Central and peripheral effects of L-citrulline on thermal physiology and nitric oxide regeneration in broilers

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ABSTRACT The mechanism that mediates L-citrulline (**L-Cit**) hypothermia is poorly understood, and the involvement of nitric oxide signaling has not been fully elucidated. Therefore, this study aimed to determine L-Cit's influence on body temperature and to ascertain the central and peripheral mechanisms associated with this response. Chicks responded to intracerebroventricular (**ICV**) injection of L-Cit with high and low body temperatures ($P < 0.05$) depending on the dose tested, for both the surface and rectal temperatures. Peripheral (i.p.) L-Cit injection did not affect body temperature responses. Nitric oxide (**NO**) concentration and NO synthase (**NOS**) were influenced with varying doses of L-Cit. Hypothalamic NO was increased at 4 μg L-Cit whereas, plasma iNOS was elevated at 2μg L-Cit treatment. However, i.p. L-Cit did not change the NO content, rather it induced higher ($P < 0.05$) plasma tNOS and iNOS activity, and further upregulated iNOS and nNOS gene expression in the hypothalamus. In addition, ICV L-Cit potentiated a pro- versus anti-inflammatory milieu with the induction of IL-8, IL-10, and TGFβ ($P < 0.05$), which may be related to the changes in body temperature. Following ICV L-Cit administration, it was observed that L-Cit caused dose variable changes in the ultrastructure of hypothalamic neurons. The lowest dose was associated with a higher number of dead or degenerating neurons, whereas the highest L-Cit dose had fewer neuronal numbers with larger sizes. Therefore, this study shows that central and peripheral L-Cit administration imposes changes in body temperature, nitric oxide production, and inflammatory responses, in a dose-dependent manner.

Key words: L-Citrulline, cytokine, body temperature, inflammation, hyperthermia

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INTRODUCTION

Nitric oxide (**NO**) is a biological signaling molecule involved in several physiological events including immune regulation, vascular function, intercellular communication, pro- and anti-inflammatory effects, feeding behavior, and thermoregulation (Choi et al., 1994; Cuzzocrea and Salvemini, 2007; Kim, 2011). In the regulation of body temperature, the role of NO has been established in mammals, where it stimulates the central nervous system (**CNS**) actions and facilitates autonomic heat dissipation during fever, heat stress, and anapyrexia (Sharma et al., 1998; Steiner and Branco, 2001). The actions of NO are important in the central and peripheral regulation of body temperature. Within the CNS, NO acts to excite and/or inhibit thermoregulatory activity, thus increasing body temperature at sites of pyrogens and temperature perception, but reduces body temperature at sites of thermointegration (Gerstberger, 1999; Lacerda et al., 2005). Peripherally administered NO enhanced skin blood flow, via convective heat transfer during blood circulation to heat-dissipating surfaces and further influenced heat generation in brown adipose tissue (**BAT**) (Monda et al., 1995; Gerstberger, 1999). Similarly, NO is necessary for the maintenance of body temperature and modulation of hyperthermia in chickens (Coleone et al., 2009). Thus, NO contributes both positively and negatively, or even simultaneously, depending on the route of administration, locale, effector, bioavailability, local concentrations, stimuli involved, as well as the downstream targets (Yuste et al., 2015). Quantitatively, plasma NO accumulation in broilers was successfully detected as NO_2^- $+NO_3^-$ to reflect the whole-body NO synthesis (Chapman and Wideman, 2006).

The role of NO in inflammation has been established in several studies (Kim, 2011; de Macchi et al., 2013; Stettner et al., 2018). Excessive NO production can be

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evoked by inflammatory signals, and in turn, NO modulates the inflammatory process by acting on key regulatory pathways (Yuste et al., 2015). At the transcriptional level, inducible NOS isoform (**iNOS**) has been implicated in the regulation of neuroinflammatory processes after its transactivation by transcription factors such as the nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-κB**) (Yuste et al., 2015). More so, NO and IL-1β have been shown to play an active role in neuroinflammation and neurodegeneration (Yuste et al., 2015). Therefore, dysregulation of NO production would influence the crosstalk between inflammatory mediators and cytokines. Central or peripheral inflammation is characterized by the upregulation of cytokines and their receptors in the brain (Galic et al., 2012). Cytokines released from both immune and non-immune cells can markedly affect regulatory brain circuits, neurotransmission and induce hormonal changes similar to changes associated with stress exposure (Gądek-Michalska et al., 2013). The pro-inflammatory response in the CNS is characterized by the complex secretion of various inflammatory mediators, including cytokines, transcription factors, prostanoids, and NO (Gądek-Michalska et al., 2013). Available evidence suggests that cytokines can influence the complex interplay of neuronal circuits involved in food intake, thermoregulation, inflammation, sleep, and behavioral patterns, via acting on the central and peripheral neuronal activity to modulate brain functions (Plata-Salamán, 1991; Besedovsky and Rey, 2008).

L-Citrulline is a non-essential amino acid that has gained research interest primarily due to its role in arginine recycling, and muscle protein synthesis (Cynober et al., 2010; Cynober et al., 2013). L-Citrulline plays an intermediate role in the urea cycle and is an essential metabolite of the Arginine-Nitric oxide cycle (Kaore and Kaore, 2014). It functions as an endogenous precursor in arginine synthesis, the substrate for nitric oxide production, in a reaction catalyzed by nitric oxide synthase (**NOS**) (Curis et al., 2005). Endogenous NO can be produced by the different isoforms of NOS namely; neuronal NOS (**nNOS**), endothelial NOS (**eNOS**), and inducible NOS (**iNOS**). Several studies have shown that L-citrulline is an effective substrate for arginine recycling and nitric oxide production using in vitro and in vivo models (Schwedhelm et al., 2008; Kurauchi et al., 2017). L-Citrulline is highly abundant in watermelon and it had been reported that impracticable amounts of food would need to be administered to expect additional biological effects of L-citrulline (Kurauchi et al., 2017). Acute L-citrulline ingestion increases de novo arginine synthesis and whole-body NO synthesis (Kim et al., 2015). Therefore, supplementary L-citrulline is considered an essential route for arginine delivery to endothelial and immune cells under certain pathological conditions, and to avoid excessive/uncontrolled NO production (Papadia et al., 2018). Recently, L-citrulline has been implicated in body temperature regulation and thermotolerance during heat stress (Chowdhury et al., 2017a; Chowdhury, 2019) and at thermoneutrality (Uyanga et al., 2021). The plasma concentration of L-citrulline was lowered during heat exposure (35 ± 1°C), suggesting amino acid alteration in heat-stressed chicks (Chowdhury et al., 2014). Another study revealed that both acute and chronic supply of a medium containing L-citrulline live bacteria reduced both the surface and rectal temperatures of chicks (Tran et al., 2019). In heat-stressed pigs, L-citrulline moderately afforded thermotolerance by decreasing the respiratory rate, with a tendency for reduced rectal temperatures (Kvidera et al., 2016). In lactating sows, dietary supplementation with 1% L−citrulline decreased the respiratory rate during heat stress but did not influence the rectal temperature (Liu et al., 2019). Together, these reports indicate the role of L-citrulline in thermal physiology and necessitates further studies to identify its mechanisms of action.

Although L-citrulline exhibits a wide range of beneficial properties, limited data are available regarding the effects of L-citrulline supplementation on physiological responses in the body, especially focusing on body temperature regulation. In the current study, central and peripheral L-citrulline was administered to chickens at varying doses, and biological responses including body temperature, nitric oxide production, and inflammation were assayed. To investigate L-Cit's role in the regulation of body temperature, we examined the changes in body temperature (core, rectal, and head), the enzyme activity and gene expression for nitric oxide synthesis, and the expression of inflammatory cytokines in the hypothalamus of broilers.

MATERIALS AND METHODS

Animals

One day-old broiler chicks (Arbor acres) were obtained locally (Liaocheng Hekangyuan Animal Husbandry Co., Ltd., China) and managed in brooding pens. The temperature was managed at 35°C for the first 7 d, then gradually reduced by 3°C every other week until it reached 24°C. The lighting schedule was constant at 24 h lights throughout the experiments. Feed was formulated to meet or exceed NRC guidelines (Table 1). Drinking water and feed were provided ad libitum during the study. L-Citrulline (**L-Cit**) was purchased from Shandong Fosun Biotechnology Co., Ltd, China. For all injections, L-Cit was freshly dissolved in 0.85% normal saline solution and vortexed to ensure uniformity. Saline was used as the control and injections were administered between 08.00 h and 10.00 h. This study was approved by the Shandong Agricultural University and performed according to the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China).

Experiment 1: Central Effects of L-Citrulline on Body Temperature

At 7 days old, 48 chicks of similar body weights (135 −140 g) were divided into 4 groups, with 12 replicates each. The ICV injections were administered according to previous reports (Liu et al., 2015; Tachibana et al.,

Table 1. Composition and nutrient levels of basal diets (as-fed basis) %.

