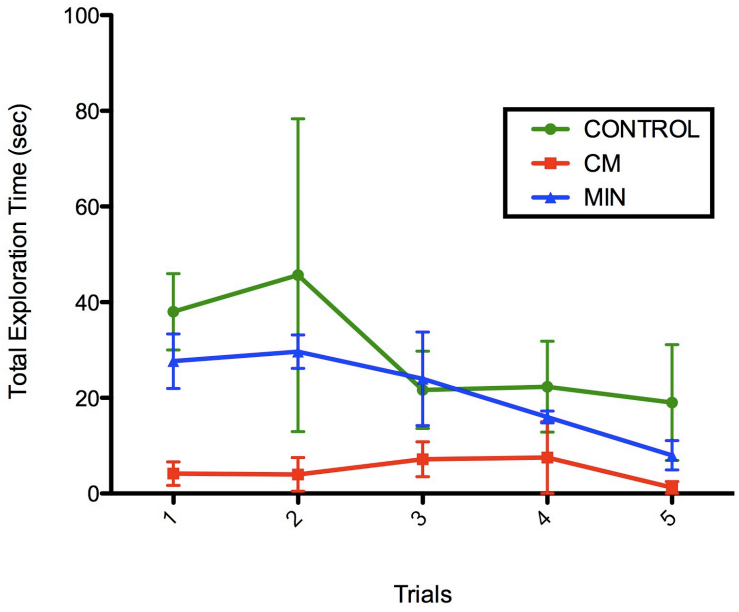

Fig.32. Graph showing the total exploration time of different experimental groups during bow-tie maze test. The experimental groups of mice were uninfected control, cerebral malaria (CM) and minocycline-treated (MIN). The experiment was repeated two times (n=5).

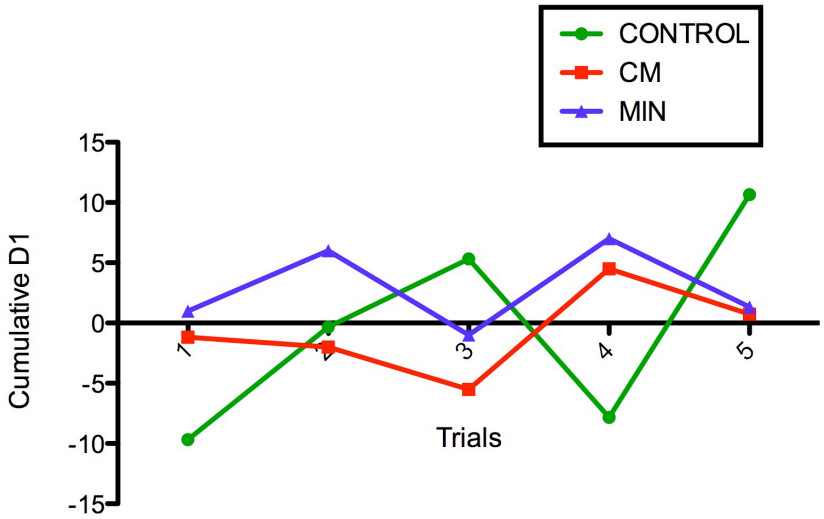

Fig.33. Graph showing the cumulative D1 different experimental groups during bow-tie maze test. Different experimental groups of mice were uninfected control, cerebral malaria (CM) and minocycline-treated (MIN). The experiment was repeated two times (n=5).

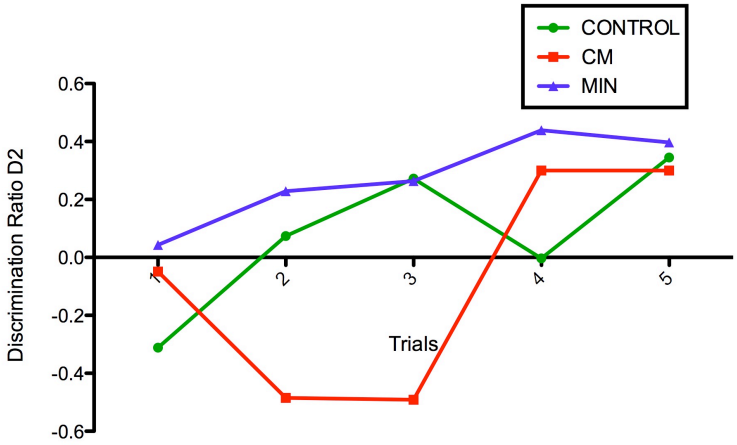

Fig.34. Graph showing the discrimination ratio (D2) of different experimental groups during bow-tie maze test. The experimental groups of mice were uninfected control, cerebral malaria (CM) and minocycline-treated (MIN). The experiment was repeated two times (n=5).

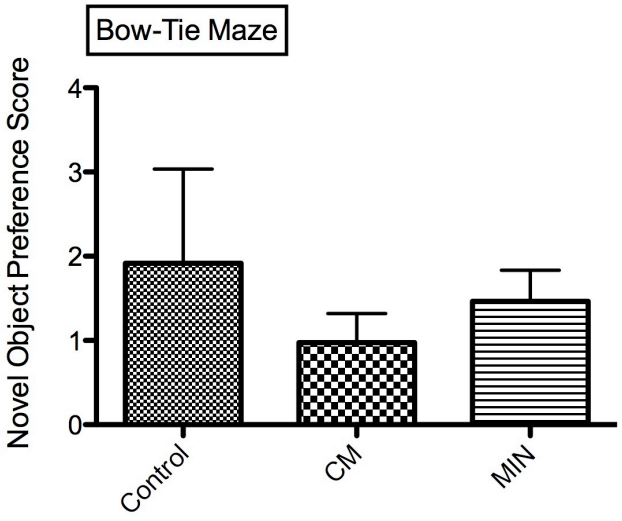

Fig.35. Graph showing the novel object preference score of different experimental groups during bow-tie maze test. The experimental groups of mice were uninfected control, cerebral malaria (CM) and minocycline-treated (MIN). The experiment was repeated two times (n=5).

4. Discussion

Cognitive impairments persistent in CM survivors demand the research on neuroprotective agents. In the present study, we measured various cellular markers to quantify neuroprotection and tried to correlate the same to behavioral studies.

We found that dentate gyrus, the most important memory-associated region of the hippocampus, was protected from hemorrhages upon minocycline treatment; pointing to the crucial neuroprotection imparted by minocycline. Our results are consistent with the study by Wu et al. **(Wu et al., 2009)** who showed that minocycline abrogated thrombin-induced intracerebral hemorrhages by decreasing TNF-α and interleukin-1β levels, and reducing microglial activation. We also found the absence of degenerating neurons in the hippocampus of minocycline-treated mice after Fluoro-Jade C staining. This result was in agreement with a study on experimental intracerebral hemorrhage where minocycline treatment led to decrease in the Fluoro-Jade C positive cells **(Zhao et al., 2011)**. Hence, the above result suggests that minocycline is highly effective in preventing neurodegeneration in cognition-associated regions of the brain.

In our study, we focused specifically on the memory-associated region hippocampus. We found no statistically significant decrease of spine densities at hippocampus region of murine CM compared to uninfected control. Even though the improvement after minocycline treatment is not statistically significant, the slight improvement might be physiologically relevant in terms of memory as minocycline has been earlier reported to improve dendritic spines and behavior in a mouse model of fragile X syndrome **(Bilousova et al., 2009)**. In conclusion, other areas of the brain needs be explored in future for checking the difference in dendritic spine densities.

Cyclooxygenase-2 is one of the proteins correlated to inflammation is associated with fever. The increase in levels of Cox-2 in brain parenchyma has been reported in murine CM **(Deininger et al., 2000)**. We found similar result of increase in Cox-2 in the CM group. Another work suggested that Cox-2 might be protective in CM as celecoxib treatment led to early induction of CM. In our study, minocycline treatment led to decrease in Cox-2 protein levels but we did not find any deleterious outcome due to this effect. As Cox-2 is an inflammation marker, we can conclude a decrease in inflammation by minocycline. Another protein, HSP-70, a stress-response protein and a cellular stress marker; was earlier reported to be elevated in the brain during murine CM **(Medana et**

al., 2001). We found that HSP-70 protein levels were decreased after minocycline treatment thus confirming a reduction in cellular stress. Previous reports have shown the elevation of pro-apoptotic protein levels in the brain during murine CM leading to neuronal apoptosis **(Apoorv and Babu, 2017; Eeka et al., 2011; Lackner et al., 2007)**. Consistent with earlier reports of caspase activity, we found a decrease in protein levels of active caspase-3 after minocycline treatment **(Apoorv and Babu, 2017)**.

To check whether neuroprotection led to cognitive improvement in infected mice, we used cognitive tests such as T-maze, for spontaneous alternation and novel object recognition tests with T-maze and bow-tie maze. We are the first to report the spontaneous alternation in *PbA*-infected mice and we did not find any statistically significant difference between infected, uninfected control or minocycline-treated mice. A previous study showed no statistically significant difference between infected and uninfected control with respect to novel object recognition, 5 days p.i. **(Desruisseaux et al., 2008)** and our study is in agreement with this work. But recent reports show a cognitive decline in the novel object recognition in *PbA*-infected mice 5 days p.i. compared to control **(Campos et al., 2015; de Miranda et al., 2015)**. This might be due to bigger sample size (n=21 and 10 respectively, repeated two times) used for the two studies compared to our work (n=5, repeated two times). As neuroprotection conferred by minocycline is not correlating to behavioral studies, we need to exercise caution while extrapolating the results to human CM. Interestingly, in the case of bow-tie maze test, we found a decline in the total exploration time of CM group compared to uninfected control. Also, minocycline improved the total object exploration by mice. This can either be due to decreased neurodegeneration or early parasite clearance by minocycline as the treatment was prophylactic. Further studies on minocycline, with increased sample sizes, are required to confirm the negative correlation and to rule out possibility of false negatives (type II errors).

5. Conclusion

Our work shows that minocycline has neuroprotective effect in the murine model of cerebral malaria at histological level but do not correlate to the behavioral tests conducted by us. Hence, it has to be validated by human clinical trials for use in human severe malaria cases. If successful in clinical trials, minocycline will help in decreasing the morbidity like cognitive impairment; and mortality associated with cerebral malaria.

CHAPTER 4

To study the association of matrix metalloproteinase-9 polymorphism 1562 C>T (rs3918242) with placental malaria

1. Introduction

In the second objective, we found that gene expressions of gelatinases are decreased in the CM group in agreement with previous study **(Van den Steen et al., 2006)**; and also pointing out a possible protective role of gelatinases in severe malaria. Hence, we wanted to check the role of gelatinases in human malaria. As procurement of human CM samples are difficult, we collaborated with Institute of Tropical Medicine and International Health, Charité-Universitaetsmedizin Berlin, Germany, to check the role of MMP-9 in placental malaria, another form of severe malaria.

Pregnant women are a particular risk group for infection with *Plasmodium falciparum* and malaria. Although commonly asymptomatic at high endemicity, malaria in pregnancy may cause anemia, abortion, stillbirth, low birth weight (LBW), and preterm delivery (PTD), and contributes to high infant mortality. The increased susceptibility of pregnant women, particularly primigravidae, is largely due to parasites expressing specific variants of the *P. falciparum* erythrocyte membrane protein-1. Parasite adhesion *via* these variant surface proteins results in the sequestration of infected red blood cells in the placental intervillous space. Sequestration frequently is accompanied by local hemozoin (malaria pigment) deposition and accumulation of inflammatory cells, including monocytes/ macrophages. Specific immune mechanisms targeting the pregnancy-associated parasites, particularly parasite-specific antibodies, are low in primigravidae. Only with successive pregnancies, these are acquired, and infection risk and manifestation decrease **(Desai et al., 2007; Mockenhaupt et al., 2006a; Rogerson et al., 2007)**.

Risk and manifestation of malaria and of malaria in pregnancy are influenced by diverse factors including host genetics **(Bedu-Addo et al., 2013; Lopez et al., 2010)**.

