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Research Article

Brain and Hepatic Glucose Utilization in Malarial Infection Does Not Depend on Cerebral Symptoms of the Disease

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Cerebral malaria causes several deaths every year. Global metabolic alteration, specifically hypoglycemia and lactic acidosis are hallmarks of severe malaria. Glucose being the major fuel source for the brain, it is important to understand cerebral glucose utilization in the host during cerebral complications of the disease that may have a significant role in cerebral pathogenesis. We have used ^{13}C NMR spectroscopy to understand glucose utilization in the brain and liver of mice with cerebral malaria (CM), noncerebral malaria (NCM), and in control mice. Animals were challenged with intravenous glucose bolus followed by metabolic profiling of brain and liver extracts. Our result suggests a differential glucose utilization in the malaria group with respect to that of controls, while no difference between CM and NCM.

1. Introduction

Malaria is a global threat that is caused by intraerythrocytic stages of parasites of *Plasmodium* sp. [1]. The parasitized RBCs consume more glucose than the normal RBCs [2]. The rate of glucose utilization by these cells is also higher than the normal RBCs [3, 4]. Intraerythrocytic *Plasmodium* parasites lack gluconeogenic enzyme, fructose 1,6 biphosphatase [5] and glycogenolytic enzymes [6], relying on host RBCs to meet for glucose requirements [7]. Both glycolysis and TCA cycle operate in *Plasmodium* sp. [8] as ATP generation pathways. However, glycolysis seems to be the major pathway in mammalian and rodent host contrary to TCA cycle in parasites. Glucose uptake from the host by parasites lead to hypoglycemia [9] and hyperlactemia [10] and are reported as major features of severe malaria. Hypoglycemia is also attributed to malabsorption of glucose, cytokine induced gluconeogenesis, and increased tissue metabolism [11]. Interestingly, occurrence of pathophysiological features such as hypoglycemia and lactic-acidosis appears to be a function of disease severity and not cerebral complications [12–17]. However, it is unknown if glucose utilization in a major metabolic tissue such as liver and brain operate differentially under CM and NCM conditions. Glucose is the major source of energy in the brain [18–20] and energy metabolism may have a key role to play in the pathophysiology of CM. Thus, it is important to understand how glucose is utilized in brain once the cerebral symptoms developed. Liver, on the other hand is one of the major sites of metabolic processes and is the primary regulatory site for blood glucose homeostasis [21]. Previous studies from our group have established significant perturbation in hepatic and cerebral glucose metabolism [7, 14, 15] in murine cerebral and noncerebral malaria. However, such studies were performed using an untargeted metabolomics approach that provided a snapshot of metabolic status [22, 23]. While such approaches are useful to delineate biomarkers and fingerprints of the tissues under certain perturbed conditions, they do not provide specific pathway level

information of precursor utilization. Isotope tracing strategies using ^{13}C NMR spectroscopy could be used to generate pathway level information by tracing metabolite isotopomers. In this report, we have used ^{13}C NMR spectroscopy to calculate the turnover of various metabolites from isotopically labeled glucose in the brain and liver.

2. Materials and Methods

2.1. Animal Handling. Animals were treated in accordance with the guidelines set forth by the Local Animal Ethics Committee of Tata Institute of Fundamental research (TIFR): IAEC approval no: TIFR/IAEC/2010-3.

2.2. Animal Experiments. Twenty young adult (6–8 weeks, weighing 24–25 g) female C57BL/6 mice were housed in 5 cages (5 mice per cage) under 22 ± 2°C and 12-hour day/night cycle. They had free access to standard food pellets and water. Fifteen of these mice were injected intraperitonially with 10^7 RBCs infected with *Plasmodium berghei* ANKA maintained in 6–8-week-old female Swiss mice. The remaining five mice served as uninfected controls. Rectal temperature of the infected mice was measured twice a day. CM and NCM were determined based on rectal temperature and neurological symptoms of mice. Mice with rectal temperature <34°C accompanied by neurological symptoms like ataxia and paralysis were considered CM, as reported previously [22, 24].

2.3. Glucose Injection. A bolus of 125 μL of 1.2M [1-^{13}C] glucose was injected intravenously into uninfected control mice and mice with CM and NCM at day 9 postinfection. After 20 min, the animals were sacrificed followed by dissection of brain and liver. The organs were snap frozen immediately after collection and stored at −80°C till further processing. The 20 minutes timepoint was chosen based on a pilot experiment (Supplementary S1) that showed optimum NMR signals of glucose metabolites between 30 and 40 min.