Items	1 to 21 d	22 to 42 d
Ingredients		
Corn (8.5% CP)	54.99	59.68
Soybean meal (43% CP)	37.02	31.65
Soybean oil	3.87	4.72
Limestone	1.19	1.27
Dicalcium phosphate	1.68	1.63
NaCl	0.30	0.28
L-Lys·HCl (99%)	0.21	0.18
DL-Methionine (99%)	0.21	0.14
L-Arginine (99%)	0.02	-
Choline chloride (50%)	0.26	0.20
Vitamin premix[1]	0.05	0.05
Mineral premix[2]	0.20	0.20
Total	100.00	100.00
Calculated nutrient levels		
Crude protein	21.0	19.0
Metabolisable energy (MJ/kg)	12.55	12.97
Calcium	0.90	0.90
Non-phytate phosphorus	0.45	0.43
Lysine	1.242	1.095
Methionine	0.537	0.449
Methionine + Cysteine	0.889	0.775
Threonine	0.861	0.774
Analyzed nutrient levels		
Dry matter	99.38	99.41
Crude protein	20.95	19.19
Crude fat	6.09	7.08
Crude fiber	5.89	5.87
Ash	6.53	5.12
Nitrogen	3.35	3.07
Total phosphorus	0.72	0.60
Calcium	1.23	0.93

[1]Vitamin premix provided the following per kilogram of diet:VA (retinyl acetate) 10,000 IU, VD_3 (cholecalciferol) 2,000 IU, VE (DL-α-tocopheryl acetate) 11.0 IU, VK 1.0 mg, VB_1 1.2 mg, VB_2 5.8 mg, VB_6 2.6 mg, VB_{12} 0.012 mg, niacin 66.0 mg, pantothenic acid (calcium pantothenate) 10.0 mg, biotin 0.20 mg, folic acid 0.70 mg.

[2]Mineral premix provided the following per kilogram of diet:Mg 100 mg, Zn 75 mg, Fe 80 mg, I 0.65 mg, Cu 8.0 mg, Se 0.35 mg.

2017). Chicks were implanted with an ICV cannula, and after recovery, the chicks were injected with saline (control) or varying concentrations of L-Cit (1, 2, or 4 μg) injections in a 10-μL volume based on a previous report (Chowdhury et al., 2015). Chicks were fasted during the experiment such that the effect on body temperature could be assessed independently of feed and water intake. The experiment was monitored for 2-h postinjection and body temperature data was obtained from 1 chick per replicate (n = 12). At the end of the experiment, 8 replicates (1 chick each) were selected for blood sampling and the chicks were exsanguinated for tissue collection. Blood was collected from the jugular veins into heparinized tubes and centrifuged at 4,000 × *g* for 15 min at 4°C to obtain plasma samples, which were stored at −20°C for further analysis. Tissue samples were snap-frozen in liquid nitrogen and transferred to −80°C until further analysis.

Experiment 2: Effects of Peripheral L-Citrulline on Body Temperature

For the peripheral experiment, 24 chickens (26-day-old) were randomized to have similar average body weights (~773 g) and allocated to 2 treatment groups. Chickens were administered intraperitoneally (**i.p.**) with saline or L-Cit (200 mg/kg BW), according to previous reports addressing physiological responses to this compound (Lee et al., 2018). Chickens were implanted with a thermochron temperature logger (iButton, DS1922L, Maxim, CA) within the abdominal cavity to continuously record the core body temperature. Prior to the experiment, the animals were fasted and maintained in individual cages at 24°C. The study lasted for 5-h postinjection. At the end of the experiment, the chickens were exsanguinated, and blood and tissue samples were collected as described in experiment 1.

Intracerebroventricular Cannula Implantation and Injection

The technique for intracerebroventricular (**ICV**) injection of chicks (Figure S1) was performed as previously reported (Liu et al., 2014). Briefly, at 7 days old, chicks were anesthetized by nasal inhalation of isoflurane (EZVET, China), and then positioned in a stereotaxic instrument (Huai Bei Zheng Hua), such that the head was restrained. A small incision was made along the midline to expose the cranium. A thin-walled stainless steel guide cannula was stereotaxically implanted into the third ventricle, using the following coordinates; 3-mm anterior to the coronal suture, 1-mm lateral from the sagittal suture, and 2- mm deep targeting the left lateral ventricle (Liu et al., 2014; Liu et al., 2015). The cannula was affixed to the cranium with jeweler's screws and dental acrylic (Denture base material, Shanghai New Century Dental Material Co. Ltd, China). The animals were returned to their cages and allowed to recover before injection.

An internal cannula was used to administer ICV injections. The internal cannula was inserted to approximately 1mm over the guide cannula, and connected by polyethylene tubing to a Hamilton microsyringe (10 μL). Chicks were injected with either saline or L-Cit treatment in a 10 uL volume, and after injection, the guide cannula was positioned for about 60 s to allow drug diffusion from the cannula tip, then a dummy cannula was reinserted (Liu et al., 2014).

Body Temperature Measurement

Rectal temperature, head temperature, and core body temperature were measured. The rectal temperature of chicks was measured immediately before ICV and i.p. injections, and the data were adopted as the baseline, followed by measurements at 30 min intervals. Rectal temperature was measured using a digital thermometer with an accuracy of ± 0.1°C (KRUUSE Vet thermometer [Cat No. 291110, China]) which was inserted to a cloacal depth of 2 to 3cm. The surface (head) temperature was measured using an infrared thermal imager (TP 160 - Suzhou Shengguang Instrument Co. Ltd, China) and core body temperature was measured using biotelemetry

(iButton, DS1922L, Maxim, CA) at 10 min intervals, and plotted over 300 min. Data were obtained using the One-wire Viewer software (Maxim Integrated Products Inc, CA).

NO Concentration and NOS Activity

Production of NO was assessed indirectly by measuring the levels of nitrate and nitrite, the stable end-product of NO (Nikami et al., 2008). Plasma and tissue NO concentrations were measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) as previously described by Uyanga et al. (2020). Absorbance was read at 550 nm using a spectrophotometer (Beijing PGeneral, Beijing, China). The total NOS (**tNOS**), and iNOS activities were determined using commercial kits (Jiancheng Bioengineering Institute). Absorbance was determined using a microplate reader (Elx808, Bio-Tek, Winooski, VT) at 530 nm.

Histology Hypothalamic tissues were excised and fixed in 4% paraformaldehyde for morphological analysis. The samples were then paraffin-embedded and sectioned into 4-μm sections on a coronal plane for staining with hematoxylin-eosin. Sections were dehydrated in alcohol, cleared with xylene and mounted on slides for visualization by light microscopy (400 ×, Olympus, Tokyo, Japan). Histological slides were examined, and digital images were obtained.

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA from the hypothalamic tissues was extracted using NcmZol reagent (NCM Biotech, China). RNA quality was determined using a spectrophotometer (Eppendorf, Germany), and RNA purity was taken as the ratio between absorbance values at 260 nm and 280 nm (A260/280 = 1.80∼2.01). RNA was reverse-transcribed using a HiFiScript cDNA synthesis kit (CWBIOtech, China) to complementary DNA (cDNA) according to the manufacturer's protocol. Reverse transcription (**RT**) reactions (20 μL) contained dNTP mix 2.5 mM, Primer mix, RNA template, 5× RT buffer, DTT 0.1M, HiFiScript 200 U/μL and RNase-Free water. Each cDNA template (2 μL) was used to prepare a 20 μL PCR reaction mix, containing 4 μL of forward and reverse primers (10 μM) each, 10 μL of 2× MagicSYBR Mixture, 0.2 μL ROX reference dye, and 7 μL ddH_2o (CWBIOtech, China).

Real-time PCR was performed using ABI QuantStudio 5 Real-Time PCR Instrument (Applied Biosystems, ThermoFisher Scientific, United States). Primers used for RT-PCR were designed using Beacon Designer 8 software and based on published target sequences (Table 2). Thermal cycling was initiated at 95°C for 30 s for predenaturation, followed by 40 cycles consisting of denaturation at 95°C for 5 s, annealing, and extension at 60°C for 30 s. Melt curve analysis was performed at 95°C for 15s, 60°C for 1 min, 95°C for 15 s, and 50°C for 30 s. A standard curve was determined to calculate the efficiency of the real-time PCR primers. Primers were normalized using the mRNA level of β-actin (ΔCT) as an internal control. The control group was used as calibrator and the relative expression of the target genes were analyzed using the $2^{-\Delta\Delta CT}$ method. Thus, gene transcription results were reported as an n-fold difference relative to the control.

