

« Research Note »

## Oral Administration of L-Citrulline, but not L-Arginine or L-Ornithine, Acts as a Hypothermic Agent in Chicks

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Some amino acids are important regulators of key metabolic pathways and necessary for several physiological functions. However, little is known about thermoregulatory functions of amino acids. In this study, therefore chicks were either centrally or orally administered with L-citrulline (L-Cit), L-arginine (L-Arg) or L-ornithine (L-Orn) to monitor changes in rectal temperature. In Experiment 1, the amino acids (L-Cit, L-Arg and L-Orn) were administered into the left ventricle of the chicks by intracerebroventricular (i.c.v.) injection at a dose of 1  $\mu\text{mol}/10 \mu\text{l}$  to monitor the effects of these amino acids on rectal temperature during 120 min of the experimental period. In Experiment 2, chicks received the same amino acids by oral administration at a dose of 15 mmol/10 ml/kg body weight. In Experiment 3, chicks received three doses of L-Cit (3.75, 7.5 or 15 mmol/10 ml/kg body weight) by oral administration. I.c.v. injection with any of the amino acids studied did not alter body temperature, but oral administration of L-Cit significantly reduced body temperature. Importantly, the highest dose effectively reduced body temperature. These results suggest that peripheral L-Cit has a hypothermic function in chicks, which may be a new candidate to minimize high body temperature in poultry during summer heat stress.

**Key words:** body temperature, chick, L-arginine, L-citrulline, L-ornithine

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### Introduction

High ambient temperature (HT) has a huge negative impact on poultry production. Maintaining normal body temperature during HT is a challenge for poultry as they lack sweat glands, relying on evaporative cooling (panting) to keep cool (Marder and Arad, 1989). There are plethora of reports referring to increased body temperature in chickens during HT (Yahav and Hurwitz, 1996; Chowdhury *et al.*, 2012a, b; 2014; Ito *et al.*, 2014) and this may cause heat stress (Soleimani *et al.*, 2010).

Several researchers have used essential amino acids in an attempt to overcome heat-stress problems in poultry (Mendes *et al.*, 1997; Rose and Uddin, 1997; Brake *et al.*, 1998; Willemsen *et al.*, 2011; Dai *et al.*, 2012). However, in addition to essential amino acids, non-essential amino acids may also reduce the effects of psychological and physiological stress in chicks (Erwan *et al.*, 2012, 2014a), since their

levels have been found to be modified under heat stress (Chowdhury *et al.*, 2014; Ito *et al.*, 2014). Recently, we reported that D-aspartate showed the potential to reduce body temperature under both thermoneutral temperature (control) and HT conditions in chicks (Erwan *et al.*, 2014b). Thus, it is interesting and important also to find out which other amino acids have thermoregulatory functions.

L-Citrulline (L-Cit) is known to enhance the bioavailability of L-arginine (L-Arg), the endothelial substrate for the production of nitric oxide (NO), and ultimately to increase endogenous NO production (Schwedhelm *et al.*, 2008). NO is well known as an endothelium-derived relaxing factor and has been shown to be a modulator of various behaviors including thermoregulation (Szabo, 1996; Monti and Jantos, 2004). Endogenous L-Cit and L-Arg are physiological amino acids in most living systems (Curis *et al.*, 2005). L-Cit was first encountered as a constituent of watermelon. It was also reported that watermelon is rich in L-Cit (Tedesco *et al.*, 1984) and it has been found that, combined with exercise, subjects consuming watermelon or synthetic L-Cit as a drink had reduced arterial blood pressure compared with those consuming a placebo (Figuroa *et al.*, 2010). As for L-ornithine (L-Orn), it was found that central injection of L-Orn has been shown to have sedative and hypnotic effects on

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neonatal chicks exposed to acute stressful conditions (Suenaga *et al.*, 2008).

In this study, we conducted experiments to examine whether central or peripheral urea cycle-related amino acids L-Cit, L-Arg and L-Orn have any thermoregulatory functions in chicks.