The latter may involve variants of matrix metalloproteinases (MMP), a family of metal-ion dependent endopeptidases, which are involved in the breakdown of extracellular matrix and tissue remodeling **(Page-McCaw et al., 2007)**. MMPs also contribute to the regulation of various cytokines and chemokines, thus playing an important role in host immune responses **(Van Lint and Libert, 2007)**. In acute malaria, increased expression of MMP-9 (gelatinase B) has been observed **(Griffiths et al., 2005)**. Moreover, *in vitro*, hemozoin phagocytosis by human monocytes and the exposure of endothelial cells to parasitized red blood cells stimulate the release of MMP-9 and TNF-α **(D'Alessandro et al., 2013; Prato et al., 2005)**. A common *MMP-9* gene polymorphism (rs3918242, replacement of C by T at location -1562) increases the promoter activity of the *MMP-9* gene 1.5 fold because the associated transcriptional-repressor protein has a reduced affinity to the *T* allelic promoter. The polymorphism, hence, has been associated with increased transcription activity and with altered risks of various diseases **(El Samanoudy et al., 2014; Ye, 2000; Zhang et al., 1999)**. We, therefore, examined whether this polymorphism affects susceptibility to or manifestation of malaria in pregnancy.

2. Materials and Methods

The characteristics of the 304 primiparous pregnant women with live singleton delivery have been reported elsewhere **(Mockenhaupt et al., 2006a)**. Informed written consent was obtained from all women, and the study protocol was approved by the Committee on Human Research Publications and Ethics, School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. Briefly, delivering women were recruited at the Presbyterian Mission Hospital in hyper- to holoendemic Agogo, Ghana (population, 30,000) in 2000 and 2001. Women were clinically examined and socio-demographic data documented. Intervillous and peripheral blood samples were collected into EDTA. Parasites were counted on Giemsa-stained blood smears per 100 high power fields and per 500 white blood cells, respectively. Leukocyte-associated hemozoin in placental samples was recorded. DNA was extracted from blood (AS1 and QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany), and nested *P. falciparum*-specific PCR assays were

performed **(Snounou et al., 1993)**. Present or past placental *P. falciparum* infection was defined by the presence of placental parasites or hemozoin in microscopy, or a positive placental *P. falciparum* PCR result. Pyrimethamine in plasma (indicating compliance with the chemoprophylaxis recommended at that time) was measured by ELISA assays with limits of detection of 10 ng/mL **(Eggelte, 1990)**. Anemia was defined as Hb <11 g/dL, measured by a hemoglobin photometer (HemoCue AB, Ängelholm, Sweden). LBW was defined as birth weight <2500 g, and PTD as gestational age <37 weeks applying the Finnström score **(Finnstrom, 1977)**.

Genotyping of rs3918242 was achieved by restriction fragment length polymorphism **(Coolman et al., 2007)**. Briefly, PCR was used to analyze MMP-9 gene promoter -1562 C/T polymorphism. Primer nucleotide sequences were as follows: FP: 5'-GCC TGG CAC ATA GTA GGC CC-3,' RP: 5'-CTT CCT AGC CAG CCG GCA TC-3.' The following recipe and cycling conditions were used for the PCR:

Table.6. The volumes of reagents used for PCR

PCR Components	**Volume**
Forward Primer	6.25 pmol
Reverse Primer	6.25 pmol
A, T, G and C	1 x 10^4 pmol each
Taq Polymerase	1.3 U
DNA	5 ng

Table.7. The cycling conditions used for PCR

Cycling Conditions	**Temperature**	**Time**
Denaturation	96 ^{o}C	4 min
33 cycles	95 ^{o}C	1 min
	53 ^{o}C	1 min
	72 ^{o}C	2 min

The amplicon was 434 bp and was subjected to restriction enzyme digestion with *SphI* (Roche, Switzerland). The digestion products were electrophoretically separated in an agarose gel (1.5%) and stained with EtBr for visualization. The patient samples were grouped according to the genotypes; identified by C allele (not digested, 434 bp) and T allele (digested; 241 bp, 193 bp).

Data were analyzed with Statview 5.0 (SAS Institute Inc., Cary, NC). Continuous variables were compared between groups by the Mann Whitney U test or Student's t test as applicable. Associations of genotypes with, e.g., *P. falciparum* infection were assessed by χ^2 test; and odds ratios (ORs) and 95% confidence intervals (95%CI) were determined. Adjusted ORs (aORs) were calculated in logistic regression models with the stepwise backward removal of factors not associated in multivariate analysis ($P > 0.05$). Graphs were plotted with GraphPad Prism 5.0 (GraphPad Software, Inc., US).

3. Results

Typing of rs3918242 was successful in 302 of 304 primiparae. The major homozygous genotype (*CC*) was observed in 82.1% (248); 17.9% of the women exhibited the minor *T* allele (heterozygous, 51; homozygous, 3; grouped henceforth). Genotypes were in Hardy-Weinberg equilibrium.

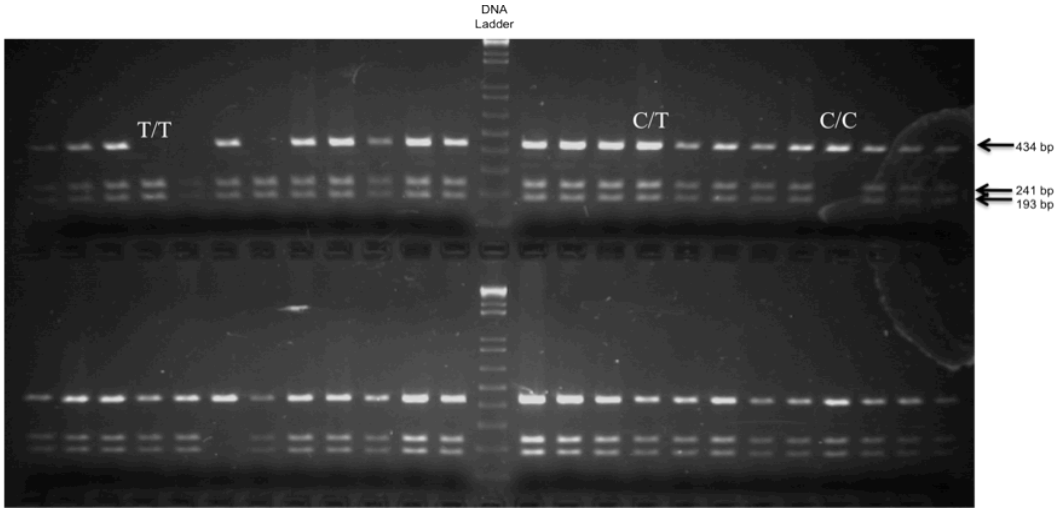

Fig.36. A representative agarose gel showing the PCR products after *SphI*-digestion. The number of digested products were used to genotype the patient samples. Samples with CC genotype were undigested showing only one band corresponding to 434 bp. Samples with CT genotype had three bands corresponding to 434 bp, 241 bp, and 193 bp whereas samples with TT genotype had two bands corresponding to 241 bp and 193 bp. The DNA ladder ranged from 100bp to 1kbp.

Table 8. Characteristics of 302 Ghanaian primiparae with live singleton delivery according to MMP-9 genotype (rs3918242)

Parameter	All	rs3918242		P*
		Major Genotype (*CC*)	Genotypes with T allele (*CT*, *TT*)	
No. (%)	302 (100)	248 (82.1)	54 (17.9)	
Age (years); median (range)	20.5 (15-36)	20 (15-36)	21 (15-30)	0.25
Rural residence (n, %)	154 (51.0)	126 (50.8)	28 (51.9)	0.89
>3 antenatal care visits (n, %)	139/295 (47.1)	106/241 (44.0)	33/54 (61.1)	0.02
Delivery in rainy season (n, %)	155 (51.3)	130 (52.4)	25 (46.3)	0.41
Pyrimethamine in plasma (n, %)	106/297 (35.7)	87/243 (35.8)	19 (35.2)	0.93
Anemia (n, %)	116 (38.4)	99 (39.9)	17 (31.5)	0.25
Low birth weight (n, %)	79 (26.2)	65 (26.2)	14 (25.9)	0.97
Preterm delivery (n, %)	80 (26.5)	69 (27.8)	11 (20.4)	0.26
P.falciparum infection (peripheral blood)				
Microscopy positive (n, %)	80 (26.5)	75 (30.2)	6 (11.1)	**0.004**
Geometric mean parasite density /µL, (95% CI)	746 (476-1171)	718 (451-1141)	1159 (173-7773)	0.58
PCR positive (n, %)	179 (59.3)	152 (61.3)	27 (50)	0.13
P.falciparum infection (placental blood)				
Microscopy positive (n, %)	139 (46.0)	122 (49.2)	17 (31.5)	**0.02**
Geometric mean parasite density /100 high power fields, (95% CI)	119 (78-181)	126 (83-191)	431 (73-2544)	0.48
Hemozoin positive (n, %)	128 (42.4)	115 (46.4)	13 (24.1)	**0.003**
PCR positive (n, %)	196 (64.9)	166 (66.9)	30 (55.6)	0.11
Present or past placental infection (n, %)	205 (67.9)	175 (70.6)	30 (55.6)	**0.03**

*, *P*-values derived from Student´s t-tests, Mann Whitney U tests, or c^2 tests, as applicable. In the three *TT* homozygous individuals, prevalence was: peripheral blood: microscopy positivity, 0/3; PCR positivity, 1/3; placental blood: microscopy positivity, 0/3; hemozoin positivity, 0/3; past or present placental infection, 1/3.

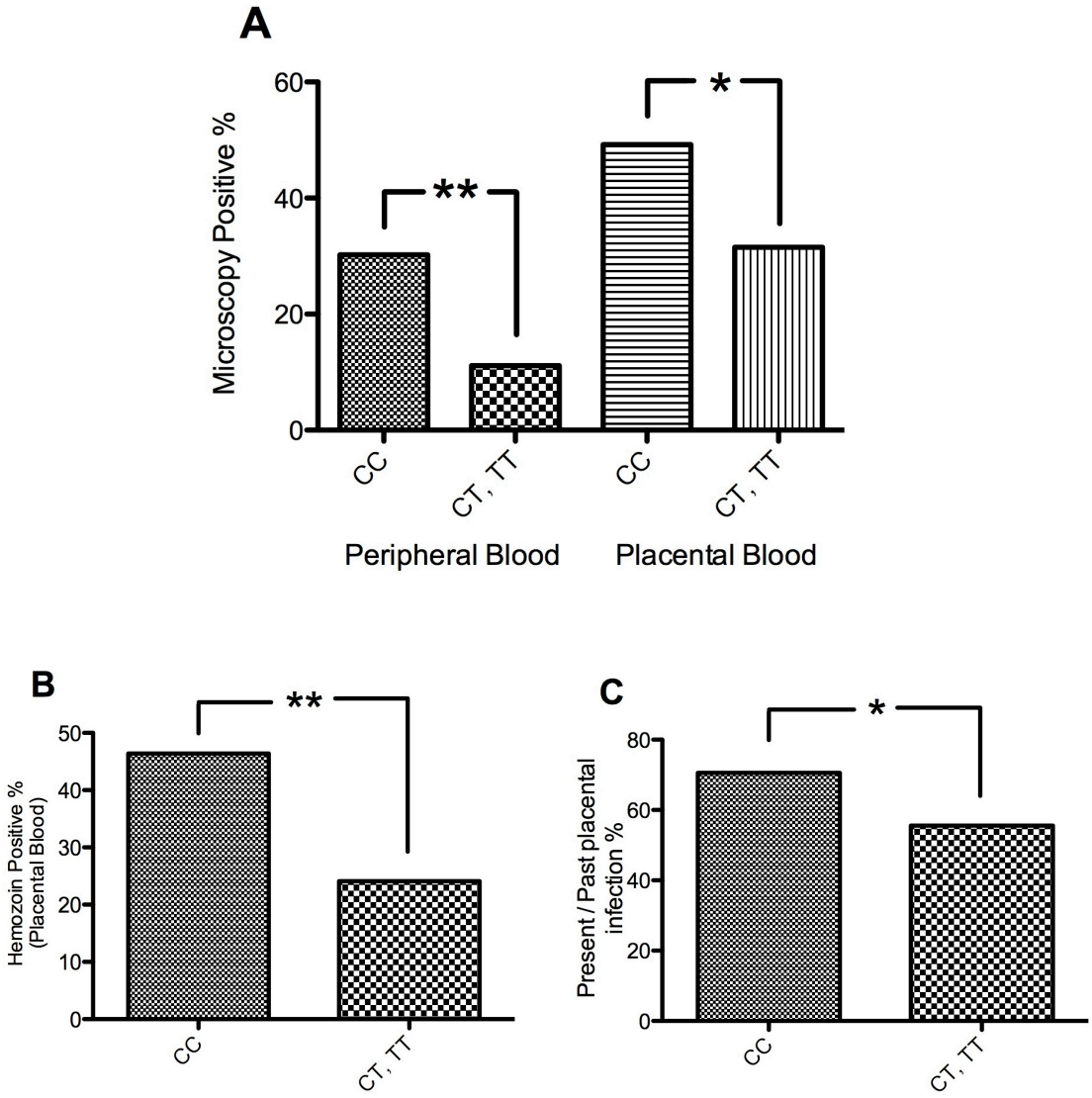

Fig.37.(A-C). The graphs show the significant results from the study on *MMP9* SNP (rs3918242). The percentage of individuals of each genotype who were microscopy positive for peripheral blood and placental blood (A), placental blood were hemozoin positive (B) and had present/past placental infections (C). The genotypes with *T* allele (*CT, TT*) had significantly less microscopy positivity (peripheral and placental blood), placental blood hemozoin positivity and present/past placental infections compared to major genotype (*CC*). [**, $P < 0.001$-0.01; *, $P < 0.05$]