Statistical Analysis

Data were analyzed using one-way ANOVA to determine the main effect of treatments, using Statistical Analysis Software (SAS version 8.1; SAS Institute Inc., Cary, NC). Tukey's test was used for mean comparisons in experiment 1 and the results are presented as means ± SEM. Orthogonal polynomial contrasts were performed to determine the linear and quadratic effects of L-Cit in experiment 1. Core body and rectal temperature data were analyzed using repeated-measures ANOVA. Charts were designed using GraphPad Prism, version 8.0.2, (GraphPad Software Inc., La Jolla, CA). Differences were considered significant at $P < 0.05$.

RESULTS

Effects of Central and Peripheral L-Citrulline on Body Temperature of Broilers

The infrared thermography (**IRT**) technique was used to detect the surface temperature (**HT**) of chicks (Figure 1A) following the 2 h ICV L-Cit injection (experiment 1). Image quantification showed that 4 μg L-Cit lowered ($P < 0.05$) HT compared to the control and 1μg L-Cit treated groups (Figure 1B). Similarly, 2 μg L-Cit treatment lowered the HT of broilers compared to the 1 μg L-Cit group. There was a significant linear ($P = 0.0004$) decline in the surface temperature with increasing L-Cit dosage. During the ICV trial, the RT had an average baseline of 40.70 ± 0.1°C. It was observed that from 30 min postinjection up to 120 min, the 1 μg L-Cit treated group had higher RT compared to other groups (Figure 2A). Interestingly, at 120 min, 4 μg L-Cit treatment had lowered RT compared to the control, 1 μg, and 2 μg L-Cit groups. There was a significant effect of treatment ($P = < 0.0001$), time ($P = < 0.0001$) and treatment × time interaction ($P = 0.0004$) on the RT of broilers. Similarly, it was observed that 1 μg L-Cit treatment increased the mean RT higher than the control, 2 μg and 4 μg L-Cit groups (Figure 2B). In addition, 2 μg L-Cit significantly increased the mean RT higher than the control and 4 μg L-Cit groups. There was a significant linear ($P = 0.027$) and quadratic ($P = 0.0001$) effect of L-Cit on the mean RT of broilers. In experiment 2, i.p. L-Cit injection did not elicit any significant changes on the HT, RT, and core body temperature of broilers (Figures 2C−2G). However, there was a significant effect of time ($P = < 0.0001$) on the core body temperature (Figure 2E). The

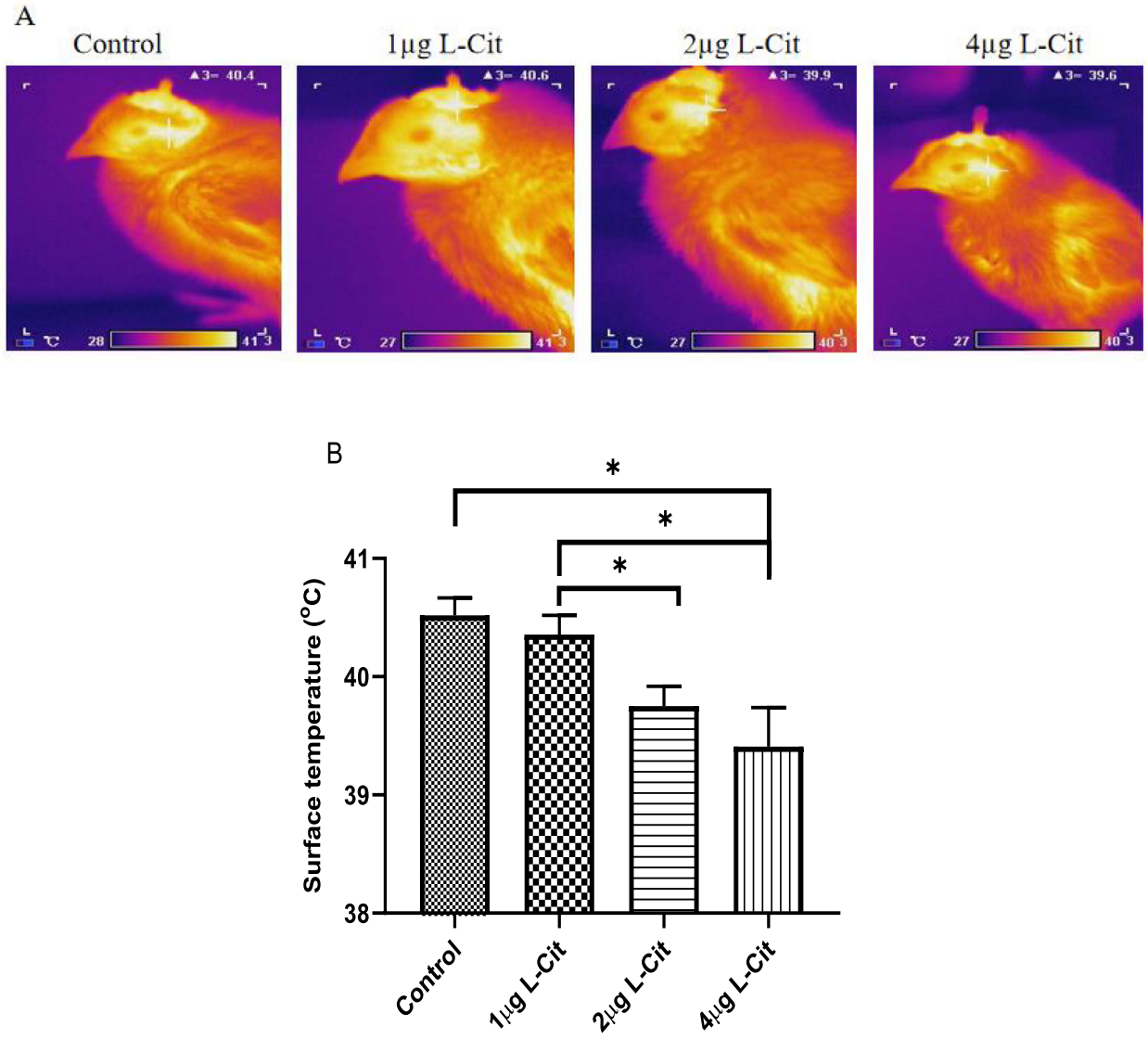

Figure 1. Surface temperature of broilers after ICV L-citrulline administration (experiment 1). (A) Thermal imagery of head measurements. (B) Image quantification of surface temperature of broiler. Data are presented as mean ± SEM. * indicate significant difference at $P < 0.05$. Abbreviation: ICV, intracerebroventricular.

control and L-Cit groups had an initial baseline of 39.8 ± 0.1°C, and it was observed that L-Cit treatment lowered the trendline at several time points compared to the control group, although without statistical significance ($P > 0.05$; Figure 2E). The periods with temperature reduction were from 10−100, 190−230, and 250−300 mins (Figure 2F).

Effects of Central and Peripheral L-Citrulline on Nitric Oxide Regeneration

Nitric oxide concentration was determined in the plasma and hypothalamic tissues after L-Cit treatment (Figure 3). Following ICV injection (experiment 1), the plasma NO did not differ between the control and L-Cit (1 μg, 2 μg, and 4 μg L-Cit) treated groups (Figure 3A). However, it was observed that the hypothalamic NO concentration was significantly increased by 4 μg L-Cit compared to 2 μg L-Cit treatment and it also tended to be higher ($P = 0.068$) than the control group (Figure 3B). There was a significant quadratic effect ($P = 0.022$) of L-Cit on hypothalamic NO concentration. From experiment 2, it was observed that i.p. L-Cit injection did not alter the plasma and hypothalamic NO concentrations ($P > 0.05$) during the study (Figures 3C and 3D).

Effects of Central and Peripheral L-Citrulline on Nitric Oxide Synthases Activity and Gene Expression

From experiment 1, the plasma tNOS activity was not affected ($P > 0.05$) by ICV L-Cit injections (Figure 5A). However, the plasma iNOS activity was upregulated ($P < 0.05$) by 2 μg L-Cit compared to the control, 1 μg, and 4 μg L-Cit groups (Figure 5B). There was a significant quadratic effect ($P = 0.017$) of L-Cit on plasma iNOS levels. In addition, the hypothalamic nNOS activity linearly ($P = > 0.0001$) decreased with an increase in L-Cit levels. It was observed that the hypothalamic