2.4. 2-Deoxyglucose Injection (2-DOG) in Mice. In a separate experiment with 12 mice (4 controls) a bolus of 125 μL of 1.2 M [1-^{13}C] 2-DOG was injected intravenously in mice with CM, NCM and uninfected control animals at day 9 post-infection. After 20 minutes they were sacrificed, and their brain were dissected out and snap frozen immediately.

2.5. Tissue Extraction of Brain and Liver. Brain and liver were subjected to methanol/water/chloroform extraction as described before [25]. Brain and liver samples were weighed and transferred to a glass homogenizer. The tissue was homogenized with ice cold methanol (4 ml/g wet weight) and ice-cold water (0.85 ml/g wet weight). To the homogenate 2 ml/g of chloroform was added and vortexed. This was followed by the addition of 2 ml/g of water and chloroform and vortexing. The homogenate was allowed to rest for 15 minutes on ice and centrifuged at 1000*g*. The supernatant was dried in a vacuum concentrator and stored at −20°C until further use for NMR experiments. The dried mass was reconstituted in D_2O containing TSP for NMR spectra acquisition.

2.6. NMR Experiments. ^{13}C {^{1}H-decoupled} NMR spectra of brain and liver extracts were acquired in 700 MHz spectrometer equipped with a triple resonance broad band inverse probe. The acquisition parameters were as follows, spectral width—218 ppm, data points—64 k, number of transient—4000, relaxation delay—1s and acquisition time—0.85s per FID. The ^{1}H decoupling was achieved by WALTZ-16 decoupling sequence. For processing, the co-added FIDs were subjected to an exponential multiplication leading to an additional line broadening of 5 Hz followed by Fourier Transformation. The spectra were manually phased, and baseline corrected. The spectral peaks were quantified and normalized with respect to entire spectrum. Data analysis was performed in Bruker TOPSPIN 2.1. Human Metabolome Database (HMDB) (http://www.hmdb.ca) and published literatures [26, 27] were used for peak assignment.

2.7. Statistical Analysis. The data are presented as box plot with line at median. The data are analyzed by ANOVA, with Bonferroni correction. *denotes statistical significance of $P < 0.05$.

3. Results

3.1. Development of Cerebral Malaria. Out of 15 mice, 14 mice survived till day 9, while 1 mouse died during experiment. 7 mice developed CM symptoms and exhibited rectal temperature <34°C. The symptoms exhibited by CM inflicted mice were ataxia, paralysis, seizures, etc. 6 mice were considered NCM. 1 mouse was kept out of analysis, (borderline rectal temperature). The parasite load was between 20% and 25% when the mice were sacrificed.

3.2. Downstream Product Formation from [1-^{13}C] Glucose. [1-^{13}C]-Glucose is metabolized to [3-^{13}C]-pyruvate that enters TCA cycle via two possible routes – pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC) pathway. The flow of ^{13}C from glucose into downstream metabolites is described in Figure 1. The labeling pattern of metabolites depends on the relative contribution of the metabolic pathways involved. Briefly, [1-^{13}C] glucose is converted to [3-^{13}C] pyruvate via glycolysis. The PDH route then leads to [2-^{13}C] acetyl–CoA which, in turn, forms C4-glutamate and C2-GABA via [4-^{13}C] 2-oxo-glutarate of TCA. Recycling of the label in TCA gives rise to [2-^{13}C] and [3-^{13}C] isotopomers of glutamate. On the other hand, the PC route, functioning as anaplerotic pathway, leads to the formation of glutamate labeled at C2 position via TCA cycle. Recycling of the label in TCA cycle results in the formation of [3-^{13}C] and [2-^{13}C] glutamate. Exact contribution of oxidative vs. anaplerotic pathway is difficult to assess. This is because C2 and C3 labeled glutamate is contributed from both routes. However, since C4 glutamate is formed exclusively via the PDH, a relative contribution of oxidative pathway may be derived by the ratio C4-/C2-glutamate.