Overall, 67.9% of the women had evidence of present or past *P. falciparum* infection. Placental hemozoin was observed in 42.4%, and PCR assays on placental samples were positive in 64.9% **(Table.8.)**. Nevertheless, fever occurred in only 2.5% (12/299). Women with the minor *T* allele had a significantly lower prevalence of present or past placental *P. falciparum* infection (OR, 0.52; 95%CI, 0.27-0.99; $P = 0.03$, **Fig.36C**), placental hemozoin (OR, 0.37; 95%CI, 0.18-0.75; $P = 0.003$, **Fig.36B**), placental parasitemia (OR, 0.47; 95% CI, 0.24-0.92; $P = 0.02$, **Fig.36A**) and peripheral blood parasitemia (OR, 0.29; 95%CI, 0.10-0.79; $P = 0.004$, **Fig.36A**). For infections detected

by PCR, the difference did not reach statistical significance, and parasite densities did not differ between women with and without the minor *T* allele **(Table.8.)**. Adjusting for factors previously identified as being associated with placental malaria, i.e., delivery in rainy season, age and presence of plasma pyrimethamine **(Mockenhaupt et al., 2006a)**, women with the minor *T* allele tended to have reduced odds of present or past *P. falciparum* infection (aOR, 0.56; 95%CI, 0.30-1.03; $P = 0.06$; age (years), aOR, 0.92; 95%CI, 0.85-0.98; rainy season, aOR, 1.76; 95%CI, 1.06-2.92; plasma pyrimethamine, 0.58; 95%CI, 0.35-0.98). Further adjustment for the difference between groups in the use of antenatal care (**Table.8.**; itself not associated with malaria) did not substantially change the estimate (aOR, 0.55; 95%CI, 0.29-1.02; $P = 0.06$). In the multivariate model, significantly reduced odds were observed for placental hemozoin (aOR, 0.38; 95%CI, 0.19-0.75; $P = 0.005$), placental parasitemia (aOR, 0.49; 95%CI, 0.26-0.93; $P = 0.003$), and peripheral blood parasitemia (aOR, 0.29; 95%CI, 0.12-0.73; $P = 0.008$).

Maternal anemia and PTD but not LBW tended to be less common in women carrying the minor *T* allele. However, there was no association of these outcomes with the polymorphism **(Table.8.)**, irrespective of stratification into infected and non-infected women (data not shown).

4. Discussion

MMP-9 has been shown to be up-regulated in acute malaria **(Griffiths et al., 2005)**, and specifically, both parasitized RBCs and hemozoin induce the release of MMP-9 by monocytes and endothelial cells **(D'Alessandro et al., 2013; Prato et al., 2005)**. Role of this endopeptidase in malaria is, nevertheless, controversial, potentially generating both protective and detrimental effects **(Geurts et al., 2012)**. Here, we show that a promoter SNP increasing MMP-9 activity reduces the odds of placental malaria. The mechanisms involved are speculative: MMP-9 has an important role in the regulation of inflammatory processes including a complex influence on various chemokines. Increased MMP-9 levels may result in both, increased and decreased chemotactic activities but in murine models, they appear to promote leukocyte

migration. Also, MMP-9 modulates the activity of several pro-inflammatory mediators, e.g., by inducing the release of TNF-α or activating pro-IL-1ß, thereby augmenting the pro-inflammatory response **(Van Lint and Libert, 2007)**. Pro-inflammatory responses in malaria are double-edged: they may contribute to pathophysiologic damage but initially increased release, in particular, contributes to accelerated parasite clearance **(Rogerson et al., 2007)**. Conceivably, increased MMP-9 activity may affect leukocyte recruitment to the intervillous space and local pro-inflammatory responses thereby enhancing parasite elimination. Notably, placental syncytiotrophoblast, the epithelium lining the intervillous space, has been shown to be immunoreactive to *P. falciparum* **(Lucchi et al., 2008)**, and also to express MMP-9 **(Xu et al., 2002)**. Moreover, part of the produced MMP-9 forms heteromers with chrondroitin sulfate proteoglycans **(Winberg et al., 2000)**. Altered *MMP-9* expression could affect heteromer formation, which in turn may decrease binding of parasitized RBCs to chondroitin sulfate (the main placental parasite ligand), resulting in reduced placental malaria.

As a limitation, this study was not *a priori* designed to assess an association between *P. falciparum* infection in pregnancy and the *MMP-9* promoter SNP, and it comprised a relatively small group of 302 primiparae. We use the term placental malaria to subsume the detection in placental blood samples of parasites by microscopy or PCR, and of hemozoin by microscopy. This does not correspond to histologic classification as suggested by, e.g., Muehlenbachs *et al.* **(Muehlenbachs et al., 2010)** but is characterized by a high sensitivity due to the inclusion of PCR assays **(Mockenhaupt et al., 2006a)**. Although infection prevalence as assessed by PCR differed between genotypes, this did not reach statistical significance. This was partly due to the fact, that the proportion of submicroscopic infections (i.e., positive by PCR only but negative by microscopy) among all infections was comparatively increased in individuals with the minor *T* allele **(Table.8.)**. Thus, infection prevalence as detected by PCR in *T* allele carriers was overall reduced even if not significantly so, and low level infections prevailed. Statistical significance might have been present in case of a larger sample size.

The same applies to the manifestation of infection, e.g., malaria-associated anemia or PTD.

5. Conclusion

The MMPs have been considered as biomarkers and therapeutic targets in malaria **(Geurts et al., 2012)**. The present study suggests that a common functional *MMP-9* polymorphism is associated with reduced odds of placental malaria, and thereby provides evidence for the *in vivo* relevance of MMP-9 in human malaria.

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SUMMARY

Severe malaria manifests where there is an inadequate or delay in treatment, which is of two types: cerebral malaria and placental malaria. Cerebral malaria (CM), a neurological complication, is caused by infection of protozoan *Plasmodium falciparum* or *P. vivax*. We used currently accepted mouse model, C57BL/6 mice infected with *Plasmodium berghei ANKA,* for studying CM.

We checked the status of an important metabolic sensor, adenosine 5'-monophosphate-activated protein kinase (AMPK), in CM. We found a decrease in gene expressions of AMPK catalytic subunits and ratio of phospho-AMPK/AMPK protein levels in the mice brains affected by CM. The reduction in protein levels was reversed upon treatment with a semi-synthetic tetracycline, minocycline (45 mg/kg). Our study suggests the possible role of AMPK in CM pathogenesis and novel AMPK activating drugs might be helpful as an adjunctive therapy conferring neuroprotection.

CM survivors are affected by long-term cognitive impairment due to inability of standard antimalarial treatments like artemisinin to prevent neuronal death in different regions of the brain associated with cognition. We investigated the effect of minocycline treatment on the survivability of mice during experimental CM. Infected mice were treated with an intra-peritoneal dose of minocycline hydrochloride, 45mg/kg daily for ten days that led to parasite clearance and mice survived until experiment ended (90 days) without parasite recrudescence. Minocycline treatment led to maintenance of blood-brain barrier integrity, decrease in tumor necrosis factor-alpha, caspase activity, gene expressions of interferon-gamma, CXCL10, CCL5, CCL2; receptors CXCR3 and CCR2; and 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 chemokine *CXCL10* and its receptor *CXCR3* makes it effective than doxycycline, a tetracycline used as chemoprophylaxis. Histological analyses showed that minocycline treatment prevented

neuronal apoptosis, hemorrhages at memory-associated hippocampal region, neurodegeneration and improved the dendritic spine densities. Hence, minocycline might be a possible candidate for use as an adjunctive therapy with standard anti-malarials. Our work shows that minocycline has neuroprotective effect in the case of murine cerebral malaria at histological level but do not correlate with the behavioral tests conducted by us. Hence, it has to be further validated by human clinical trials for use in human severe malaria cases. If successful in clinical trials, minocycline will help in decreasing the morbidity like cognitive impairment; and mortality associated with cerebral malaria.

Pregnant women are a particular risk group for the infection of *Plasmodium falciparum* and other forms of malaria. Phagocytosis of hemozoin induces increased activity of matrix metalloproteinase (MMP)-9, an endopeptidase involved in cytokine regulation. We examined whether a common functional *MMP-9* promoter polymorphism (rs3918242) affects *Plasmodium falciparum* infection in pregnancy. Eighteen percent of Ghanaian primiparae carried the minor *T* allele. It was associated with reduced odds of placental hemozoin and of placental as well as peripheral blood parasitemia. Our study indicates that a common *MMP-9* polymorphism protects against placental malaria indicating that this endopeptidase is involved in susceptibility to *P. falciparum*.

List of Publications

Original Research Articles

1. **Apoorv, T.S.,** Babu, P.P., Meese, S., Gai, P.P., Bedu-Addo, G., Mockenhaupt, F.P., 2015. Matrix Metalloproteinase-9 Polymorphism 1562 C > T (rs3918242) Associated with Protection Against Placental Malaria. ***The American Journal of Tropical Medicine and Hygiene.*** 93(1), 186-188.

2. **Apoorv, T.S.,** Babu, P.P., 2017. Minocycline prevents cerebral malaria, confers neuroprotection and increases survivability of mice during *Plasmodium berghei ANKA* infection. ***Cytokine.*** 90, 113-123.

Manuscript under review

Apoorv TS*, Karthik Ch.* & Babu PP: **AMP-activated protein kinase (AMPK) is decreased in the mouse brain during experimental cerebral malaria** (*equal authorship, submitted to *Neuroscience Letters*, Manuscript ID: NSL-S-17-00539)

Manuscript prepared

Kunal Jain*, **Thittayil Suresh Apoorv***, Sumeet Sood, Phanithi Prakash Babu & K. Gowthamarajan: Intraperitoneal artemether nanoformulation rescues mice from experimental cerebral malaria. (*Equal authorship, to be submitted to *Antimicrobial Agents and Chemotherapy*)

Manuscripts under preparation

Apoorv TS & Babu PP: Apoptosis and cerebral malaria (Review)

Apoorv TS & Babu PP: Minocycline confers neuroprotection during murine cerebral malaria by prevention of apoptosis and neurodegeneration

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

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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.

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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

phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, 20 mM β-glycerophosphate and 20 mM NaF] using Omni Tip™ tissue homogenizer (Omni International, US). The cytosolic fractions from mice brain homogenates were acquired using the protocol as described by Timmons et al. [29]. The homogenate was centrifuged (Kubota, Japan) at 2000*g*, 3 min, 4 °C to get supernatant 'A' and nuclear pellet 'N'. The pellet N was then re-suspended in isolation buffer and centrifuged again at 2000*g*, 3 min, 4 °C. This supernatant 'B' was combined with supernatant 'A' and centrifuged at 20,000*g*, 10 min, 4 °C to obtain supernatant: the complete cytosolic fraction. The protein concentration of complete cytosolic fraction was estimated using Bradford method [30]. Cytosolic fractions from each experimental group (100 μg protein) were separated using 10% and 15% sodium-dodecyl-sulphate polyacrylamide gels, and transferred to nitrocellulose membrane. The nitrocellulose membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) and incubated with anti-TNF-α and anti-β-tubulin (Cell Signaling Technologies, US) primary rabbit antibodies (dilution 1:1000) overnight at 4 °C. Membranes after washing were incubated with alkaline phosphatase-labeled anti-rabbit secondary antibodies (dilution 1:30,000) (Sigma-Aldrich, US). Protein bands were detected using 5-bromo-4-chloro-3-indolyl-phosphate - nitro blue tetrazolium (BCIP-NBT) method. Protein levels were quantified by densitometry using ImageJ software (NIH, US).