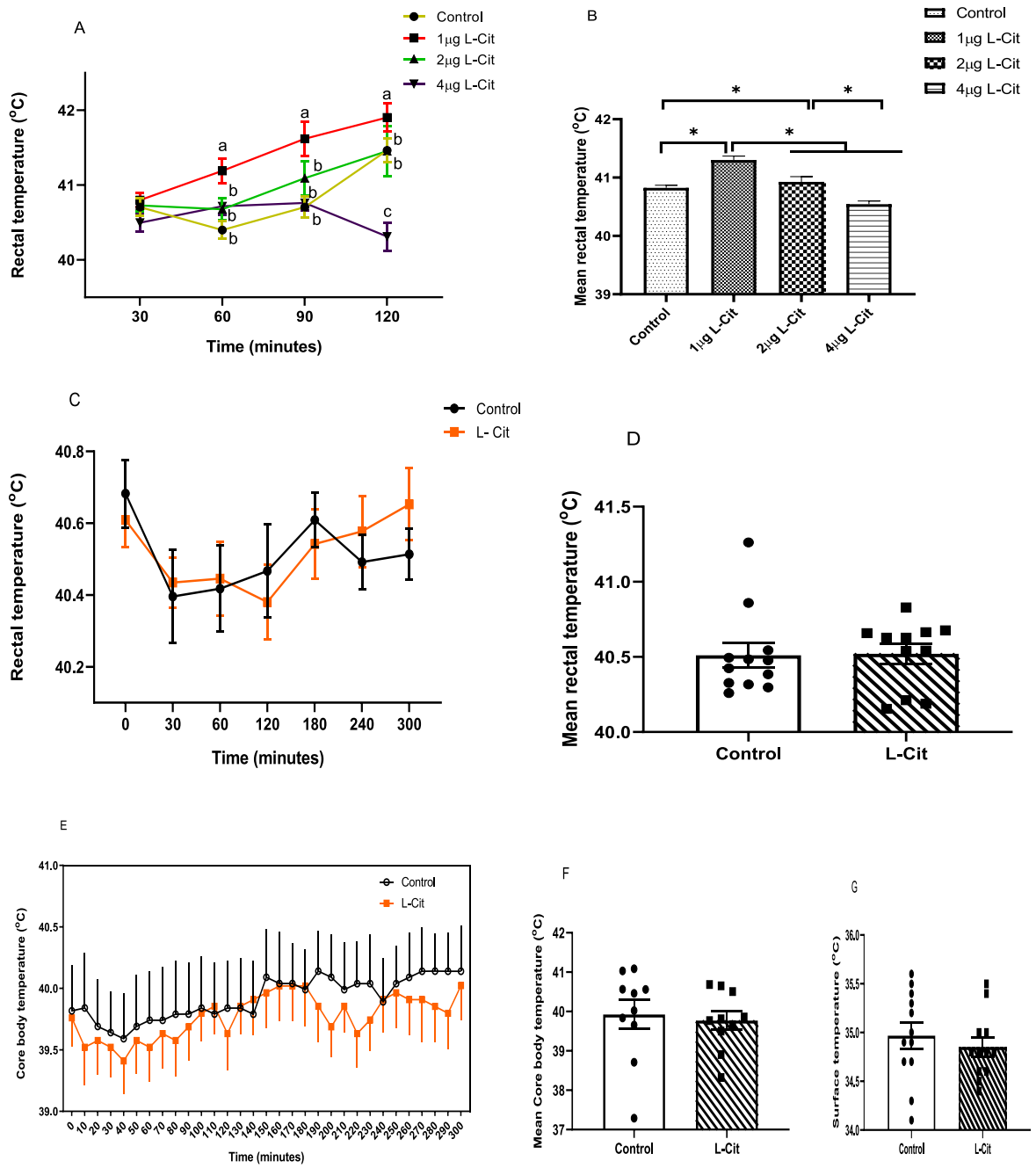

Figure 2. Body temperature of broilers following ICV (experiment 1) and I.P. (experiment 2) L-citrulline administration. (A) Rectal temperature of broilers during ICV experiment. (B) Mean rectal temperature of broilers during ICV experiment. (C) Rectal temperature of broilers during IP experiment. (D) Mean rectal temperature of broilers during IP experiment. (E) Core body temperature of broilers during IP experiment. (F) Mean core body temperature of broilers during IP experiment. (G) Surface temperature of broilers during IP experiment. Data are presented as mean ± SEM (n = 9 to 12). * indicate significant difference at $P < 0.05$. Abbreviation: ICV, intracerebroventricular.

nNOS activity tended ($P < 0.10$) to decline at 4 μg L-Cit compared to the control and 1 μg L-Cit treatments (Figure 4C). In experiment 1, the hypothalamic mRNA expressions for all three NOS isoforms (nNOS, iNOS, and eNOS) were not significantly different ($P > 0.05$) among treatment groups (Figures 4D−4F).

The results from experiment 2 show that the plasma tNOS and iNOS enzymes were increased ($P < 0.05$) by i. p. L-Cit treatment compared to the control group (Figures 6A and 6B). However, the hypothalamic nNOS activity was not significantly affected by i. p. L-Cit administration (Figure 6C). In addition, the relative mRNA expression of the hypothalamic nNOS and iNOS were significantly upregulated ($P < 0.05$) by i.p. L-Cit administration compared to the control group, however, the hypothalamic eNOS expression was not changed (Figures 6D−6F).

Effects of Central L-Citrulline on the Hypothalamic Morphology

Histology results showed that the number of hypothalamic neurons and their morphology were influenced by

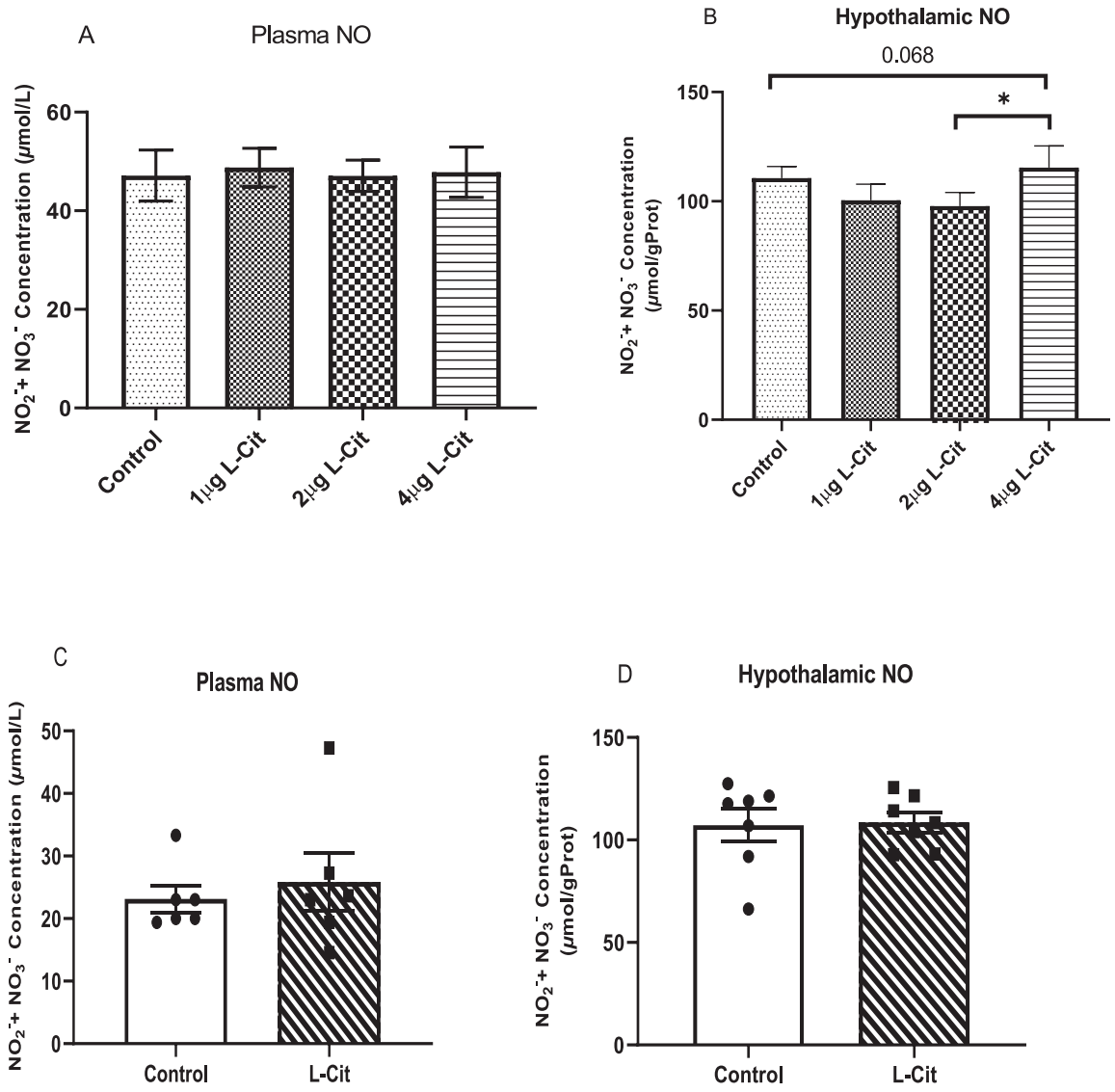

Figure 3. Nitric oxide (NO) concentration of broilers after central and peripheral L-citrulline administration. (A) Plasma NO following ICV experiment (B) Hypothalamic NO following ICV experiment (C) Plasma NO following IP experiment (D) Hypothalamic NO following IP experiment . Data are presented as mean ± SEM (n = 6 to 8). * indicate significant difference at $P < 0.05$. Abbreviation: ICV, intracerebroventricular.