## Materials and Methods

### Animals

A total of 100 one-day-old male layer chicks (Julia) (*Gallus gallus domesticus*) were purchased from a local hatchery (Murata hatchery, Fukuoka, Japan) and housed in wire-meshed cages (5 cages; 50×35×33 cm per cage) in a group (20 birds per cage) at a constant temperature of 30±1°C and with continuous light. Chicks were all of the same age and were housed without any adult present. Food (Commercial starter diet (metabolisable energy: 12.77 MJ/kg and protein: 24%; food ingredients: grain 61% (mainly maize), defatted meal 25% (soybean meal and maize gluten meal), fish meal 9%, rice bran 1% and others 4%); AX, Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were provided *ad libitum*. For acclimatization, 4 and 5 days old chicks were reared individually and assigned for treatment and control groups for the Experiment 1, and Experiments 2 or 3, respectively prior to the day of the experiment on the basis of their body weight (59.6±0.6 g for 4 days chicks and 64.7±1.0 g for 5 days chicks) in order to produce uniform groups. The chicks had free access to food and water during the whole experimental period. The number of animals used in each group was kept to a minimum ( $n=5-9$ ). This study was performed in accordance with the guidelines for animal experiments carried out in the Faculty of Agriculture and on the Graduate Course of Kyushu University, and adhered to Law no. 105 and Notification no. 6 of the government.

### Preparations of Drugs

L-Cit, L-Arg and L-Orn were purchased from Wako Pure Chemical Industries (Osaka, Japan). In order for them to be injected intracerebroventricularly, these amino acids were dissolved in 0.85% saline containing 0.1% Evans Blue solution. The solutions were dissolved by stirring them in a vortex, and they were sonicated in a bath sonicator for 10 min. The resulting dissolved amino acids were kept on ice during the experiment. For oral injections, the same amino acids were suspended in 0.25% methyl cellulose solution, and these suspensions were stirred well on a vortex. These solutions were kept at room temperature (30±1°C) during the experiments.

### Experimental Design

In Experiment 1, following an acclimatization period, a total number of 36 chicks were selected and divided into four groups again on the day of the experiment based on their initial rectal temperature in order to produce uniform groups. The amino acids (L-Cit, L-Arg and L-Orn) were administered into the left ventricle of the chicks by intracerebroventricular (i.c.v.) injection via a microsyringe, according to the method described elsewhere (Davis *et al.*, 1979). The dose

of the amino acids for i.c.v. injection was based on the findings of our previous reports (Erwan *et al.*, 2012; 2014a). In brief, chicks (5 days old) were intracerebroventricularly injected either with 1 μmol/10 μl of the above-mentioned amino acids or, for the control, with the same volume of saline. This injection method is not stress-causing (Furuse *et al.*, 1999; Koutoku *et al.*, 2005).

In Experiment 2, a total number of 36 chicks were selected and divided into four groups as described above for Experiment 1. Chicks (6 days old) were orally administered with amino acid solutions (L-Cit, L-Arg or L-Orn) using an elastic plastic needle on a small syringe or, for the control, with 0.25% methyl cellulose solution. Chicks received the amino acids (L-Cit, L-Arg or L-Orn) by oral injection at a dose of 15 mmol/10 ml/kg body weight, based on the findings of our recent report (Erwan *et al.*, 2014b). In Experiment 3, a total number of 28 chicks (6 days old) received three doses of L-Cit (3.75, 7.5 or 15 mmol/10 ml/kg body weight) by oral administration.

### Measurement of Rectal Temperature

The rectal temperature of chicks was measured with a digital thermometer with an accuracy of ±0.1°C (Thermalert TH-5, Physitemp Instruments Inc., USA) by inserting the thermistor probe into the cloaca to a depth of around 2 cm. Rectal temperature was measured at 0, 30, 60, 90 and 120 min after i.c.v. or oral injections.

### Statistical Analysis

Data were statistically analyzed by a paired t-test between 0 min and each time point after injection for all treatments. Significant differences were denoted as  $P<0.05$ . Data were analyzed using the statistical program Statview Version 5.0 (SAS Institute, Cary, USA, 1998). Values are presented as means±S.E.M.

## Results

As shown in Fig. 1, rectal temperatures of chicks did not change significantly ( $P>0.05$ ) between 0 min and each time point after i.c.v. administration of L-Cit, L-Arg or L-Orn. Changes in rectal temperatures following oral administration of L-Cit, L-Arg and L-Orn are shown in Fig. 2. L-Cit significantly ( $P<0.05$ ) decreased rectal temperature at 60 min. Conversely, rectal temperature significantly ( $P<0.05$ ) increased at 90 and 120 min with L-Arg administration and at 60 min with L-Orn administration compared with 0 min. Fig. 3 shows the effect of different doses of orally administered L-Cit on rectal temperature during 120 min of the experimental period. Rectal temperature significantly ( $P<0.05$ ) decreased at 30, 60 and 120 min after injection of the highest dose of L-Cit.