3.3. Alterations in Cerebral Glucose Utilization. Representative 1-dimensional ^{13}C-NMR spectrum from brain extract of

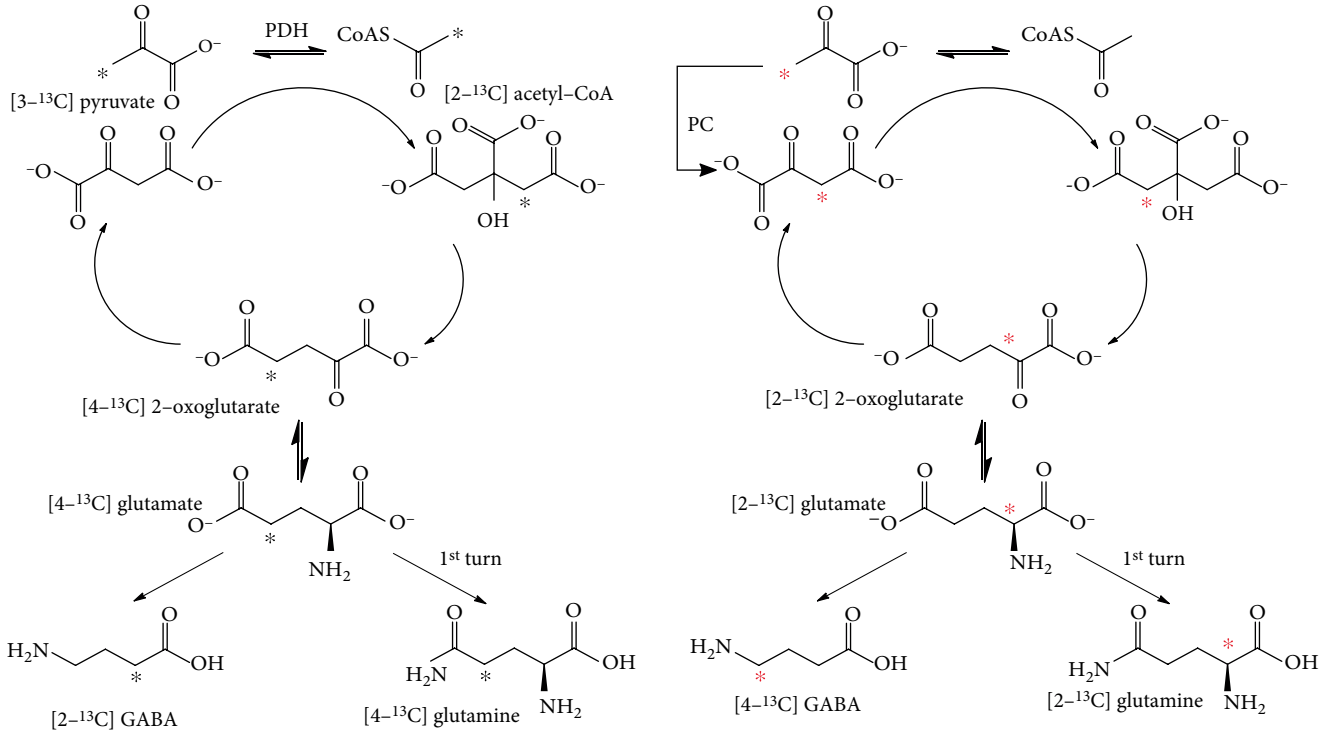

Figure 1: Schematic representation of the labeling of glucose via pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC). The asterisk represents the labeling of the metabolite with ^{13}C.