ICV L-Cit administration (Figure 6). The control groups displayed normal hypothalamic architecture compared to the L-Cit treated groups. It was observed that 1 μg and 2 μg L-Cit had increased cell numbers, which appeared to be smaller and atrophic. Several of the neurons observed at 1 μg L-Cit appeared as degenerating neurons with pyknotic nuclei. However, the 4 μg L-Cit treatment tended to induce larger-sized neurons with visible nuclei, nucleoli, and prominent Nissl substance of neurons.

Effects of Central and Peripheral L-Citrulline on Inflammatory Cytokines Gene Expression

From experiment 1, the effects of ICV L-Cit administration on the mRNA expression of pro-inflammatory and anti-inflammatory cytokines are shown in Figure 7. The expression of IL -2 and IL-6 was not affected ($P > 0.05$) by L-Cit administration (Figures 7A and 7B). However, IL-8 and IL-10 expression were upregulated ($P < 0.05$) by 1 μg L-Cit treatment compared to the 4 μg L-Cit group (Figure 8C and 8D). There was a significant linear effect ($P = 0.009$) of L-Cit on IL-10 expression. The hypothalamic TGFβ expression was significantly increased ($P < 0.05$) by 4 μg L-Cit compared to other treatment groups of control, 1 μg and 2 μg L-Cit (Figure 7E). There was a significant linear ($P = 0.004$) and quadratic ($P = 0.024$) effect of L-Cit on hypothalamic TGFβ expression. However, there were no significant ($P > 0.05$) changes in the mRNA expression of TLR4, NF-kB, and TNF-α in the hypothalamic tissues of broiler chicks (Figures 7F−7H).

Peripheral administration of L-Cit did not ($P > 0.05$) elicit any changes in the mRNA expression of inflammatory cytokines in the hypothalamus of broilers following 5 h i.p. L-Cit injection. The gene expression for Interleukins (IL-2, IL-6, IL-8, IL-10), NF-kB, TLR4, TNF-α, and TGFβ was similar between the saline (control) and the L-Cit treated groups (Figures 8A−8H)

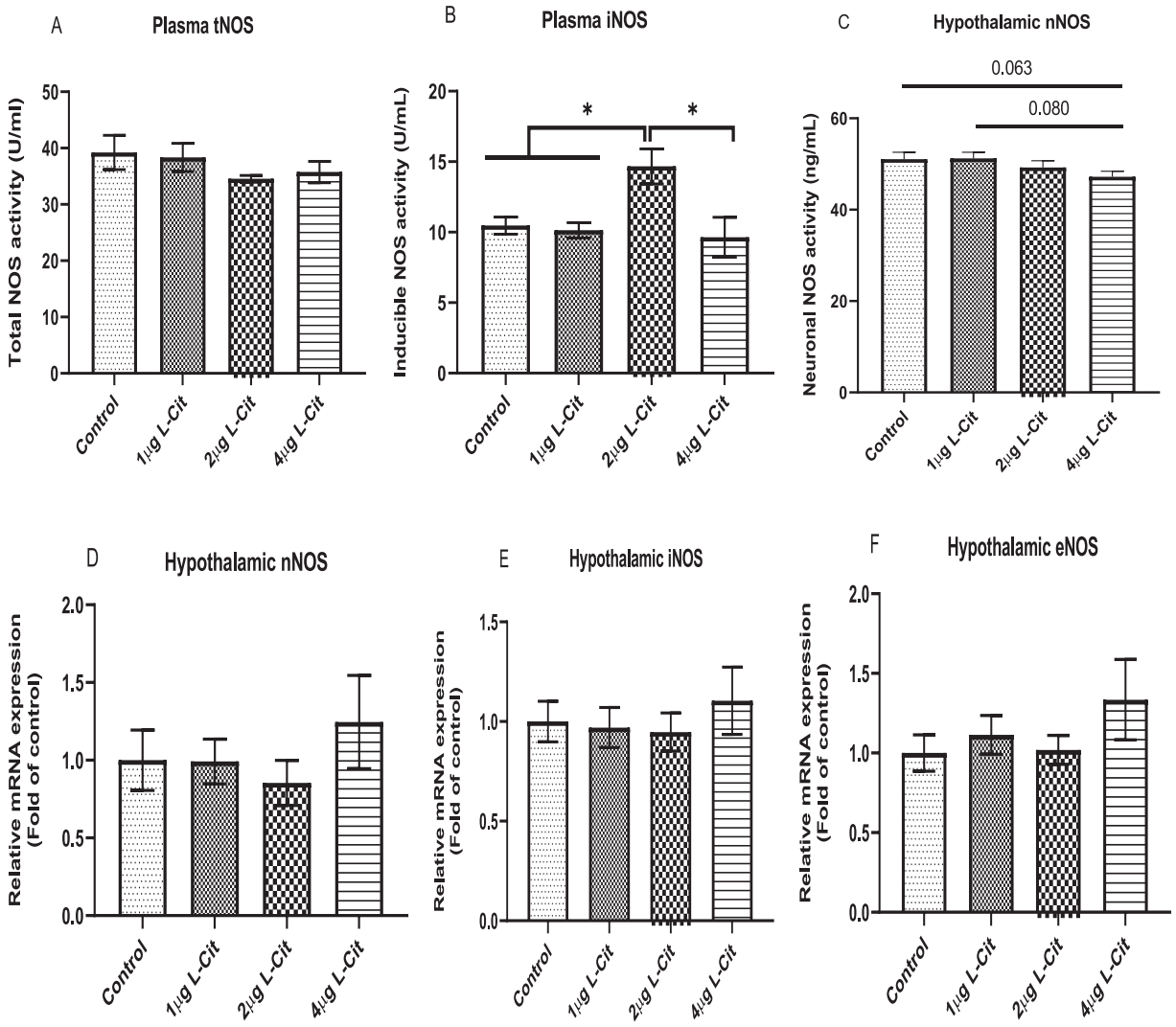

Figure 4. Enzyme activities and mRNA expression profiles for nitric oxide synthase (NOS) isoforms following central L-citrulline administration (experiment 1). (A) Plasma tNOS activity. (B) Plasma iNOS activity. (C) Hypothalamic nNOS activity. (D) Hypothalamic nNOS expression. (E) Hypothalamic iNOS expression. (F) Hypothalamic eNOS expression. Data are presented as mean ± SEM (n = 8). * indicate significant difference at $P < 0.05$.

DISCUSSION

This study was designed to provide evidence on the influence of L-citrulline on body temperature regulation, with insights into the involvement of nitric oxide signaling. Previous studies have reported that L-citrulline induces a hypothermic effect in chicks (Chowdhury et al., 2017a; Uyanga et al., 2021), however, the mechanism of action remains elusive. In the present study, we have demonstrated that L-citrulline can potentiate actions centrally in the hypothalamus to influence body temperature regulation, and this may be partly related to the induction of peripheral nitric oxide synthesis.

Monitoring of the surface temperature in broilers can be used as a parameter to determine peripheral blood flow, heat exchange, thermal comfort, or stress conditions (Cangar et al., 2008). In the present study, it was observed that the HT linearly decreased with an increase in L-Cit levels. The lowest L-Cit dose (1 μg) had higher HT similar to the control group. In contrast, the higher L-Cit doses (2 and 4 μg) significantly reduced the HT of chicks. Similarly, central injection of L-Cit elicited a dose-dependent response on the RT of chicks. At the lowest dose of 1 μg, L-Cit induced sustained hyperthermic condition in chicks which lasted until 120 min. Interestingly, the highest L-Cit dose (4 μg) elicited a significant drop in RT over a longer duration (at 120 min). This dose differential effects of L-Cit on RT was further ascertained using the mean RT findings. It was found that the lowest L-Cit (1 μg) had the highest mean RT and that the 2 μg L-Cit still had a higher mean RT compared to the highest L-Cit (4 μg) treatment. Previous studies in chicks have demonstrated L-citrulline's ability to decrease the rectal temperature, and core body temperature in chickens (Chowdhury et al., 2017a; Uyanga et al., 2021). Changes in cloacal temperature can be used to evaluate heat exchange, attainment of thermal equilibrium, and the range of thermal comfort in broilers (Nascimento et al., 2012). Regression analysis between the cloacal and body surface temperature (measured using a clinical thermometer and IRT