## Discussion

The objective of the current study was to reveal whether the amino acids (L-Cit, L-Arg and L-Orn) of the so-called urea cycle have thermoregulatory functions in chicks. Oral administration, but not central administration, of these amino acids altered rectal temperature. Oral administration of L-Cit strongly depressed rectal temperature conversely, L-Arg

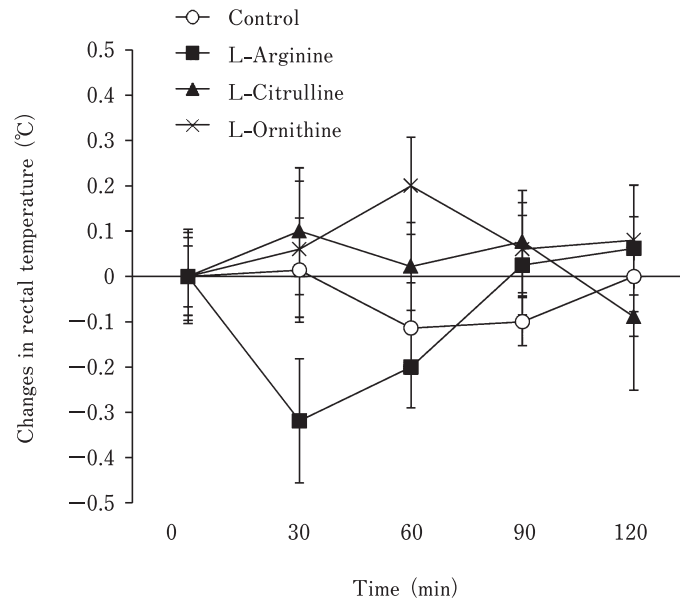


Fig. 1. Effect of intracerebroventricular (i.c.v.) injection of L-Cit, L-Arg or L-Orn on rectal temperatures in chicks during 120 min of the experimental period. The number of chicks used in each group ranged between 5-9. Values are means  $\pm$  S.E.M.

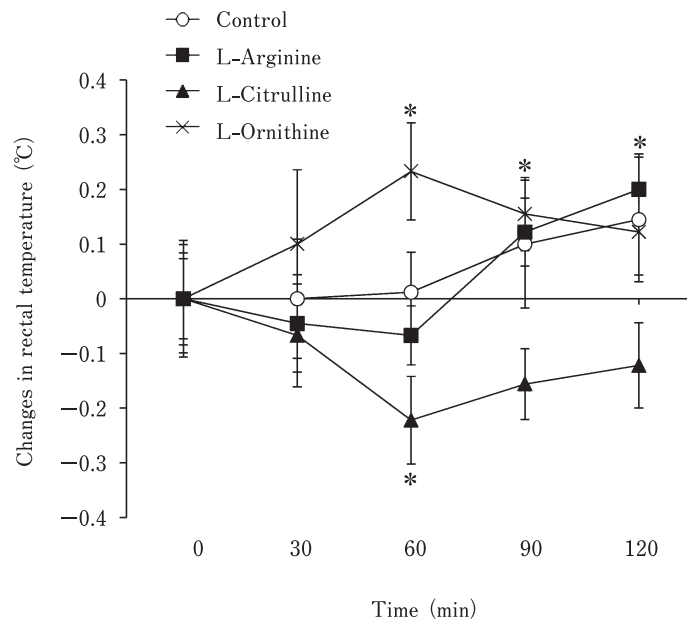


Fig. 2. Effect of oral administration of L-Cit, L-Arg or L-Orn on rectal temperatures in chicks during 120 min of the experimental period. The number of chicks used in each group was 9. Values are means  $\pm$  S.E.M. \*,  $P < 0.05$  vs. 0 min, by paired t-test.

and L-Orn slightly increased it.