2.6. Hematoxylin & Eosin (H&E) staining

H&E staining helps in assessing the extent of cell death in tissue sections. Mice were first anesthetized with 10% pentobarbital i.p. The brains of each group were perfused with saline and then 4% paraformaldehyde in PBS (pH 7.4). The brains were resected, fixed in 4% paraformaldehyde for 48 h and embedded in paraffin wax. Brain samples were cut into 20 μm sections using microtome (Leica RM 2145, Germany). For H&E staining, the sections were first deparaffinized in xylene. Sections were then hydrated in alcohol series, stained with Meyer's hematoxylin and eosin; dehydrated in alcohol series, cleared in xylene and mounted with DPX. Brain sections were visualized under a light microscope (Olympus BX-51, Japan) and photographed at 400× magnification.

2.7. Fluoro-Jade® C staining

Fluoro-Jade® C (Millipore, US) stains the degenerating neurons and is used to assess the degree of neuronal degeneration and cell death. Staining protocol was followed as of Schmued et al. except Fluoro-Jade® C was used at a concentration of 0.001% [31]. Fluoro-Jade® C-stained brain sections were imaged using FITC filter in laser scanning confocal microscope (Carl-Zeiss, Germany). Fluoro-Jade® C-positive cells were counted per field using ImageJ software (NIH, US).

2.8. Caspase activity assay

The fluorescence emitted during cleavage of false caspase substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin relates to the aggregate enzymatic activity of pro-apoptotic effector caspases 3, 6 and 7. The cytosolic fractions from mice brain homogenates were acquired as in immunoblotting (sub-section 2.5). Cytosolic fractions from each experimental group (100 μg protein) were made up to 100 μL using caspase assay buffer (20 mM HEPES pH 7.4, 10% sucrose, 100 mM NaCl, 10 mM DTT, 0.1% CHAPS, 1 mM EDTA) and incubated at 37 °C for 1 h. Caspase substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Sigma-Aldrich, US) was added to all reaction tubes to a final concentration of 5 μM, and caspase assay buffer added to make final reaction volume 1 ml. The tubes were incubated again at 37 °C for 1 h. The solutions were subjected to spectrofluorometry (FluoroMax®). Fluorometric detection was done at excitation λ = 400 nm and emission λ = 450–500 nm.

2.9. Real-time PCR

The cDNA for real-time PCR was synthesized using same method aforementioned in semi-quantitative PCR section (sub-section 2.3). Quantitative PCR for the genes *TNF-α, IFN-γ, CXCL10, CXCR3,* and *GAPDH* was done using SYBR® *Premix Ex Taq*™ [Tli RNase H Plus] (Takara, Japan) as per manufacturer's protocol in 7500 Fast Real-time PCR system (Applied Biosystems, US). Primers (Eurofins, India) were used at a concentration of 0.1 pmol, and 1 μl of cDNA from each experimental group was used for PCR. Nucleotide sequences of primers used: *TNF-α* (5′-CCA ACG GCA TGG ATC TCA AAG ACA-3′, 5′-ATG AGA TAG CAA ATC GGC TGA CGG-3′); *IFN-γ* (5′-TCC TCA TGG CTG TTT CTG GCT GTT-3′, 5′-TGT CAC CAT CCT TTT GCC AGT TCC-3′); *CXCL10* (5′-CAA GGG ATC CCT CTC GCA AGG AC-3′, 5′-GGC AAT GAT CTC AAC ACG TGG GCA-3′); *CXCR3* (5′-CAA GGG ATC CCT CTC GCA AGG AC-3′, 5′-GGC AAT GAT CTC AAC ACG TGG GCA-3′); *CCL5* (5′-ATA TGG CTC GGA CAC CAC TC-3′, 5′-GTG ACA AAC ACG ACT GCA AG-3′); *CCL2* (5′-CCA GAT GCA GTT AAC GCC CCA-3′, 5′-CCT CTC TCT TGA GCT TGG TGA CAA A-3′) and *CCR2* (5′-CAC ACC CTG TTT CGC TGT AGG AAT-3′, 5′-CTG CAT GGC CTG GTC TAA GTG CT-3′). *GAPDH* primers, described in semi-quantitative PCR section (sub-section 2.3), were used for quantitative PCR for use as an internal control. Relative changes in gene expression were calculated using $\Delta\Delta C_t$ values.

2.10. Statistical analysis

All results are shown as the mean ± standard error of the mean. All data were analyzed using one-way ANOVA with Holm-Sidak post-test except for Kaplan-Meir survival curve, and graphs were plotted using GraphPad Prism 5.0 software (GraphPad Software, Inc., US). Significance was represented by asterisks: *** indicate $P < 0.001$, ** indicate $P = 0.001–0.01$, * indicate $P < 0.05$, and n.s indicate not significant, $P > 0.05$.

3. Results

3.1. Minocycline treatment prevents CM, leads to parasite clearance and increases survivability of mice

Sixty percent of mice of infected group succumbed to CM on days 6–8 whereas the rest 40% died of severe anemia on days 11–14. Mice that received both doses of minocycline (45 mg/kg and 22.5 mg/kg) survived for 90 days corresponding to end of the experiment (Fig. 1A). The parasitemia in CM mice was 10–15% during the day of death, i.e. day 7 (p.i); whereas the parasites were cleared in both minocycline groups on day 7 p.i (Fig. 1B). Parasitemia was checked in both minocycline groups after 90 days to rule out chances of recrudescence. Interestingly during days 1 and 2 p.i, parasitemia was found to be lower in minocycline group that received 22.5 mg/kg compared to the group that received 45 mg/kg. However, the minocycline group that received 22.5 mg/kg showed increased reticulocytosis compared to 45 mg/kg group (Fig. 1C and D). As reticulocytosis is linked to anemia [32], the dose 45 mg/kg was considered to be optimal and continued for rest of the study. Semi-quantitative PCR, a more sensitive method than microscopy, showed the absence of parasite-specific *18S rRNA* in blood, brain, liver and spleen of minocycline treated group confirming parasite clearance (Fig. 1E).

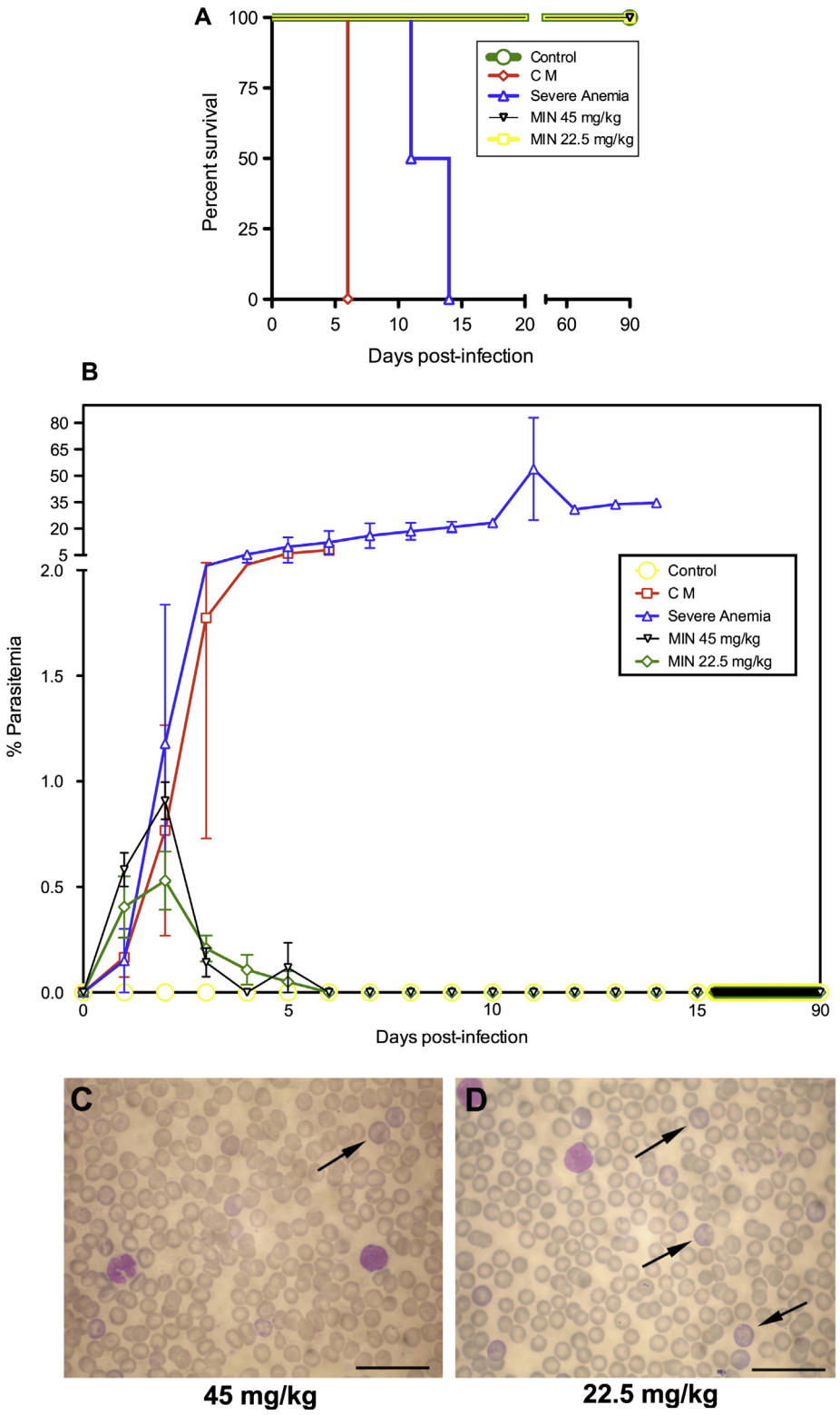


Fig. 1. Minocycline treatment prevents CM, leads to parasite clearance and increases the survivability of mice. (A) Kaplan-Meier survival curve showing day-wise percent survival of experimental groups upon minocycline treatment: control (circle symbol, green line), CM (diamond symbol, red line), severe anemia (triangle symbol, blue line), minocycline dose 45 mg/kg (MIN 45 mg/kg, inverted-triangle symbol, black line) and minocycline dose 22.5 mg/kg (MIN 22.5 mg/kg, square symbol, yellow line). The experiment was repeated three times (n = 15) and representative data expressed as mean ± SEM is shown. (B) Representative parasitemia curve showing day-wise parasitemia of experimental groups: control (circle symbol, yellow line), CM (square symbol, red line), severe anemia (triangle symbol, blue line), minocycline dose 45 mg/kg (MIN 45 mg/kg, inverted-triangle symbol, black line) and minocycline dose 22.5 mg/kg (MIN 22.5 mg/kg, diamond symbol, green line). The experiment was repeated three times (n = 15) and representative data expressed as mean ± SEM is shown. (C and D) Giemsa-stained blood smears of experimental groups, minocycline dose 45 mg/kg (C) and minocycline dose 22.5 mg/kg (D) taken on 8th day p.i. Arrows indicate reticulocytes. There was an increased reticulocytosis in 22.5 mg/kg group compared to 45 mg/kg group (bars, 50 μm). (E) Gene expression of parasite-specific *18S rRNA* in brain, liver, spleen and blood that correspond to parasite load. Lanes CON, CM, and MIN correspond to control, cerebral malaria and minocycline treated respectively. Lane '−ve' corresponds to 'no template negative control.' Housekeeping gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as loading control. No amplification was found in minocycline treated group confirming parasite clearance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

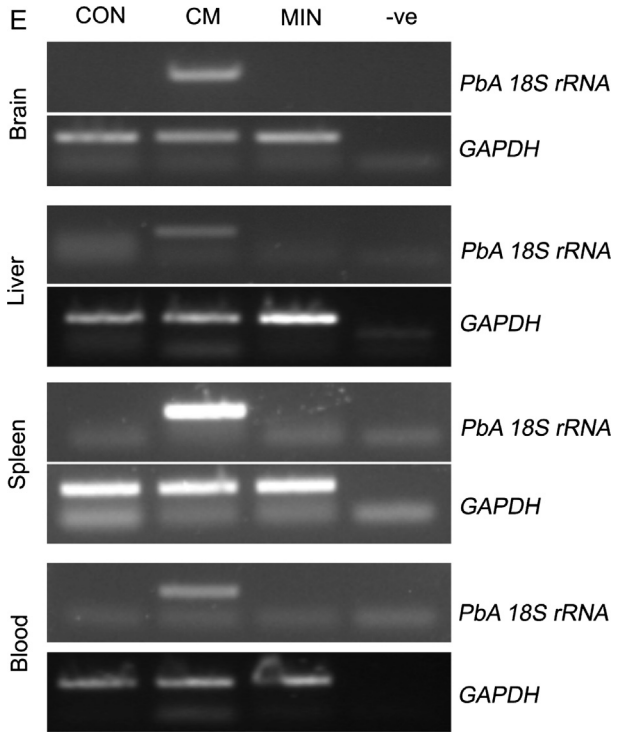
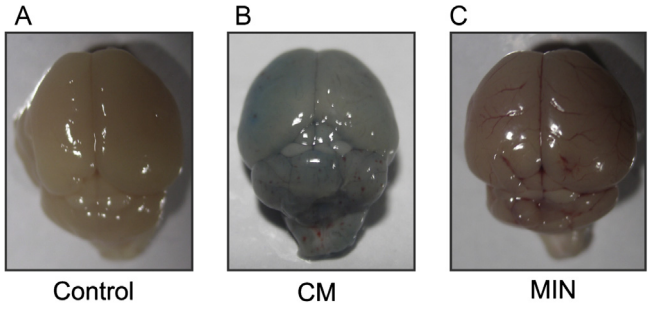