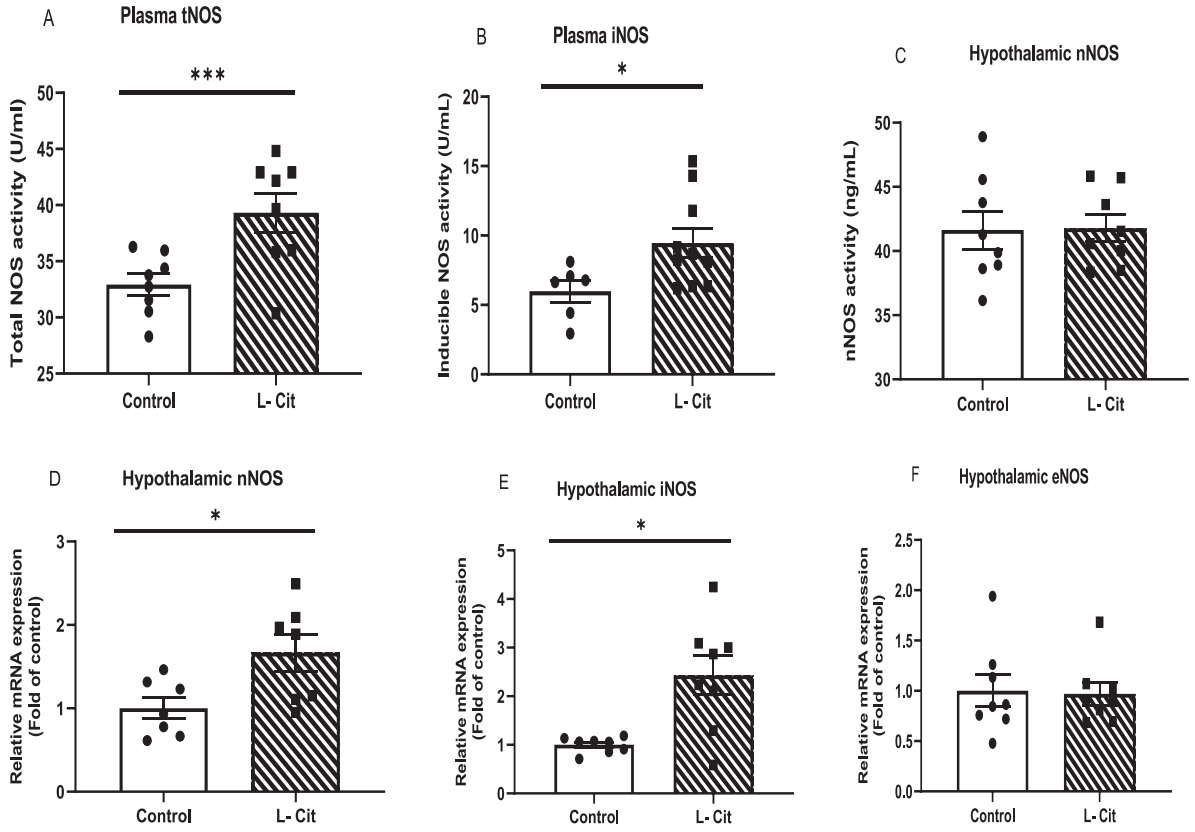

Figure 5. Enzyme activities and mRNA expression profiles for nitric oxide synthase (NOS) following I.P. L-citrulline administration (experiment 2). (A) Plasma tNOS activity. (B) Plasma iNOS activity. (C) Hypothalamic nNOS activity. (D) Hypothalamic nNOS expression. (E) Hypothalamic iNOS expression. (F) Hypothalamic eNOS expression. Data are presented as mean ± SEM (n=8). * indicate significant difference at $P < 0.05$.

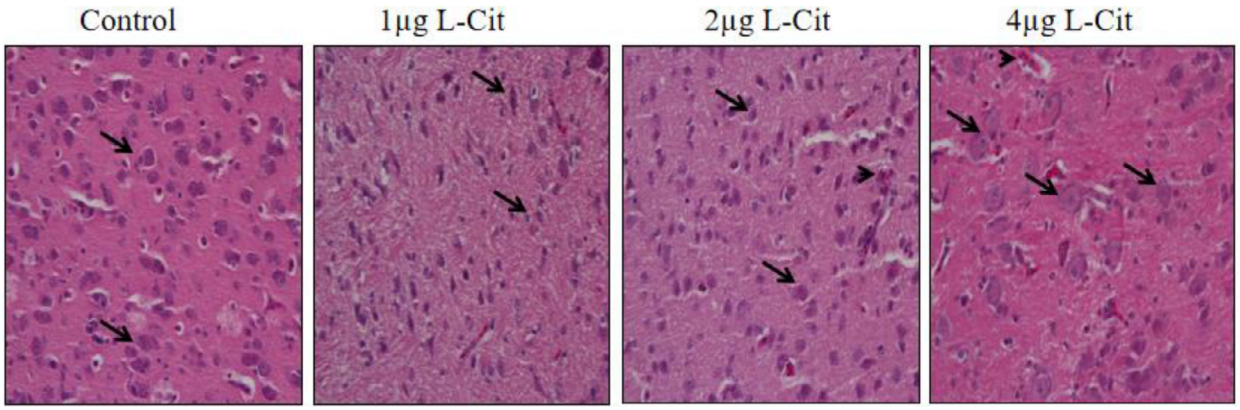

Figure 6. Hematoxylin and Eosin staining of hypothalamic tissues of broilers after ICV L-citrulline administration (experiment 1) for histological observation (400×). Abbreviation: ICV, intracerebroventricular.

respectively) indicated a 95% predictive accuracy between the two variables (Candido et al., 2020). Therefore, these findings from the rectal and head temperature measurements during the ICV experiment affirms that central L-Cit affects body temperature in a dose-responsive manner, such that higher L-Cit concentrations would lower body temperature and vice versa. Previous studies have shown that oral administration of urea cycle amino acids including L-citrulline, L-arginine, and L-ornithine, exhibited significant changes in the rectal temperature of chicks, with L-citrulline strongly initiating hypothermic effects (Chowdhury et al., 2015, 2017a), suggesting its role as a new candidate for body temperature regulation. However, a single ICV administration (1 μmol/10 μL) of L-citrulline did not elicit any changes in rectal temperature (Chowdhury et al., 2017b). In line with our findings, a single peripheral injection of L-Cit showed no significant changes to both rectal and core body temperature. However, it was evident that the core body temperature was lowered intermittently at several time points after i.p. L-Cit administration. Therefore, our results show that in line with the hypothermic role associated with L-citrulline, central L-Cit administration may exert dual effects on body temperature regulation depending on the dosage administered.

