Potential Efficacy of Citicoline as Adjunct Therapy in Treatment of Cerebral Malaria

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Cerebral malaria (CM) is characterized by a dysregulated immune response that results in endothelial membrane destabilization and increased microparticle (MP) production. Citicoline (CTC) is a membrane stabilizer used for the treatment of neurological disorders. We evaluated the efficacy of CTC as adjunct therapy to aid recovery from experimental CM. We show that CTC reduces MP production *in vitro*; in combination with artesunate *in vivo*, confers partial protection against CM; and prolongs survival.

The incidence of cerebral malaria (CM) is highly dependent on the immune status of the population and the frequency of *Plasmodium falciparum* transmission. This debilitating syndrome is characterized by coma, seizures, and respiratory distress. In children, the coma usually develops rapidly after a seizure, whereas in adults its development is slower and takes 2 to 3 days (1).

During infection, the sequestration of mature-stage-parasitized erythrocytes, platelets, and leukocytes within cerebral microvessels, coupled with cytokine overproduction, leads to hypoxia (2), endothelial activation, and blood-brain barrier disruption (3). During the acute phase of pediatric CM, several pathophysiological mechanisms are involved, including CD8$^+$ T cell activation, (4) and the complement cascade (5), although disruption of the endothelium seems to be a supported mechanism of cerebral dysfunction.

Evidence of a destabilized endothelium is the increased release of endothelial-cell-derived microparticles (MP). In CM patients, high titers of MP are detected in the blood coinciding with a rise in the tumor necrosis factor (TNF) level, which returns to the baseline postinfection (p.i.) (6). These elevated levels do not occur in uncomplicated malaria or severe anemia (7). TNF plays a major role in this mechanism, as it strongly induces endothelial vesiculation (8).

Current therapies for CM are designed to target the pathogen rather than the underlying pathogenic mechanisms responsible for the manifestation of the pathology (9–11). In the last 5 years, several novel compounds have been tested in animal models or in humans as adjunct therapy to prevent tissue and brain alterations during infection, including erythropoietin (EPO) (2, 12), defibrotide (13), atorvastatin (14, 15), the exogenous nitric oxide (NO) donor dipropylenetriamine-NONOate (16), and others (17, 18). EPO, atorvastatin, and NO have undergone U.S. FDA review and been approved for other indications. However, most of these compounds are new and have a long way to go from the benchtop before being implemented as a line of treatment at the bedside. Targeting membrane alteration in the same approach as for ischemia-reperfusion disorders paves the way for the use of a treatment already used for patients. Citicoline (CTC) is used in the United States and Europe by adults and children to improve brain recovery after ischemic stroke and neurological and vascular disorders (19–22). It works efficiently on damage after focal ischemia (neuronal damage, blood-brain barrier dysfunction, behavioral dysfunction) and brain edema (for a review, see reference 23) in

mice, but its benefit for humans is still controversial (24). CTC is a natural compound found in eukaryotic cells that regulates membrane fluidity and is synthesized mainly by the CDP-choline pathway (23–26).

We evaluated the efficacy of CTC, a membrane-stabilizing agent, as adjunct therapy to enhance recovery from experimental CM. In this study, we used both *in vitro* and *in vivo* models of CM. Mouse models using *Plasmodium berghei* ANKA infection can mimic CM pathology. *P. berghei* ANKA-infected mice develop a lethal syndrome 7 days p.i. with a significant increase in MP in their plasma at the time of onset of the neurological syndrome (27, 28).

CTC confers protection against neurological syndrome in murine CM. All of the mice used in this study were handled under University of Sydney Animal Ethics Committee approval (approval number K20/7-2006/3/4434). Mice were infected with *P. berghei* ANKA by following protocols in previously published studies (29). Eight-week-old female CBA mice received intraperitoneal injections of 1×10^6 infected red blood cells. The cerebral syndrome usually occurred during the neurological phase, day 7 to 14 p.i. Mice that surpassed the neurological syndrome died during the hyperparasitemia phase (day 14 onward) because of severe anemia. Parasitemia was determined by light microscopy with thin Diff-Quick-stained smears of blood collected from the tail vein. Clinical presentation was monitored daily.