Fig. 1 (*continued*)



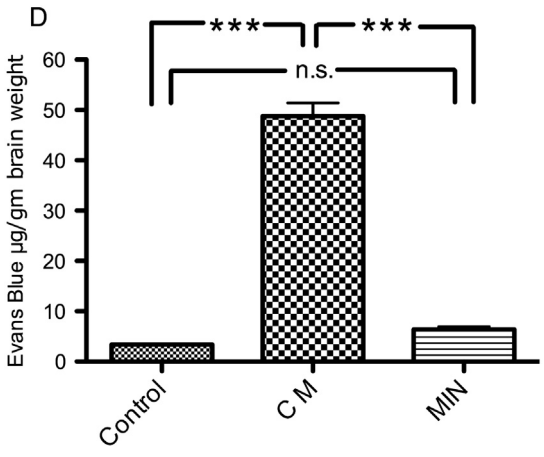


Fig. 2. Minocycline maintains BBB integrity. (A–C) Representative images of the brain of different experimental groups, control, CM and minocycline-treated (MIN), after Evans blue injection. The experiment was repeated three times and representative data is shown. (D) Graph indicating the amount of Evans blue extravasated into brains of different experimental groups. The experiment was repeated three times; results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM (*** indicate $P < 0.001$ and n.s indicate not significant, $P > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Minocycline maintains blood-brain barrier (BBB) integrity

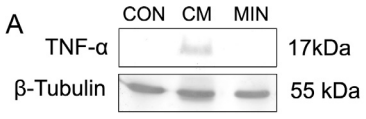
The brains of CM mice after Evans blue injection were blue in color indicating that BBB is compromised (Fig. 2B). In the case of control and minocycline group, brains were normal pinkish in color (Fig. 2A and C). The formamide extraction revealed that the amount of Evans blue extravasated into the brain was significantly decreased ($P < 0.001$) in minocycline treated group compared to CM mice (Fig. 2D). Low Evans blue extravasation implies that minocycline maintains BBB integrity during infection.

3.3. Minocycline decreases the protein expression of pro-inflammatory cytokine tumor necrosis factor-α (TNF-α)

The brain cytosolic fractions of CM mice showed high protein expression of TNF-α compared to control group ($P < 0.001$). There was a highly significant reduction in the protein levels of TNF-α in minocycline-treated group comparison to CM group ($P < 0.001$; Fig. 3A and B).

3.4. Minocycline contributes to decreased apoptosis and hemorrhage

Hematoxylin & Eosin (H&E) staining of the mouse brain sections showed increased apoptosis (identified by condensed cytoplasm with dark shrunken nuclei and large interstitial spaces) and hemorrhages in CM mice (Fig. 4B, E, and H). Apoptosis was decreased and hemorrhages absent in minocycline group (Fig. 4C, F, and I). Most importantly, there were no hemorrhages in the dentate gyrus, the primary memory-associated region of the hippocampus (Fig. 4F).



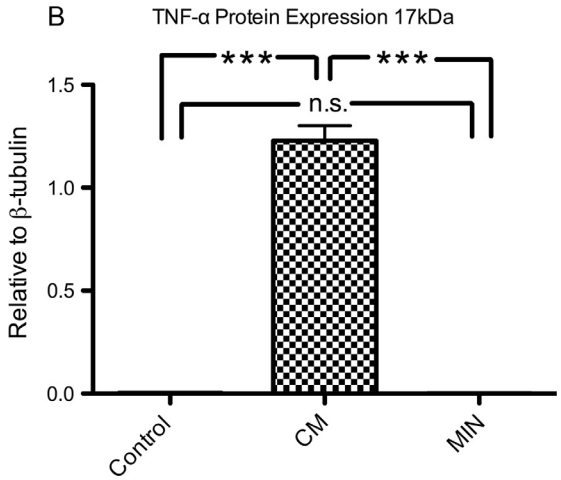


Fig. 3. Minocycline decreases the protein expression of pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-alpha). (A) Immunoblot showing the TNF-alpha protein levels in brain samples of different experimental groups: control (CON), cerebral malaria (CM) and minocycline (MIN). The experiment was repeated three times. (B) Graph showing the TNF-alpha protein expression in relation to β-tubulin. The results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM (*** indicate $P < 0.001$, ** indicate $P = 0.001$–0.01 and n.s indicate not significant, $P > 0.05$).

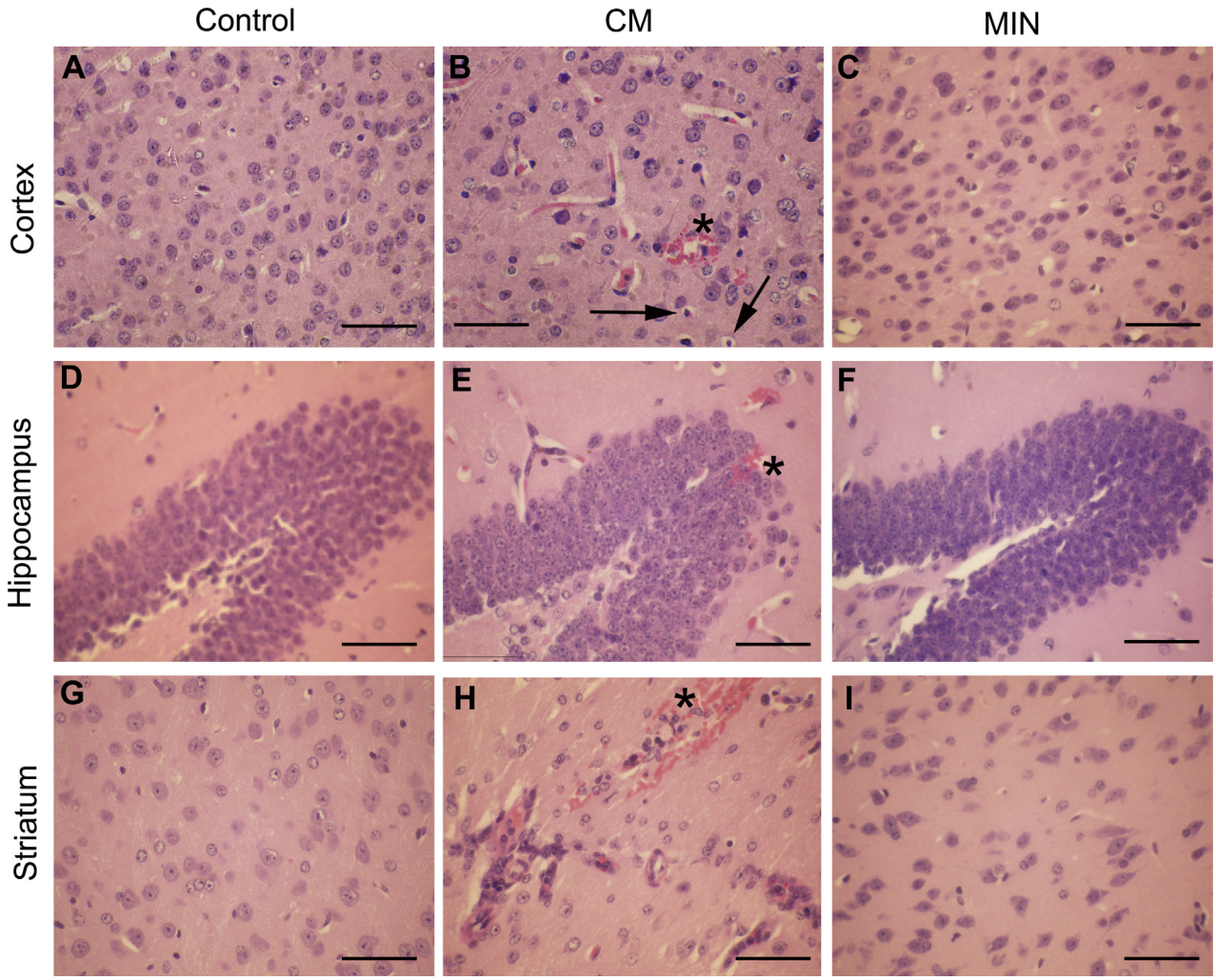


Fig. 4. Minocycline treatment leads to decreased apoptosis and hemorrhage. (A–I) Representative images of brain regions (cortical, hippocampal and striatal) of experimental groups control, CM and minocycline-treated (MIN) after H&E staining (Magnification, 400×). In hippocampus, dentate gyrus, the primary region associated with memory is especially shown. Arrows indicate the cells undergoing apoptosis and asterisks indicate hemorrhages (bars, 50 μm).

3.5. Minocycline prevents neurodegeneration

Fluoro-Jade C (FJC) staining revealed increased number of FJC-positive cells in CM mice brains (Fig. 5B, E, and H). In contrast, brains of minocycline group showed a decrease in FJC-positive cells (Fig. 5C, F, and I) as comparable to that of control (Fig. 5A, D, and G). Specifically, there was a significant reduction of FJC-positive cells in the cortex and striatal regions of the minocycline group ($P < 0.01$). Most importantly, we found the absence of FJC-positive cells in memory associated-hippocampal region of the brain ($P < 0.001$; Fig. 5F).

3.6. Minocycline decreases effector caspase activity

The brain cytosolic fractions of CM mice showed high caspase (effector caspases 3, 6 and 7) activity compared to control group. Minocycline treatment led to inhibition of caspase activity ($P < 0.001$; Fig. 6). Interestingly, the caspase activity level in minocycline group was even less than in control group, but this was not statistically significant ($P > 0.05$).

3.7. Minocycline decreases gene expressions of inflammatory mediators

Consistent with previous reports, gene expressions of *IFN-gamma*, *CXCL10*, *CXCR3*, *CCL5*, *CCL2* and *CCR2* were elevated in the brains of CM mice With exception of *TNF-alpha,* minocycline treatment led to significant decrease in the gene expressions of *IFN-γ* ($P < 0.01$; Fig. 7B), *CXCL10* ($P < 0.001$; Fig. 7C), *CXCR3* ($P < 0.001$; Fig. 7D), *CCL5* ($P < 0.001$; Fig. 7E), *CCL2* ($P < 0.001$; Fig. 7F) and *CCR2* ($P < 0.01$; Fig. 7G). Interestingly, *CXCL10* gene expression was almost nullified in minocycline group (relative gene expression = 0.0105).

3.8. Minocycline decreases inflammatory cytokine and chemokine gene expression irrespective of its anti-parasitic activity

In another batch of infected mice treated with minocycline, the mice were sacrificed before parasite clearance. There was no significant change among the minocycline-treated groups compared to CM group ($P > 0.05$). This indicates that the parasite loads in minocycline-treated groups were identical to that of CM group and incomplete parasite clearance by minocycline upon tissue sampling. Gene expression of *IFN-γ* was significantly decreased ($P < 0.01$) in the case of all minocycline-treated groups compared to CM group (Fig. 8B). There was a highly significant reduction in the *CXCL10* gene expression in experimental groups, 4th day p.i. after two minocycline doses ($P < 0.001$), 5th day p.i. after two minocycline doses ($P < 0.01$), and 6th day p.i. after three minocycline doses ($P < 0.001$). In the case of *CXCR3* gene expression, there was a highly significant reduction in experimental group 6th day p.