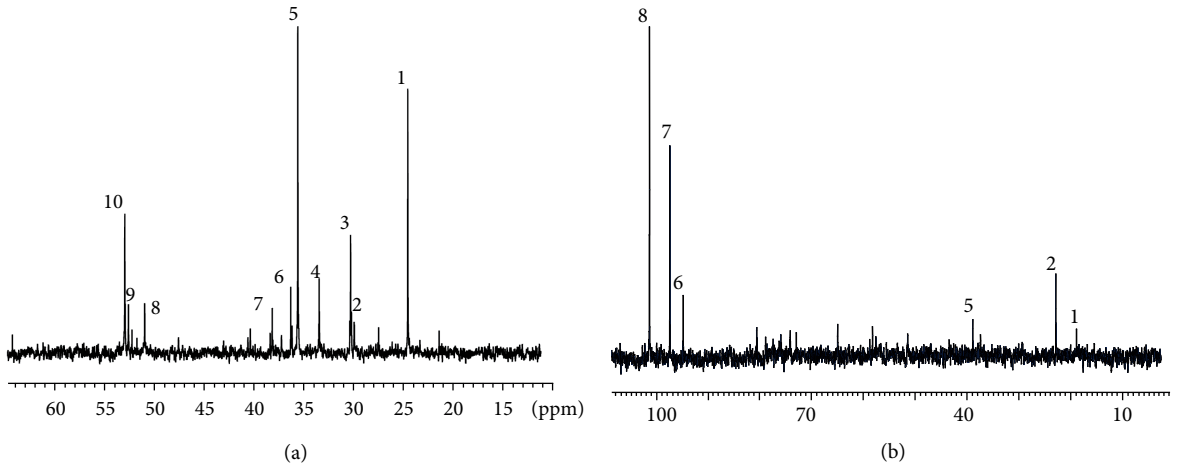

Figure 2: A typical 175 MHz ^{13}C NMR spectrum of the brain and liver extract of the control mouse. Assignment for brain: 1—C3-lactate, 2—C3-glutamine, 3—C3-glutamate, 4—C4-glutamine, 5—C4-glutamate, 6—C2-GABA, 7—C3-aspartate, 8—C2-aspartate, 9—C2-glutamine, 10—C2-glutamate. Assignment for liver: 1—C3-Alanine, 2—C3-lactate, 5—C4-glutamate, 6—not assigned, 7 & 8—C1-glucose. (a) Brain and (b) liver.

a control mouse postlabeled glucose injection is shown in Figure 2. Although not as information rich as ^{1}H NMR due to relative insensitivity, the spectrum identifies key energy metabolites with isotopomer level information. Interestingly, no ^{13}C multiplets were detected. Specifically, we detected lactate (3-^{13}C), glutamine (3-^{13}C, 4-^{13}C, 2-^{13}C), GABA (2-^{13}C), glutamate (3-^{13}C, 4-^{13}C, 2-^{13}C), and aspartate (3-^{13}C, 2-^{13}C). Normalized intensities of the detected peaks were analyzed and shown in Figure 3. Significant differences with respect to control animals were observed in the following isotopomers—[3-^{13}C]-lactate was elevated ($p < 0.05$), while all three detected isotopomers of glutamate and [2-^{13}C]-GABA were decreased ($p < 0.05$). The ratio of [4-^{13}C] to [2-^{13}C]- was not significant in any malaria inflicted groups. Interestingly, none of the isotopomers were differentially altered in CM animals compared to NCM.

Further inspection of the spectra suggested residual [1-^{13}C]-glucose signals in the brain extracts of CM and NCM animals, while the controls had none (Figure 4). We sought to investigate whether this difference is a result of altered glucose transport or downstream utilization. [1-^{13}C]- 2- deoxyglucose