Starting on day 4 p.i., mice received intraperitoneal injections of CTC (n = 28), artesunate (ART) (Sigma) (n = 21), or a combination of CTC and ART (n = 14). CTC was solubilized in phosphate-buffered saline (PBS) and administered daily for 3 days. Two doses (500 and 1,000 mg/kg) were used in separate experiments. The regimens were chosen in accordance with literature on mouse stroke models. A subeffective dose of ART (40 mg/kg) was
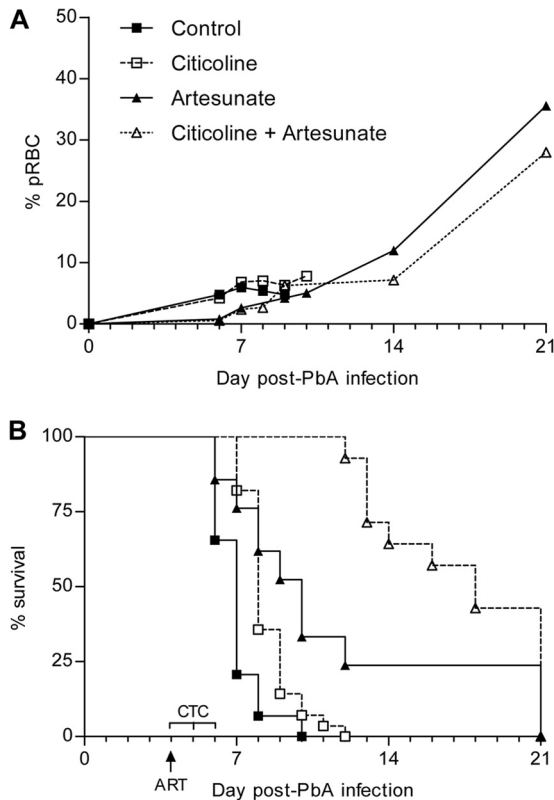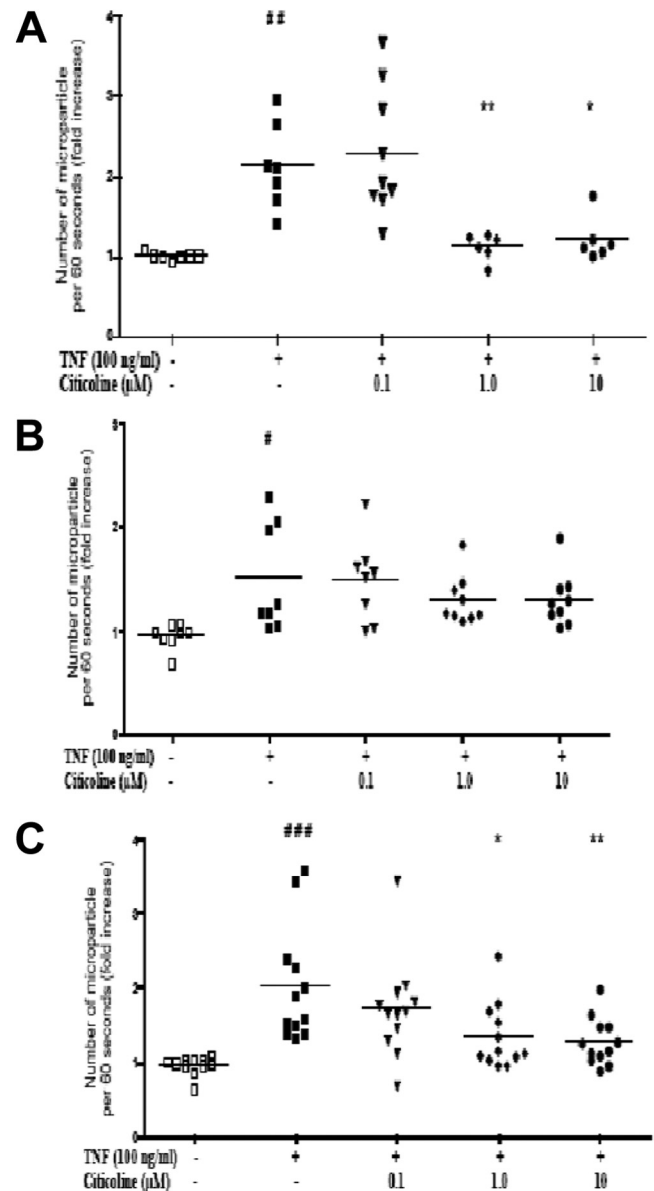
FIG 1 CTC as adjunct therapy for mice with CM. Shown are the parasitemia levels (A) and percentages of survival (B) of infected mice following treatment with CTC alone (n = 28; open squares), ART (n = 21; closed triangles), a combination of CTC and ART (n = 14; open triangles), or PBS (n = 28; closed squares). Parasitemia was calculated from Diff-Quick-stained blood smears, and data are presented as means ± standard deviations. pRBC, parasitized red blood cells.