It was found in the current study that a single dose of i.c.v. injection of L-Cit, L-Arg or L-Orn did not cause any change in rectal temperature. Because we have used only a single

dose for i.c.v. injection in the current study, we cannot preclude the possibility of central effect of the three amino acids on the thermal center of the brain in thermoregulation. Thus, further study is needed to clarify this matter using at

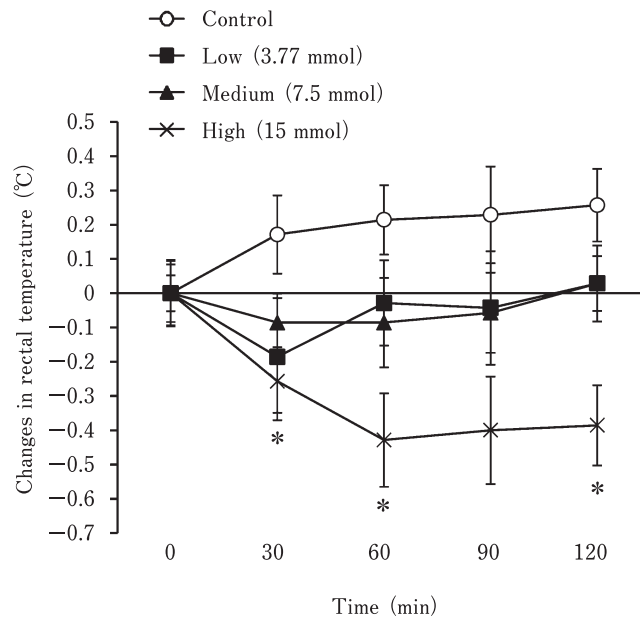


Fig. 3. Effect of different doses of orally administered L-Cit on rectal temperatures in chicks during 120 min of the experimental period. The number of chicks used in each group was 7. Values are means  $\pm$  S.E.M. \*,  $P < 0.05$  vs. 0 min, by paired t-test.

least four doses on a log scale.

In Experiment 2, it was clearly observed that, when it was orally administered, L-Cit decreased the rectal temperature, but L-Arg and L-Orn increased it. Although it is still unknown how the so-called urea cycle amino acids could regulate the body temperature in chicks, we can nonetheless speculate on it. In mammals, almost all of the L-Arg coming from the food supply is withdrawn from the portal blood by the liver to convert it to urea (Curis *et al.*, 2005). However, L-Cit can bypass the liver, since the liver is unable to uptake L-Cit from the portal circulation (Windmueller and Spaeth, 1981). This bypassed L-Cit is then converted to L-Arg by the kidney and released into the blood to make it available for the whole body. However, birds lack carbamyl phosphate synthetase, one of the enzymes of the urea cycle necessary for the synthesis of L-Cit from L-Orn in the liver and kidney (Tamir and Ratner, 1963). Therefore, birds cannot synthesize L-Cit or L-Arg, but can synthesize L-Orn from L-Arg (Suenaga *et al.*, 2008). After being synthesized in the cytosol, L-Orn is transported to the mitochondria. Mitochondria are important organelles in the cell which are involved with heat production and energy generation (Alberts *et al.*, 2014). However, still there is no report available whether ornithine has any involvement in the process of thermogenesis in the mitochondria. NO produced in the conversion of L-Arg to L-Cit by the enzyme NO synthase (Palmer *et al.*, 1987) may play some roles as a hypothermic agent in chicks because thermoregulation has been proposed as one of the main physiological functions of NO (Szabo, 1996). However, not only NO but also L-Cit itself and/or its metabolites argi-

ninosuccinate, L-Arg or other metabolites of L-Cit may be involved in the process of hypothermia through influencing the thermal center of the brain and/or peripheral tissues. Therefore, further research is needed to determine the concentration of these amino acids and their metabolites in the central and peripheral tissues as well as in the plasma to reveal the functional mechanism of hypothermia induced by L-Cit.

It was further confirmed in this study that, compared with the other two lower doses, the highest dose (15 mmol/ 10 ml/ kg body weight) was the most effective dose for inducing the hypothermic action. Based on our recent findings, plasma of chicks contain on an average 32.2 to 35.4 pmol/ $\mu$ l L-Cit (Chowdhury *et al.*, 2014). Thus, the used dose of L-Cit in the current study is a pharmacological dose which was much higher than the physiological range. Rimando and Perkins-Veazie (2005) reported that watermelon rind contained more L-Cit than flesh on a dry weight basis which indicates that rind, an underutilized agricultural waste, could be used as a natural source of L-Cit to control high body temperature.

In conclusion, peripheral L-Cit has a hypothermic function in neonatal chicks. This fact indicates that L-Cit could be used to control high body temperature during heat stress following further research. A future study will focus on elucidating the mechanisms of the peripheral functions of L-Cit in thermoregulation.

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