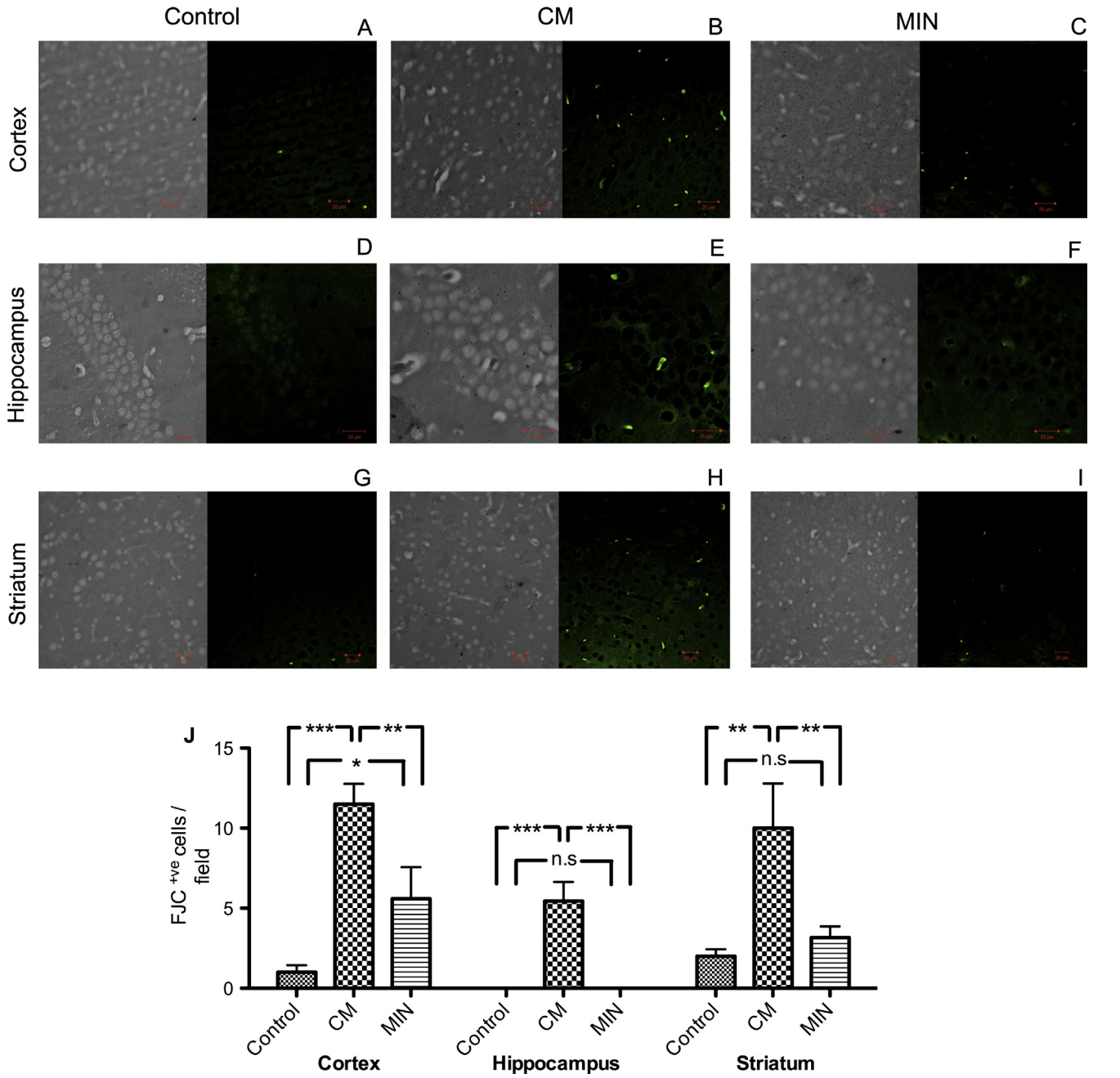


Fig. 5. Minocycline prevents neurodegeneration. (A–I) Representative images of brain regions (cortical, hippocampal and striatal) of experimental groups control, CM and minocycline-treated (MIN) after Fluoro-Jade® C staining. Cells stained green indicate degenerating neurons (bars, 20 μm). (J) Graph showing the number of Fluoro-Jade C positive cells per field (n = 5 fields) in different experimental groups control, CM and minocycline-treated (MIN). Results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM (*** indicate $P < 0.001$, ** indicate $P = 0.001–0.01$, * indicate $P < 0.05$, and n.s indicate not significant, $P > 0.05$).

i. after three minocycline doses compared to CM. There was no significant reduction of *CXCR3* gene expression in 4th day p.i. after two minocycline doses, and 5th day p.i. after two minocycline doses ($P > 0.05$).

4. Discussion

Most of the knowledge regarding human CM has been from autopsy reports that represent the terminal stages and hence, cannot reveal much about the events that lead to CM. As it is unethical to experiment on humans, animal models have been developed for suitable interventions before the terminal stages. For our study, we used the currently accepted model of CM: C57BL/6 mice infected with *PbA* [8].

In the case of rodents, the dose of minocycline which gives neuroprotective effect is reported to be in the range 10–90 mg/kg [13]. A study in the mouse model of Japanese Encephalitis Virus infection was done using two dose regimens, 45 mg/kg and 22.5 mg/kg [33]. In our study, the effective dose of minocycline was decided on a trial-and-error method. Both doses of 45 mg/kg and 22.5 mg/kg were found to increase the survivability of *PbA*-infected mice treated with minocycline. However, we found increased reticulocytosis in the mice group that received 22.5 mg/kg. As reticulocytosis is linked to anemia [32], we considered the dose of 45 mg/kg to be favorable and was continued through rest of the study.

Minocycline, like any other tetracycline, is anti-parasitic [14,15,34]. Tetracyclines bind to the 30S subunit of bacterial ribosomes and prevent binding of aminoacyl-tRNA, leading to inhibi-

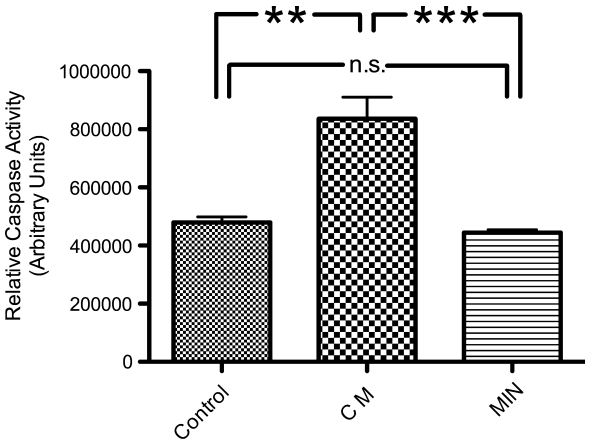


Fig. 6. Minocycline decreases effector caspase activity. The graph shows caspase activity levels in brain samples of different experimental groups: control, CM and minocycline-treated (MIN). The experiment was repeated three times; results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM (*** indicate $P < 0.001$, ** indicate $P = 0.001–0.01$ and n.s indicate not significant, $P > 0.05$).

tion of protein synthesis [11]. *Plasmodium* contains apicoplasts (apicomplexan plastid), a cyanobacteria-derived endosymbiotic plastid. The absence of apicoplasts does not result in the death of parasites, but leads to an error in the development of parasite progenies and thereby an impairment of successful invasion of uninfected blood cells [35]. As apicoplasts are of bacterial origin, tetracyclines affect apicoplast of the *Plasmodium* [11,34]. In our study, the parasites exposed to minocycline showed morphological irregularities, probably due to impaired protein synthesis, which decreased its virulence. Also, minocycline has been shown to decrease the activities of *Plasmodium* mitochondria and plastids [36]. These might be the reasons for parasite clearance in minocycline-treated mice and mice continued to survive for 90 days (mice monitored for 90 days and experiment ended).

Autopsy studies have shown that TNF-α and interleukin-1β levels are elevated in the human CM brains [37]. Activation of microglia (resident macrophages in the brain) during infection, leads to the release of inflammatory mediators like pro-inflammatory cytokine TNF-α, reactive oxygen intermediates, reactive nitrogen intermediates; and other products like matrix metalloproteinases, glutamate and quinolinic acid [38]. Jambou et al. [39] showed that TNF-α enhances the transfer of *P. falciparum* antigens from iRBC to human brain endothelial cells, leading to an opening of intercellular junctions and compromising BBB integrity. BBB breakdown occurs via soluble guanylyl cyclase and protein tyrosine kinase activation [39,40] contributing to CM pathogenesis. In our study, we found that minocycline was able to effectively decrease TNF-α protein levels in brain and maintain BBB integrity. Minocycline, with its ability to inhibit T-cells from producing inflammatory cytokines like TNF-α and interferon-γ [41], might have prevented BBB disruption in minocycline-treated mice.

Dentate gyrus, the primary memory-associated region of the hippocampus, was protected from hemorrhages upon minocycline treatment; pointing to the significant neuroprotection imparted by minocycline. Our results are consistent with the study by Wu et al.

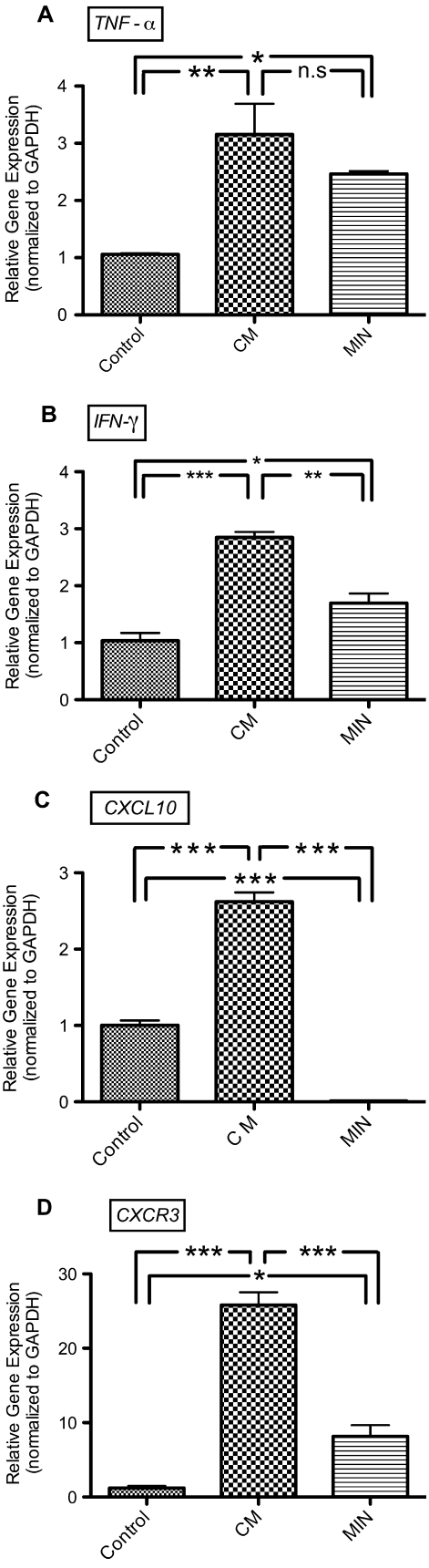


Fig. 7. Minocycline decreases inflammatory cytokine and chemokine gene expression. Graphs showing gene expressions of *TNF-α* (A), *IFN-γ* (B), *CXCL10* (C), *CXCR3* (D), *CCL5* (E), *CCL2* (F) and *CCR2* (G) in brains of different experimental groups control, CM and minocycline-treated (MIN). The experiment was repeated three times; results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM (*** indicate $P < 0.001$, ** indicate $P = 0.001–0.01$, * indicate $P < 0.05$, and n.s indicate not significant, $P > 0.05$).