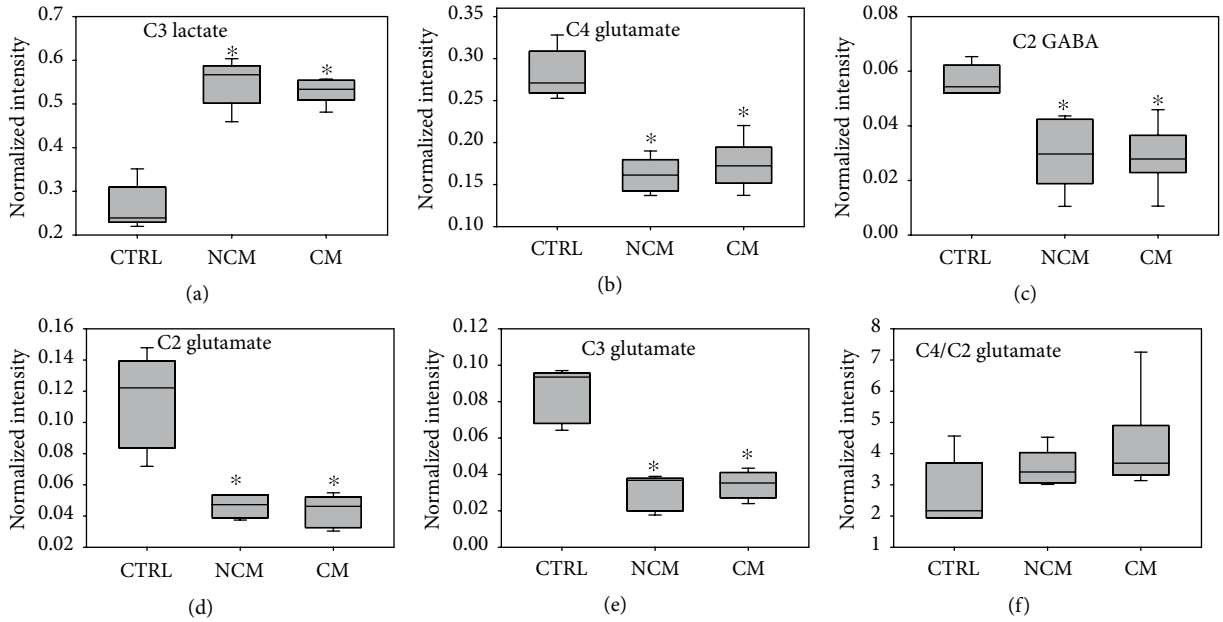

FIGURE 3: Relative levels of ^{13}C isotopomers of glucose in brain of CM ($N = 7$), NCM ($N = 6$) and control mice ($N = 5$). (a) C3 lactate, (b) C4 glutamate, (c) C2 GABA, (d) C2 glutamate, (e) C3 glutamate, and (f) C4/C2 glutamate. *Significant with respect to controls ($p < 0.05$).

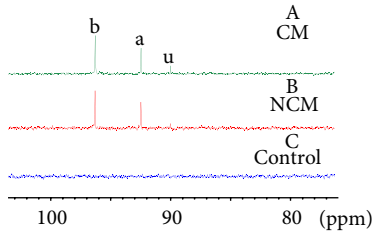

FIGURE 4: Expanded region of ^{13}C NMR spectrum of brain of CM, NCM and control mice. a, b and refer to α glucose, β glucose and u refer to unassigned peak.

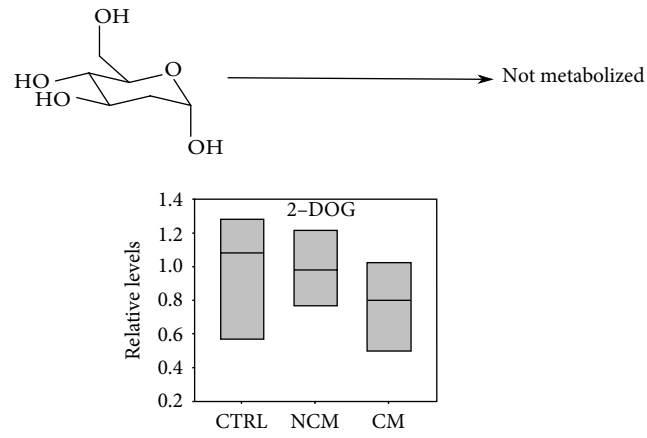

FIGURE 5: Relative levels of 2 deoxyglucose in brains of control, NCM and CM mice: (a) CM (b) NCM (c) control.

(2-DOG) was injected into the animals and the levels were checked using ^{13}C NMR. No significant differences (Figure 5) were observed suggesting depletion in downstream flux, primarily through TCA cycle, leadings to the residual glucose signals.

3.4. Alterations in Hepatic Glucose Metabolism. Figure 2 shows a representative ^{13}C NMR spectrum from liver extract of a control mouse post-labeled glucose injection. In addition to peaks from labeled glucose, [4-^{13}C]-glutamate, [3-^{13}C]-alanine, and [3-^{13}C]-lactate were also observed. Significant depletion of the [1-^{13}C]-glucose was observed in the malaria infected animals (Figure 6, $p < 0.05$). [3-^{13}C]-lactate and alanine were significantly increased in the malaria infected animals (Figure 6, $p < 0.05$). [4-^{13}C]-glutamate did not show any change between control and infected animals.

4. Discussion

Alterations in glucose metabolism are a hallmark of malarial infection. The parasite utilizes host resources, including glucose, to sustain its life cycle. Therefore, host glucose metabolism is altered in malarial infection. *P. falciparum* infection has been shown to alter glycolytic flux in the nonparasitized RBCs from infected RBC culture [28]. Further studies have demonstrated that specific host metabolic reprogramming is needed for the sustainability of the parasite [29, 30]. In addition to this, dietary alterations, such as high fat diet and caloric restrictions, have been shown to modify the effects of *P. falciparum* infection [31]. Further, inhibition of glycolysis confers protection from CM [32]. Glucose is the primary dietary source of energy in brain. Thus, changes in substrate utilization postglucose challenge may provide clues about specific metabolic reprogramming in CM. In this study of tracking the flow of ^{13}C label into various metabolites after the injection of [1-^{13}C] glucose in CM, NCM, and control mice, some important observations were made. Both groups of malaria inflicted mice exhibited a relative increased glycolytic activity, discernable by formation of