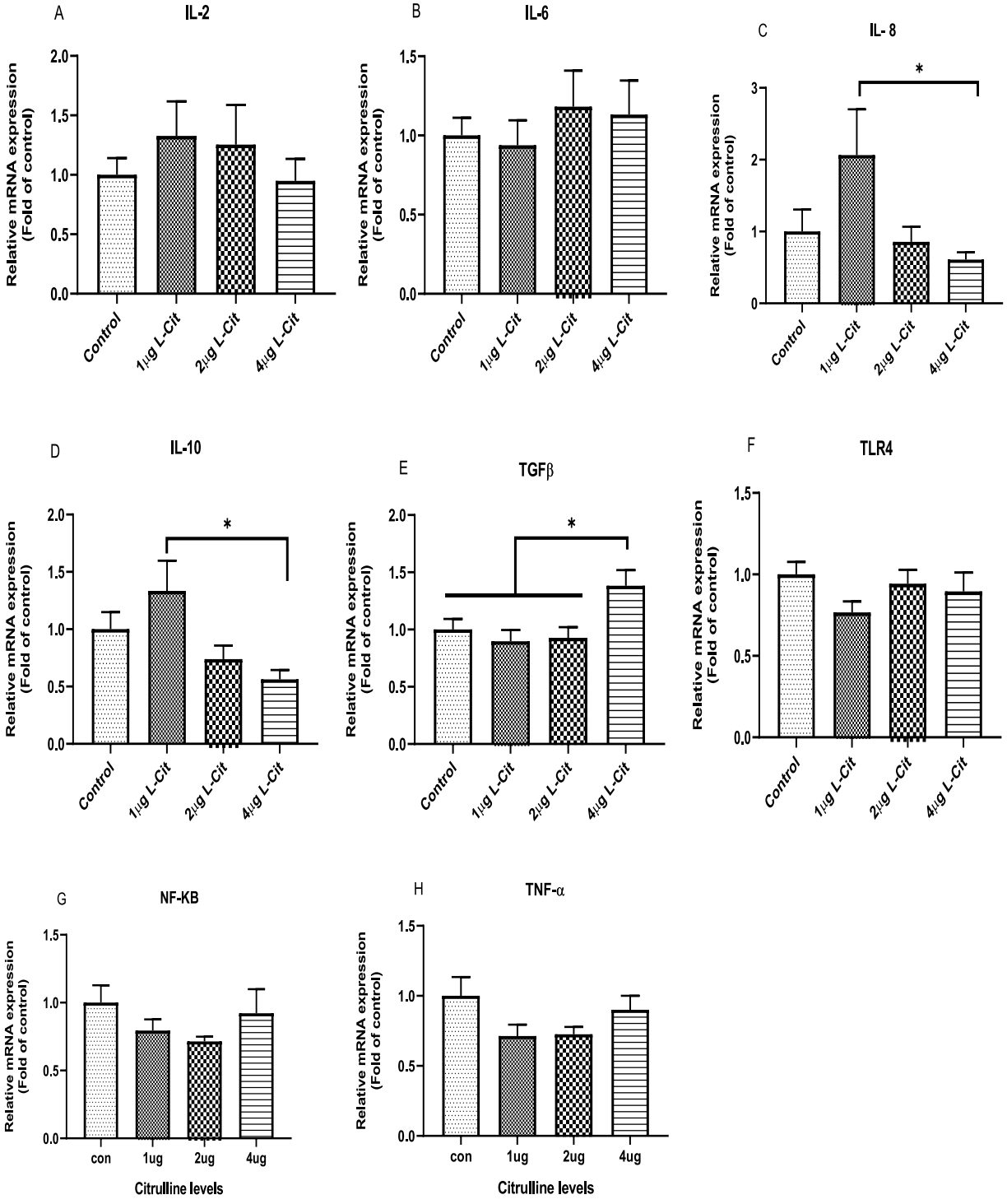

Figure 7. Relative mRNA expression of inflammatory cytokines in the hypothalamic tissues of broilers following ICV L-citrulline administration (experiment 1). (A) IL-2, (B) IL-6, (C) IL-8, (D) IL-10, (F) TGFβ, (G) TLR4, (H) NF-κB, (I) TNF-α . Data are presented as mean ± SEM (n = 8). * indicate significant difference at $P < 0.05$.

Several studies using in vitro and in vivo models have demonstrated that L-citrulline is an efficient substrate for arginine recycling and nitric oxide production (Schwedhelm et al., 2008; Kurauchi et al., 2017). Short-term effects (4 h) of L-citrulline in combination with L-arginine was reported to upregulate the citrulline-arginine recycling and rapidly potentiate NO-cGMP dependent reactions (Morita et al., 2014). Under physiological conditions, the state of the L-arginine/NO pathway can be characterized quantitatively by determining NOS enzyme activity (Tsikas, 2004). Also, due to the short half-life of NO, its concentration is reliably assessed analytically using the levels of its oxidative metabolites, that is, nitrite and nitrate (Tsikas et al., 2006). In this study, it was found that the highest L-Cit dose (4 μg) tended to increase hypothalamic NO concentration. This suggests a moderate effect of central L-Cit on NO generation, which may be effective at higher doses. Previous reports had shown that oral L-citrulline administration increased brain concentration of L-arginine and

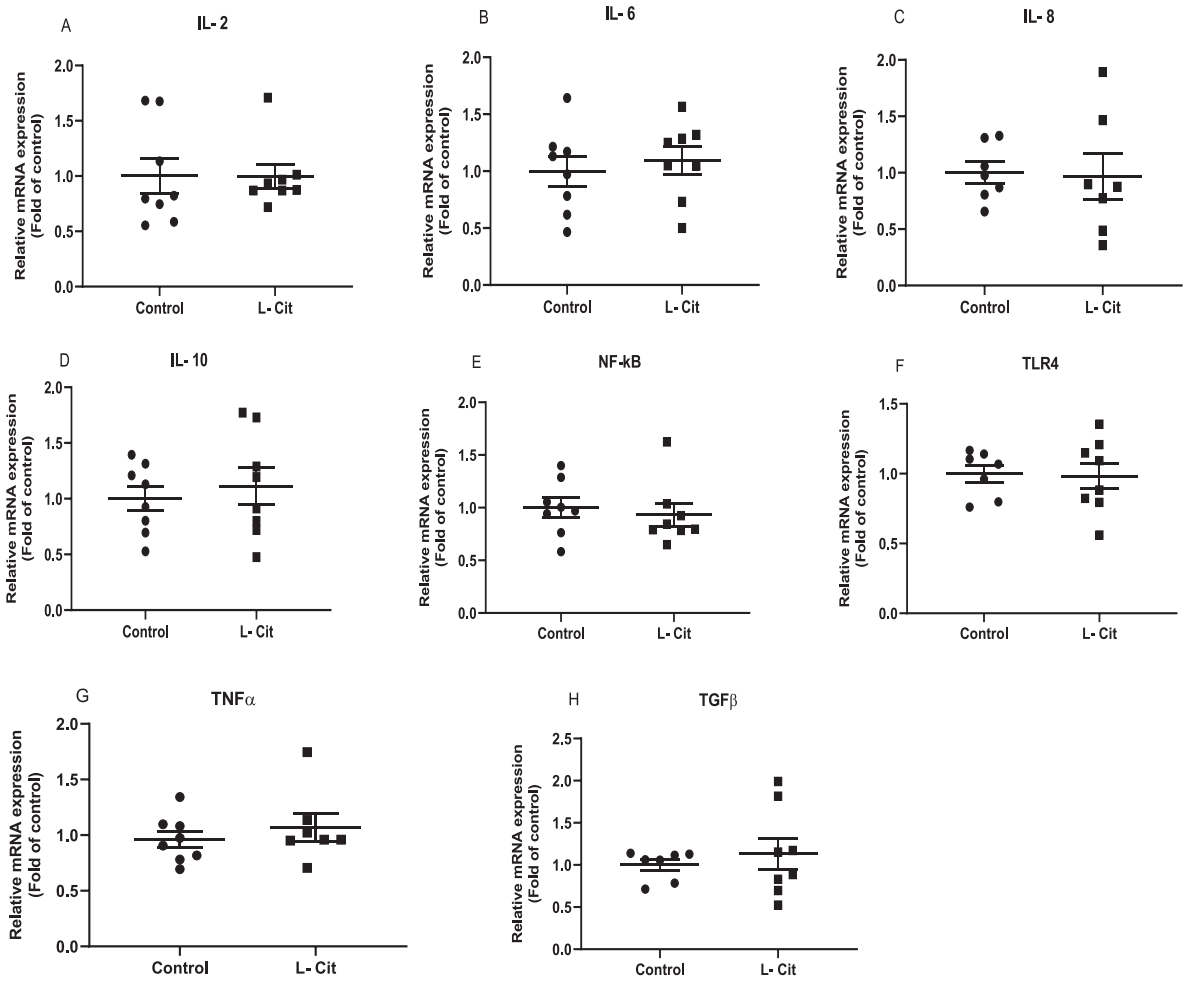

Figure 8. Relative mRNA expression of inflammatory cytokines in the hypothalamic tissues of broilers following I.P. L-citrulline administration (experiment 2). (A) IL-2, (B) IL-6, (C) IL-8, (D) IL-10, (F) NF-κB (G) TLR4, (H) TNF-α, (I) TGFβ. Data are presented as mean ± SEM (n = 8).

L-citrulline (Kurauchi et al., 2017), thus, enhancing substrate availability for NOS activity and NO production. We had proposed that central L-Cit injection would similarly modulate NO and NOS concentrations as previously reported (Uyanga et al., 2020). However, our results revealed that the plasma NO was unchanged whereas, only the highest L-Cit dose could modulate hypothalamic NO level. Interestingly, higher plasma iNOS activity was initiated following the drop in NO levels by 2 μg L-Cit, probably to replenish NO deficiency from iNOS catalysis. Moreover, although the total NOS activity was unchanged, the variability observed with iNOS isoform (which is typically induced with several inflammatory stimuli), was consistent with the changes in hypothalamic NO concentrations. From experiment 2, the peripheral administration of L-Cit, (200 mg L-Cit/kg BW) did not influence NO production in the plasma and hypothalamus, but rather it modulated the various NOS isoforms. In the plasma, the tNOS and iNOS were elevated, whereas, in the hypothalamus,

Table 2. List of primers for RT-PCR.