[42] who showed that minocycline abrogated thrombin-induced intracerebral hemorrhages by decreasing TNF-α and interleukin-1β levels, and reducing microglial activation. We also found the

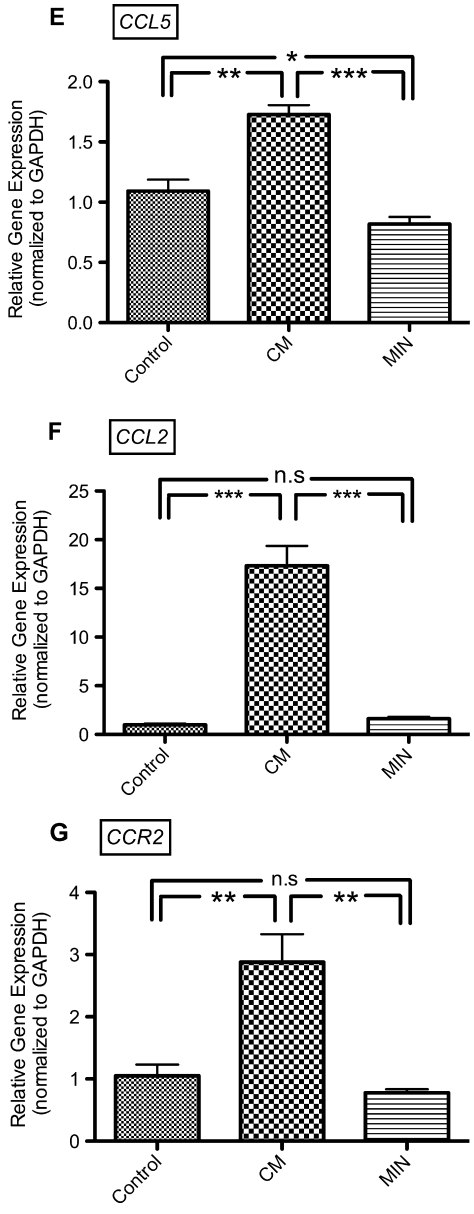


Fig. 7 (*continued*)

absence of degenerating neurons in the hippocampus of minocycline-treated mice after Fluoro-Jade C staining. Our results suggest that minocycline is highly effective in preventing neurodegeneration in cognition-associated regions of the brain.

The release of cytochrome-c, Smac/Diablo (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI) and AIF (apoptosis-inducing factor) from the mitochondria are the molecular events ultimately leading to apoptosis. Minocycline has earlier been shown to inhibit these steps. Also, minocycline has also been

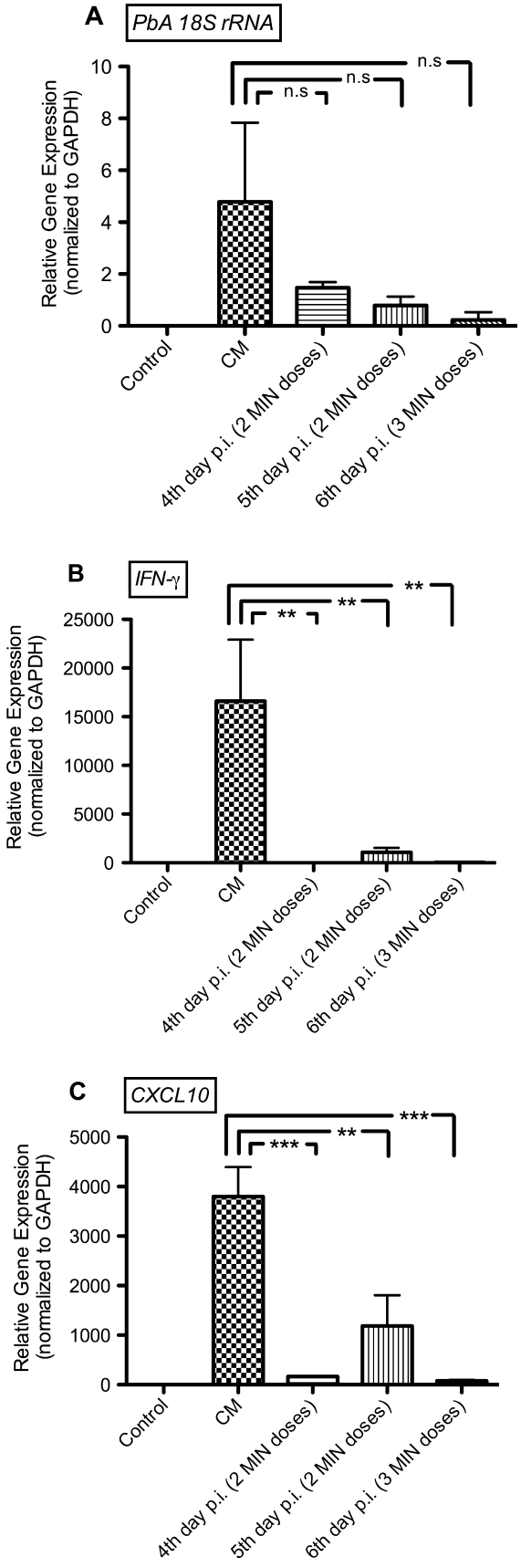


Fig. 8. Minocycline decreases inflammatory cytokine and chemokine gene expression independent of anti-parasitic activity. Graphs showing gene expressions of *PbA I8S rRNA* (A), *IFN-γ* (B), *CXCL10* (C) and *CXCR3* (D), in brains of different experimental groups control, CM, 4th day p.i. after two minocycline doses, 5th day p.i. after two minocycline doses, and 6th day p.i. after three minocycline doses. The experiment was repeated three times; results were subjected to one-way ANOVA with Holm-Sidak post-test and expressed as mean ± SEM (*** indicate $P < 0.001$, ** indicate $P = 0.001–0.01$, * indicate $P < 0.05$, and n.s indicate not significant, $P > 0.05$). MIN = minocycline dose, 45 mg/kg daily.

reported to downregulate caspase-1 and caspase-3 expression; and upregulate the anti-apoptotic protein Bcl-2 levels [18,20,43]. Minocycline has been shown to inhibit both caspase-dependent

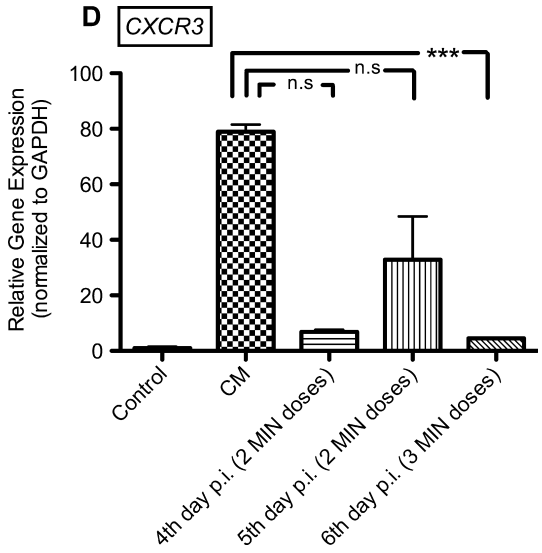


Fig. 8 (*continued*)

and caspase-independent apoptosis [44,45]. Similarly, we found a decrease in apoptosis and inhibition of effector caspase activity in the minocycline-treated group compared to the CM group. The inhibition of apoptosis implies that minocycline effectively inhibits neuronal cell death and provides neuroprotection during CM.

The murine cerebral malaria pathology has been correlated to T cell homing in the brain and chemokine receptor CXCR3 [23,24,46]. Several studies have demonstrated the ability of minocycline to decrease the production of *CXCR3* mRNA [25,26]. Bakmiwewa et al. showed that IFN-γ induces production of CXCL10 by astrocytes during murine cerebral malaria [47]. Consistent with this study, our gene expression studies in mouse brain showed that minocycline decreased the mRNA levels of *IFN-γ*, accompanied by the decline in expressions of *CXCL10* and *CXCR3*. It is probable that lowered production of CXCL10 in the brain might have contributed to high splenic retention of $CD4^+$ T cells, reduced chemoattraction towards the brain and decreased cerebral inflammation explaining the neuroprotection conferred to infected mice upon minocycline treatment. Also, it is well known that $CD4^+$ T cells play a major role in immunity against malaria via production of cytokines that improve phagocytosis by macrophages [24]. The rise in splenic $CD4^+$ T cell population might have also contributed to parasite clearance, in addition to the anti-parasitic property of minocycline. Moreover, the leukocyte migration to the brain is also dependent on the increased gene expressions of CCL5 and CCR2 [48]. Our study shows that minocycline treatment also decreases the gene expressions of *CCL5* and *CCR2* in the mouse brain; consequently preventing cerebral inflammation.

During pathological conditions, CXCL10 is expressed by neurons and astrocytes to attract $CXCR3^+$-microglia. Increased expression of CXCL10 and CXCR3 is correlated with microglial activation and dendrite loss [49]. Minocycline might decrease dendrite loss and impart neuroprotection by reducing the production of CXCL10 and CXCR3. Wilson et al. [50] showed *in vitro* that CXCL10 induces caspase-dependent apoptosis of human brain microvascular endothelial cells and inhibition of CXCL10 using atorvastatin as an adjunctive therapy in experimental cerebral malaria increased the survivability of mice up to 30%. Lowered production of CXCL10 would also have decreased the caspase-dependent apoptosis of endothelial cells associated with BBB.

Limitation of our study is the lack of *Plasmodium* strain resistant to minocycline. Neuroprotective role of minocycline may be further confirmed using a resistant *Plasmodium* strain. The resistance of *Plasmodium* to tetracyclines has been associated to copy numbers of *P. falciparum GTPase TetQ gene* (*pfTetQ*) and *P. falciparum metabolite drug transporter gene* (*pfmdt*) [51]. Extra passaging of *PbA* in mice with increasing doses of minocycline can result in a resistant parasite strain [16]. Future experiments with resistant strain including behavioral analyses can further confirm cognitive improvement in mice. In our study, we showed that minocycline is neuroprotective in murine cerebral malaria. Although there is a chance of parasite developing resistance to minocycline in future, only the anti-parasitic effect of the minocycline will be affected. The neuroprotective action of minocycline will be intact and will help in preventing cognitive deficits during infection or post-infection.

Another batch of infected mice was treated with daily doses of minocycline (45 mg/kg), two to four days before the onset of cerebral symptoms and mice were sacrificed before complete parasite clearance. Our gene expression studies of the mice brains revealed that minocycline reduced the mRNA levels of inflammatory mediators IFN-γ and CXCL10, and the reduction was irrespective of the parasite load in the brain. Also, minocycline was able to affect *CXCR3* gene expression when three doses of minocycline were given before 6th day (p.i.). Therefore, our gene expression studies confirm that the neuroprotective activity of minocycline was not a consequence of parasite clearance. Although doxycycline, another tetracycline, has been in use as chemoprophylaxis with standard antimalarials, doxycycline lacks the ability to modulate *CXCR3* gene expression and confer neuroprotection [25]; which points out that minocycline is more effective than doxycycline for preventing CM.

When we tried rescue treatment during final stages of CM, mice succumbed to CM and rescue treatment was a failure. It has already been discussed that minocycline might be affecting the parasite apicoplasts [35]. As mice during the final stage of CM survive only for 3–6 h, minocycline might not be having sufficient 'time window' to affect the parasite division. Hence, except for final stages of CM, minocycline might be a suitable candidate as an adjunctive therapy for conferring neuroprotection and preventing CM.

Considering the pharmacokinetics of minocycline, its half-life in humans is 12–18 h [52] whereas 2–3 h in mice [53,54]. During i.p administration, the peritoneal cavity acts as a reservoir, slowly releasing and maintaining the levels of minocycline in plasma, thus aiding parasite clearance [55]. In addition, mice are given only a single 'high' minocycline dose daily. However, this 'high' dose can be considered to be low dose taking into consideration the high liver metabolism in rodents. Hence, the mice are in reality, are under-dosed [19]. However, even after under-dosing, we got favorable outcomes of increased survivability in mice, and most importantly, neuroprotection.

5. Conclusions

Our study shows a promising role of minocycline in experimental cerebral malaria. As minocycline has a neuroprotective action in CM, it might have a major role to play as adjunctive therapy with other antimalarials. However, it has to be validated by human clinical trials for use as a neuroprotective agent in human severe malaria cases. If successful in clinical trials, minocycline will help in decreasing the morbidity and mortality associated with this dreadful neurological complication.

Acknowledgements

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Matrix Metalloproteinase-9 Polymorphism 1562 C > T (rs3918242) Associated with Protection against Placental Malaria

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Abstract. Phagocytosis of malaria pigment (hemozoin) induces increased activity of matrix metalloproteinase (MMP)-9, an endopeptidase involved in cytokine regulation. In this study, we examined whether a common functional *MMP-9* promoter polymorphism (rs3918242) affects *Plasmodium falciparum* infection in pregnancy. Eighteen percent of Ghanaian primiparae carried the minor *T* allele. It was associated with reduced odds of placental hemozoin and of placental as well as peripheral blood parasitemia. The results indicate that a common *MMP-9* polymorphism protects against placental malaria indicating that this endopeptidase is involved in susceptibility to *P. falciparum*.