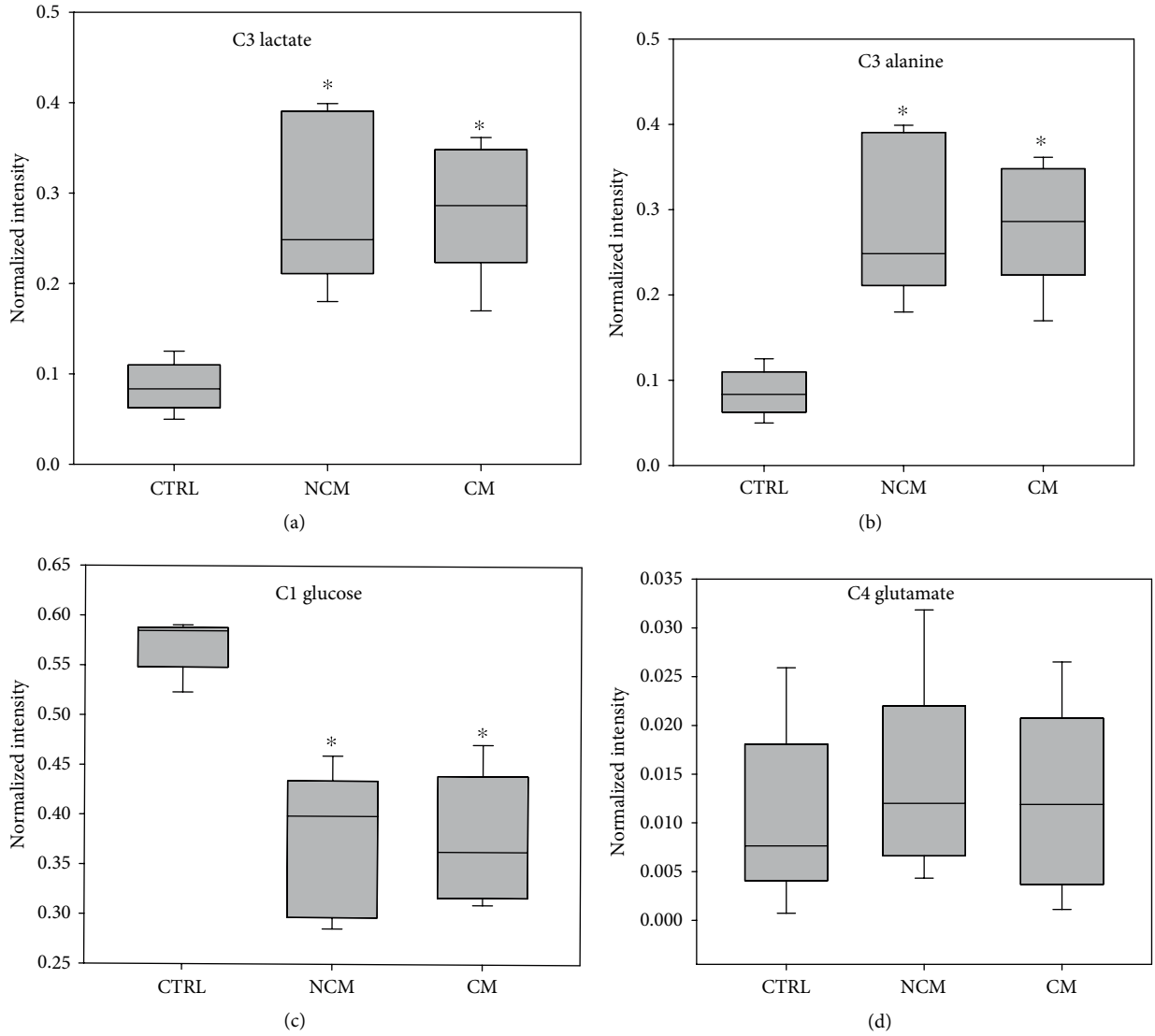

FIGURE 6: Relative levels of ^{13}C isotopomers in liver of CM ($N = 7$), NCM ($N = 6$) and control mice ($N = 5$). (a) C3 lactate, (b) C3 alanine, (c) C1 glucose, and (d) C4 glutamate. *Significant with respect to controls ($p < 0.05$).

elevated lactate, with respect to control while a relatively reduced flux via TCA cycle, indicated by reduced amounts of ^{13}C label in all the three isotopomers (C2, C3, and C4) of glutamate. These observations are not dependent on differential glucose transport across to brain across malarial groups (CM and NCM) and controls, suggested by similar 2-DOG levels in brain of the three groups of mice, implying only the downstream metabolism of glucose is altered in malaria infection.