Gene	Forward sequence (5'to 3')	Reverse sequence (3'to 5')	Accession number
IL-2	GAACCTCAAGAGTCTTACGGGTCTA	ACAAAGTTGGTCAGTTCATGGAGA	XM_015276098.2
IL-6	CTCCTCGCCAATCTGAAGTC	AGGCACTGAAACTCCTGGTC	HM179640.1
IL-8	CTGCGGTGCCAGTGCATTAG	AGCACACCTCTCTTCCATCC	HQ739087.1
IL-10	CGCTGTCACCGCTTCTTCA	TCCCGTTCTCATCCATCTTCTC	NM_001004414.2
TGF-β	AGGATCTGCAGTGGAAGTGGAT	CCCCGGGTTGTGTTGGT	M31160
TNF-α	GAGCGTTGACTTGGCTGTC	AAGCAACAACCAGCTATGCAC	NM_204267
NF-κB	TCAACGCAGGACCTAAAGACAT	GCAGATAGCCAAGTTCAGGATG	XM_015285418.2
TLR4	GAAGGGAAGGCTGGAATAA	GTGGGAGACAGGACAGAAGT	AY064697.1
nNOS	CTCGGATGCACGGAAGTCCT	CGTGAACCCAGCCCAAACAC	XM_004934480.1
iNOS	GTGGTATGCTCTGCCTGCTGTTG	GTCTCGCACTCCAATCTCTGTTCC	NM_204961
eNOS	GGATGTGCTGCACGGTCTGC	AGGACGTGCTGCGGACACAG	JQ434761.1
β-Actin	TGCGTGACATCAAGGAGAAG	TGCCAGGGTACATTGTGGTA	L08165.1

both the nNOS and iNOS isoforms were upregulated following L-Cit administration. This suggests that modulation of peripheral and hypothalamic NOS activities may be largely responsible for NO production during central L-Cit administration, rather than changes at the transcriptional level. In line with this findings, L-citrulline was shown to induce plasma iNOS activity both during normal and under pathological conditions (Lange et al., 2019; Uyanga et al., 2020). Studies revealed that C2C12 myotubes treated with L-citrulline substantially increased iNOS mRNA expression (~3.5 and ~35 fold higher than control and serum-free treatments), without altering eNOS or nNOS expression. These effects were attributed to the established role of iNOS in inflammatory signaling (Ham et al., 2015).The distinct properties associated with each NOS isoform determines their physiological or pathophysiological effects, such that NO synthesis from constitutive NOS is essential for normal physiological functioning, in contrast to iNOS induction, which occurs in response to inflammatory stimuli (cytokines and endotoxins), allowing for prolonged NO synthesis and several pathological cascades (Cuzzocrea and Salvemini, 2007). Therefore, L-Cit induction of iNOS and nNOS may be suggestive of an immune response following central and peripheral L-Cit administration.

Cytokines are involved in several aspects of CNS functioning, however, there has been increased attention toward understanding their roles as mediators of dysregulated CNS function during inflammatory conditions (Galic et al., 2012). It was reported that the precise mechanisms responsible for cytokine synthesis are yet to be entirely ascertained since brain cytokine production can occur via various routes including microglia, astrocytes, pericytes, inflammatory cells, endothelial cells, and, constitutively expressed sources such as neurons (Galic et al., 2012). Upregulation of inflammatory cytokines such as IL-1 and TNF-α, along with NO production via iNOS and nNOS synthesis is associated with neuronal damage, neuritic plaques, and implicated in the pathogenesis of neurodegenerative diseases (Katsuse et al., 2003). In this study, central treatment with L-Cit did not influence the expression of several cytokines including IL-2, IL-6, TLR4, NF-kB, and TNF-α expression. Similarly, in splenocytes stimulated to induce innate and adaptive immune cytokines, it was reported that IL-6, TNF-α, IFN-γ, IL-4, and IL-13 concentrations were unchanged regardless of L-citrulline supplementation (Lee et al., 2018). In another study, L-citrulline regulated cytokine production in peritoneal macrophages by decreasing TNF- α production, causing an anti-inflammatory effect (Breuillard et al., 2015). These reports indicate that L-Cit administration may provide an anti-inflammatory response, although inconsistencies may arise from study models (in vitro vs. in vivo, or disease vs. healthy state), different species, and age of subjects (Lee et al., 2018).

In this study, ICV treatment with L-Cit influenced pro- and anti-inflammatory cytokines such as IL-8, IL-10, and TGFβ expression in the hypothalamus. It was observed that the low dose (1 μg) L-Cit upregulated IL-8 and IL-10 expression, whereas the highest dose (4 μg) increased TGFβ expression. IL-8 is a pleiotropic chemokine associated with neuroprotective and neurotoxic roles (Willette et al., 2013). It is found both under normal and pathological conditions, and although not constitutively produced by several cell types, it is rapidly induced in disease states (Romero et al., 2006; Kumar et al., 2015). Therefore, L-Cit's downregulation of IL-8 and IL-10 at higher doses may be related with the body temperature changes elicited by increased L-Cit dose. Also, the spike in IL-6 and IL-8 expression by 1 μg L-Cit treatment may occur as a consequence of an ongoing immune response, since it has been revealed that the hypothalamus is receptive to immune stimuli, and can engage in a neuroendocrine-immunoregulatory cascade when the magnitude of insult is high (Rey et al., 2009). The balance between pro- and anti-inflammatory cytokines can influence the degree of inflammation (Kumar et al., 2015). L-Cit's induction of IL-10 and TGFβ connotes an anti-inflammatory milieu, to establish a protective effect against neuroinflammation. L-citrulline supplementation modulated the immune responses of regulatory T cells via increased IL-10 and TGF-β1 levels (Lee et al., 2018), and established an anti-inflammatory profile, characterized by increased IL-10 production (Romero et al., 2013). Moreover, given that the expression of cytokine genes in chickens treated with i.p. L-Cit was unaffected (IL-2, IL-6, IL-8, IL-10, NF-κB, TLR4, TNF-α, and TGFβ), this suggests that peripheral L-Cit was not responsible for stimulating cytokine production.

Histological studies further revealed evidence of L-Cit-induced changes in the hypothalamus. It was observed that at a high dose, the number and size of hypothalamic neurons were altered, such that they had fewer neurons with enlarged nuclei, suggesting neuronal swelling. Vacuoles in the brain parenchyma may develop from an accumulation of fluid, neuronal injury leading to cell death, infections associated with neuronal degeneration, or artifacts during tissue processing (Purcell et al., 2018). The number of neurons, neuronal densities, and the sizes of neuronal and non-neuronal cells are key factors that influence brain functions (Farrow et al., 2021). It was observed that at the lowest L-Cit dose, the hypothalamus showed a gradient of condensed neurons of higher numbers with poorly visible nuclei. They displayed cell body shrinkage, loss of Nissl substance, intensely stained eosinophilic cytoplasm, and small/shrunken darkly stained (pyknotic) nuclei under light microscopy, which typically characterizes degenerating neurons (Garman, 2010). Since the hypothalamic nuclei are involved in the regulation of body temperature, and several other physiological responses, L-Cit influenced these nuclei by causing structural changes in neuronal cells, which will modify the functional relevance of the hypothalamic nuclei. Additionally, it is noteworthy to mention that this study did not include a sham control that could have provided additional information on L-Cit-specific effects. Therefore, site-specific studies are

warranted to ascertain the role of L-Cit in hypothalamic inflammation.

Tentatively, it may also be explained that although i.p. L-Cit elicited no changes in the hypothalamic cytokine mRNA expression levels, this does not concretely reflect on the effects of L-Cit on immune cells or circulating cytokines production, as our investigation was limited to the hypothalamus. Due to the lack of responsiveness of the peripheral L-Cit experiment, the 200mg/kg BW dosage adapted for this study was of concern. These amounts are within the range reported from previous studies in rats, where it elicited immune responses (Lee et al., 2018). Moreover, this dose is considered safe, nontoxic, and largely tolerable since L-citrulline and arginine are widely adopted as dietary supplements (<2.5% of dry matter; Wu et al., 2007; Lee et al., 2018). In line with this, chicks orally administered L-Cit at varying doses (0, 3.75, 7.5, and 15 mmol/kg BW) did not show any changes in rectal temperature, or plasma metabolites (Erwan et al., 2020). However, due to the paucity of research on L-Cit administration in chickens for comparative studies, the bioavailability of L-Cit at different physiological states should be thoroughly investigated because lower citrulline to arginine flux would limit the efficiency of NOS and NO production. From the results presented, it is evident that L-citrulline influences the central regulation of core body temperature and that this effect may be mediated along with changes in inflammatory cytokine signaling. In addition, central and peripheral L-Cit could modulate NO synthesis at the transcriptional and systemic levels but afforded no changes to the plasma NO levels. Thus the role of NO in L-citrulline's actions may be further validated using NO inhibition/ ablation models. Also, studies designed for temperature conditioning (hot or cold treatments) and/or inflammation models may provide further insights into the relevance of L-citrulline in body temperature regulation.

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Ethics approval: The study was reviewed and approved by the Institutional Animal Care and Use Committee at the College of Animal Science of Shandong Agriculture University, and was carried out following the "Guidelines for Experimental Animal" of the Ministry of Science and Technology (Beijing, P. R. China).

Data availability: Data obtained and analyzed in this research are included within this article and can be obtained from the corresponding author upon request.

Authors' contributions: The study was designed by VAU and HL. The authors, VAU, and LL performed the animal experiments, and data collection. VAU analyzed, interpreted and presented the data. JZ, XW, and HJ contributed to the research design and results interpretation. All authors read, reviewed and approved the final manuscript.

DISCLOSURES

The authors declare that they have no competing interests financial or otherwise regarding the publication of this paper.

SUPPLEMENTARY MATERIALS

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