FIG 2 Effect of CTC on MP production by D3 HBEC. HBEC were stimulated with 100 ng/ml of TNF. The MP in the supernatant were counted in a flow cytometer after annexin V-FITC labeling. (A) Incubation with CTC 2 h prior to TNF stimulation. (B) Incubation with CTC concomitantly with TNF stimulation. (C) Incubation with CTC 6 h after TNF stimulation.

administered once at day 4 p.i. Control mice received PBS treatment (n = 28).

Brain, lung, liver, and spleen tissue samples were collected from mice that exhibited clinical signs of CM or were moribund. The tissue was prepared for thin paraffin sectioning. The hematoxylin-eosin-stained sections analyzed had no histopathological features associated with CM to widespread hemorrhage, a high degree of edema and leukocyte and parasitized red blood cell adherence to the endothelium throughout the brain, plugging of microvessels, and some necrosis of microvessels (30). In this study, histopathology was mainly used to confirm CM.

At day 7, the parasitemia of the two groups treated with ART differed significantly from that of the control group (5.96, 2.6, and 2.35% in controls, ART, and ART plus CTC, respectively, [P = 0.0051 and P < 0.0001]) (Fig. 1). However, no significant difference in parasitemia between controls and the CTC-alone group was found (P = 0.4081). After day 7, no significant difference in parasitemia between the ART and ART-plus-CTC groups was found either, suggesting that CTC by itself has no impact on the parasite.

Control mice developed clinical CM between days 6 and 10 p.i. When CTC was administered at 1,000 mg/kg, the onset of clinical CM was delayed by at least 24 h. Thus, when mice were checked at day 7, treatment with CTC alone had enhanced survival from 20.6 to 82.1% but all of the mice died before day 14. After day 14,

survival increased from 0% (controls and CTC) or 23.8% (ART alone) to 64.2% in the combined ART-plus-CTC group. Combination of CTC and ART prolonged survival by more than 6 days. When used at 500 mg/kg, the trend of the CTC effect was similar to that observed at the 1,000-mg/ml treatment but statistical tests fell under the threshold of significance. Statistics were performed with GraphPad Prism software. Survival curves were compared by using the Kaplan-Meier (log rank) test; P < 0.05 was considered significant. Parasitemia in the control and other groups was compared by using the Mann-Whitney t test.

CTC decreases the production of MP by TNF-stimulated HBEC. MP production by human brain endothelial cells (HBEC) was studied *in vitro* (Fig. 2). Immortalized HBEC (D3 line) (31)

were grown to confluence in EBM-2 medium (Lonza CC-3156) in 24-well plates at 37°C. MP production was induced by incubation with TNF (100 ng/ml) overnight. CTC was added 2 h prior to, concomitantly with, or 6 h after stimulation with TNF (doses, 0.1, 1, 10, 30, and 100 μM). Culture supernatants were collected, and MP were labeled with annexin V-fluorescein isothiocyanate (FITC), a known marker of MP, prior to flow cytometric analysis.

Negative controls for the experiment were obtained from resting cells. Positive controls were obtained from cells stimulated with TNF (100 ng/ml) for 18 h, which induced a 2-fold increase in MP vesiculation compared to that of resting cells. CTC at 0.1 μM showed no apparent inhibition of MP production. However, when added 2 h prior to TNF stimulation, CTC (1 to 10 μM) reduced the formation of MP to the baseline level. When added 6 h after TNF stimulation, CTC (1 to 10 μM) induced a 70% reduction in MP production compared to that of the positive control ($P < 0.05$ and $P < 0.01$, respectively). Again, at the lowest dose, CTC had no significant effect.

In conclusion, data obtained *in vivo* clearly demonstrate that CTC can confer partial protection against experimental CM. This improvement of survival also fits in with a decrease in MP production when HBEC are treated with CTC *in vitro*. These data support a role for CTC in reducing MP production by stabilizing the microvascular endothelium, similar to what is seen in other vascular pathologies (32–35). CTC therapy can be proposed for clinical studies of human patients to improve recovery from CM, as it is already used as a food supplement for malnourished children and has low toxicity.

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