In malarial group, increased production of lactate from pyruvate could thus result in relatively reduced flux towards TCA cycle as it is the feed to TCA cycle. In this regard, a recent literature reported rescue of CM in mice by targeting glutamine metabolism [29]. This can be due to altered glutamine formation due to TCA cycle flux.

The lactate levels and levels of various isotopomers of glutamate are not different in CM and NCM, suggesting that the fluxes through anaerobic and aerobic pathways were not different in the two groups of infected animals. A relatively reduced TCA cycle flux compared to control mice might indicate a reduced production of ATP in the brain of both CM and NCM mice, however, this observation needs further validation. Moreover, the relative operation of two routes (PDH and PC) of the entry of pyruvate into TCA cycle is similar in all three classes of animals namely CM, NCM, and control. This is clear from the ratio of C4-/C2-glutamate in the brain. Further, from our study, it is also clear that metabolism of glucose is reduced in both CM and NCM. Brain is a much less efficient glucogenic organ than liver. Malarial infection perhaps reduce brain glucose utilization to avoid a state of hypoglycemia which perhaps is a host response to modulate the disease manifestations [32]. The neurological symptoms of malaria did not affect the downstream utilization of glucose with respect to normal malaria. This result corroborates with our previously published results [14] demonstrating no difference in molecular fingerprints of brain glucose in CM and NCM.

Also, an important conclusion from the experiment is that the glucose transport is not perturbed in CM and NCM

compared to controls as there is no difference in the uptake of 2-DOG in brain of CM, NCM and control mice. The difference in the levels of glucose in CM/NCM w.r.t controls is due to perturbation in its downstream utilization. The presence of the unutilized glucose has been reported for the CM and not the NCM [33]. However, the parasite used in that study was *P. berghei* K173 for NCM. Thus, this study suggests that glucose utilization is also dependent on the nature of parasites used for the study.

Hepatic glucose utilization in cerebral malaria is virtually unexplored. However, systemic glucose utilization is known to be altered during cerebral malarial complications [23]. The study of the metabolite pool in the liver suggests an increased glucose utilization as evidenced from lower levels of residual [1-^{13}C] glucose. The increased levels of labelling in lactate and alanine suggest that there is enhanced anaerobic glycolysis in liver of both CM and NCM inflicted animals. Alanine is formed from pyruvate by the action of alanine transaminase. Increased concentration of pyruvate, therefore, leads to the formation of alanine in the case of both CM and NCM. Therefore, elevated systemic glycolysis in malaria is also partly due to altered hepatic glucose utilization.

Overall, we observed significantly impacted glucose metabolism postmalarial infection; however no significant difference of glucose utilization by the liver and brain of CM and NCM was observed. It is to be noted, however, that these experiments mimic a condition of glucose challenge and do not reflect steady state glucose utilization, which could be dependent on the cerebral status of the infection.

We should also note the limitation of our study. Specifically, the experimental design did not allow us to separate metabolite of parasite and host origin. Nevertheless, due to the relatively insensitive nature of NMR, detection of metabolites from parasites is unlikely. This is particularly true for liver metabolites since parasite load is comparable between CM and NCM groups.

5. Conclusion

Malaria significantly alters tissue level glucose utilizations post glucose challenge. Specifically, brain glycolysis seems to be elevated while TCA cycle decreases. Hepatic glycolysis is also elevated, which could regulate systemic glycolysis as well. However, no differential effects were observed between CM and NCM animals. Further investigation of steady state glucose utilization may shed light into the link between glucose metabolism and cerebral malaria.

Data Availability

The data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

^{13}C NMR spectrum of liver harvested after ^{13}C glucose bolus injection in mice. Liver is harvested at different time points after bolus injection to understand the optimum time point. (*Supplementary Materials*)

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