Pregnant women are a particular risk group for infection with *Plasmodium falciparum* and malaria. Although commonly asymptomatic at high endemicity, malaria in pregnancy may cause anemia, abortion, stillbirth, low birth weight (LBW), and preterm delivery (PTD), and contributes to high infant mortality. The increased susceptibility of pregnant women, particularly primigravidae, is largely due to parasites expressing specific variants of the *P. falciparum* erythrocyte membrane protein-1. Parasite adhesion via these variant surface proteins results in the sequestration of infected red blood cells (RBCs) in the placental intervillous space. Sequestration frequently is accompanied by local hemozoin (malaria pigment) deposition and accumulation of inflammatory cells, including monocytes/macrophages. Specific immune mechanisms targeting the pregnancy-associated parasites, particularly parasite-specific antibodies, are low in primigravidae. Only with successive pregnancies, these are acquired, and infection risk and manifestation decrease.[1–3]

Risk and manifestation of malaria and of malaria in pregnancy are influenced by diverse factors including host genetics.[4,5] The latter may involve variants of matrix metalloproteinases (MMPs), a family of metal ion-dependent endopeptidases that are involved in the breakdown of extracellular matrix and tissue remodeling.[6] MMPs also contribute to the regulation of various cytokines and chemokines, thus playing an important role in host immune responses.[7] In acute malaria, increased expression of MMP-9 (gelatinase B) has been observed.[8] Moreover, in vitro, hemozoin phagocytosis by human monocytes and the exposure of endothelial cells to parasitized RBCs stimulate the release of MMP-9 and tumor necrosis factor α (TNF-α).[9,10] A common *MMP-9* gene polymorphism (rs3918242, replacement of C by T at location 1562) increases the promoter activity of the *MMP-9* gene 1.5-fold because the associated transcriptional repressor protein has a reduced affinity to the *T* allelic promoter. The polymorphism, hence, has been associated with increased transcription activity and with altered risks of various diseases.[11–13] We therefore examined whether this polymorphism affects susceptibility to or manifestation of malaria in pregnancy.

The characteristics of the 304 primiparous pregnant women with live singleton delivery have been reported elsewhere.[2] Informed written consent was obtained from all women, and the study protocol was approved by the Committee on Human Research Publications and Ethics, School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. In brief, delivering women were recruited at the Presbyterian Mission Hospital in hyper- to holoendemic Agogo, Ghana (population, 30,000), in 2000 and 2001. Women were clinically examined and sociodemographic data were documented. Intervillous and peripheral blood samples were collected into ethylenediaminetetraacetic acid (EDTA). Parasites were counted on Giemsa-stained blood smears per 100 high-power fields and per 500 white blood cells, respectively. Leukocyte-associated hemozoin in placental samples was recorded. Deoxyribonucleic acid (DNA) was extracted from blood (AS1 and QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany), and nested *P. falciparum*–specific polymerase chain reaction (PCR) assays were performed.[14] Present or past placental *P. falciparum* infection was defined by the presence of placental parasites or hemozoin in microscopy, or a positive placental *P. falciparum* PCR result. Pyrimethamine in plasma (indicating compliance with the chemoprophylaxis recommended at that time) was measured by enzyme-linked immunosorbent assays (ELISAs) with limits of detection of 10 ng/mL.[15] Anemia was defined as hemoglobin (Hb) < 11 g/dL, measured by a hemoglobin photometer (HemoCue AB, Ängelholm, Sweden). LBW was defined as birth weight < 2,500 g, and PTD as gestational age < 37 weeks applying the Finnström score.[16] Genotyping of rs3918242 was achieved by restriction fragment length polymorphism.[17] Data were analyzed with Statview 5.0 (SAS Institute Inc., Cary, NC). Continuous variables were compared between groups by the Mann–Whitney *U* test or Student's *t* test as applicable. Associations of genotypes with, for example, *P. falciparum* infection were assessed by χ^2 test, and odds ratios (ORs) and 95% confidence intervals (95% CI) were determined. Adjusted ORs (aORs) were calculated in logistic regression models with stepwise backward removal of factors not associated in multivariate analysis ($P > 0.05$).

Typing of rs3918242 was successful in 302 of 304 primiparae. The major homozygous genotype (*CC*) was observed in

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TABLE 1
Characteristics of 302 Ghanaian primiparae with live singleton delivery according to MMP-9 genotype (rs3918242)

Parameter	All	rs3918242		*P**
		Major genotype (*CC*)	Genotypes with *T* allele (*CT*, *TT*)	
No. (%)	302 (100)	248 (82.1)	54 (17.9)	
Age (years); median (range)	20.5 (15–36)	20 (15–36)	21 (15–30)	0.25
Rural residence (*n*, %)	154 (51.0)	126 (50.8)	28 (51.9)	0.89
>3 antenatal care visits (*n*, %)	139/295 (47.1)	106/241 (44.0)	33/54 (61.1)	0.02
Delivery in rainy season (*n*, %)	155 (51.3)	130 (52.4)	25 (46.3)	0.41
Pyrimethamine in plasma (*n*, %)	106/297 (35.7)	87/243 (35.8)	19 (35.2)	0.93
Anemia (*n*, %)	116 (38.4)	99 (39.9)	17 (31.5)	0.25
LBW (*n*, %)	79 (26.2)	65 (26.2)	14 (25.9)	0.97
PTD (*n*, %)	80 (26.5)	69 (27.8)	11 (20.4)	0.26
Plasmodium falciparum infection (peripheral blood)				
Microscopy positive (*n*, %)	80 (26.5)	75 (30.2)	6 (11.1)	**0.004**
Geometric mean parasite density/μL, (95% CI)	746 (476–1,171)	718 (451–1,141)	1,159 (173–7,773)	0.58
PCR positive (*n*, %)	179 (59.3)	152 (61.3)	27 (50)	0.13
P. falciparum infection (placental blood)				
Microscopy positive (*n*, %)	139 (46.0)	122 (49.2)	17 (31.5)	**0.02**
Geometric mean parasite density/100 high-power fields, (95% CI)	119 (78–181)	126 (83–191)	431 (73–2,544)	0.48
Hemozoin positive (*n*, %)	128 (42.4)	115 (46.4)	13 (24.1)	**0.003**
PCR positive (*n*, %)	196 (64.9)	166 (66.9)	30 (55.6)	0.11
Present or past placental infection (*n*, %)	205 (67.9)	175 (70.6)	30 (55.6)	**0.03**

CI = confidence interval; LBW = low birth weight; MMP = matrix metalloproteinase; PCR = polymerase chain reaction; PTD = preterm delivery.
*P values derived from Student's t tests, Mann–Whitney U tests, or χ^2 tests, as applicable. P value in bold indicate significance < 0.05. In the three *TT* homozygous individuals, prevalence was peripheral blood: microscopy positivity, 0/3; PCR positivity, 1/3; placental blood: microscopy positivity, 0/3; hemozoin positivity, 0/3; past or present placental infection, 1/3.

82.1% (248); 17.9% of the women exhibited the minor *T* allele (heterozygous, 51; homozygous, 3; grouped henceforth). Genotypes were in Hardy–Weinberg equilibrium.

Overall, 67.9% of the women had evidence of present or past *P. falciparum* infection. Placental hemozoin was observed in 42.4%, and PCR assays on placental samples were positive in 64.9% (Table 1). Nevertheless, fever occurred in only 2.5% (12/299). Women with the minor *T* allele had a significantly lower prevalence of present or past placental *P. falciparum* infection (OR = 0.52; 95% CI = 0.27–0.99; $P = 0.03$), placental hemozoin (OR = 0.37; 95% CI = 0.18–0.75; $P = 0.003$), placental parasitemia (OR = 0.47; 95% CI = 0.24–0.92; $P = 0.02$), and peripheral blood parasitemia (OR = 0.29; 95% CI = 0.10–0.79; $P = 0.004$). For infections detected by PCR, the difference did not reach statistical significance, and parasite densities did not differ between women with and without the minor *T* allele (Table 1). Adjusting for factors previously identified as being associated with placental malaria, that is, delivery in rainy season, age, and presence of plasma pyrimethamine,[2] women with the minor *T* allele tended to have reduced odds of present or past *P. falciparum* infection (aOR = 0.56, 95% CI = 0.30–1.03, $P = 0.06$; age (years), aOR = 0.92, 95% CI = 0.85–0.98; rainy season, aOR = 1.76, 95% CI = 1.06–2.92; plasma pyrimethamine, aOR = 0.58, 95% CI = 0.35–0.98). Further adjustment for the difference between groups in the use of antenatal care (itself not associated with malaria, Table 1) did not substantially change the estimate (aOR = 0.55, 95% CI = 0.29–1.02, $P = 0.06$). In the multivariate model, significantly reduced odds were observed for placental hemozoin (aOR = 0.38, 95% CI = 0.19–0.75, $P = 0.005$), placental parasitemia (aOR = 0.49, 95% CI = 0.26–0.93, $P = 0.003$), and peripheral blood parasitemia (aOR = 0.29, 95% CI = 0.12–0.73, $P = 0.008$).

Maternal anemia and PTD but not LBW tended to be less common in women carrying the minor *T* allele. However, there was no association of these outcomes with the polymorphism (Table 1), irrespective of stratification into infected and noninfected women (data not shown).

MMP-9 has been shown to be upregulated in acute malaria,[8] and specifically, both parasitized RBCs and hemozoin induce release of MMP-9 by monocytes and endothelial cells.[9,10] The role of this endopeptidase in malaria is, nevertheless, controversial, potentially generating both protective and detrimental effects.[18] Here, we show that a promoter single nucleotide polymorphism (SNP) increasing MMP-9 activity reduces the odds of placental malaria. The mechanisms involved are speculative: MMP-9 has an important role in the regulation of inflammatory processes including a complex influence on various chemokines. Increased MMP-9 levels may result in both increased and decreased chemotactic activities but in murine models they appear to promote leukocyte migration. In addition, MMP-9 modulates the activity of several pro-inflammatory mediators, for example, by inducing the release of TNF-α or activating pro-interleukin (IL)-1β, thereby augmenting the pro-inflammatory response.[7] Pro-inflammatory responses in malaria are double edged: they may contribute to pathophysiologic damage but initially increased release in particular contributes to accelerated parasite clearance.[3] Conceivably, increased MMP-9 activity may affect leukocyte recruitment to the intervillous space and local pro-inflammatory responses thereby enhancing parasite elimination. Notably, the placental syncytiotrophoblast, the epithelium lining the intervillous space, has been shown to be immunoreactive to *P. falciparum*,[19] and also to express MMP-9.[20] Moreover, part of the produced MMP-9 forms heteromers with chrondroitin sulfate proteoglycans.[21] Altered *MMP-9* expression could affect heteromer formation, which in turn may decrease binding of parasitized RBCs to chondroitin sulfate (the main placental parasite ligand), resulting in reduced placental malaria.

As a limitation, this study was not a priori designed to assess an association between *P. falciparum* infection in

pregnancy and the *MMP-9* promoter SNP, and it comprised a relatively small group of 302 primiparae. We use the term placental malaria to describe the detection in placental blood samples of parasites by microscopy or PCR and of hemozoin by microscopy. This does not correspond to histologic classification as suggested by, for example, Muehlenbachs and others[22] but is characterized by a high sensitivity due to the inclusion of PCR assays.[2] Although infection prevalence as assessed by PCR differed between genotypes, this did not reach statistical significance. This was partly because the proportion of submicroscopic infections (i.e., positive by PCR only, but negative by microscopy) among all infections was comparatively increased in individuals with the minor *T* allele (Table 1). Thus, infection prevalence as detected by PCR in *T* allele carriers was overall reduced even if not significantly so, and low-level infections prevailed. Statistical significance might have been present in case of a larger sample size. The same applies to the manifestation of infection, for example, malaria-associated anemia or PTD.

The MMPs have been considered as biomarkers and therapeutic targets in malaria.[18] This study suggests that a common functional *MMP-9* polymorphism is associated with reduced odds of placental malaria, and thereby provides evidence for the in vivo relevance of MMP-9 in human malaria.

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- MMP-9 polymorphism 1562 C > T (rs3918242) associated with protection against placental malaria.

Apoorv & Babu - Cytokine J (2017) 90, 113-123.
- Minocycline prevents cerebral malaria & confers neuroprotection during Plasmodium berghei infection

Apoorv et al. (under review - Neurosci Lett)
- AMP-activated protein kinase (AMPK) is decreased in the mouse brain during experimental cerebral malaria

Kunal*, Apoorv* et al. (manuscript to be submitted to Antimicrob Agents Chemother)
- Artemether nanoformulation rescues mice from murine cerebral malaria

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AMP-ACTIVATED PROTEIN KINASE (AMPK) AND MATRIX METALLOPROTEINASE - 9 (MMP-9) IN SEVERE MALARIA AND ROLE OF MINOCYCLINE AS A THERAPEUTIC AGENT

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