

«Research Note»

Oral Administration of a Medium Containing L-Citrulline-producing Live Bacteria Reduces Body Temperature in Chicks

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Recently, we showed that oral administration of crystallized L-citrulline (L-Cit) caused hypothermia under a control thermoneutral temperature (CT) and provided thermotolerance under high ambient temperature (HT) in chicks. The aim of this study was to clarify whether oral administration of a medium containing L-Cit-producing live bacteria can reduce body temperature in chicks under CT. In Experiment 1, 7-day-old chicks were orally administered either a medium (containing mainly L-Cit-producing live bacteria and 277 mM L-Cit) or an equimolar amount of L-Cit to determine their effects on body temperature (acute treatment). In Experiment 2, chicks were subjected to the same treatment from 7 to 13 days of age (chronic treatment). Rectal and surface body temperatures were recorded daily after 1 h of treatment. Both acute and chronic oral administration of the medium, but not of the equimolar amount of L-Cit, significantly reduced the rectal and surface body temperatures of the chicks. Chronic administration of the medium resulted in consistently low rectal and surface body temperatures during the entire experimental period. In conclusion, acute or chronic administration of the medium containing L-Cit-producing live bacteria, but not of the equimolar amount of L-Cit, reduced the rectal and surface body temperatures of the chicks. Our results suggest that medium containing L-Cit-producing live bacteria can be used as a new feed supplement for lowering the body temperature of chicks.

Key words: chick, L-citrulline, medium, rectal temperature, surface temperature

J. Poult. Sci., 56: 285–289, 2019

Introduction

Chicks are susceptible to high ambient temperature (HT) because of their limited ability to regulate heat loss (Nicol, 2011). Reports show that the body temperature of chicks increases rapidly under HT (Chowdhury *et al.*, 2012a, b; Ito *et al.*, 2014, 2015). Exposure of chicks to extreme HT induces heat stress (Ito *et al.*, 2015), characterized by severe increase in body temperature and heightened stress response, which decreases food intake and feed efficiency, reducing meat production in broilers (Howlader and Rose, 1987) and

egg production in laying hens (Sterling *et al.*, 2003). Behavioral, physiological, and molecular responses to heat stress have been described previously (Etches *et al.*, 1995; Chowdhury *et al.*, 2012a, b). Recently, alteration in the levels of brain and plasma free amino acids was reported to be one of these responses (Chowdhury *et al.*, 2014; Ito *et al.*, 2014, 2015). For instance, L-citrulline (L-Cit) level was reduced in the plasma of heat-exposed chicks (Chowdhury *et al.*, 2014). Chowdhury *et al.* (2015, 2017) further showed that oral administration of L-Cit produced hypothermic effects in young chicks. Supplementation of essential amino acids to reduce the negative effects of heat stress and maintain steady production performance has currently attracted the attention of the chicken farming industry. However, utilization of a non-essential amino acid, L-Cit, was a novel approach for attenuating heat stress response in chicks.

L-Cit, a non-protein amino acid, is a physiological amino acid which plays an important role in both cellular metabolism and organ function in most living systems (Curis *et al.*, 2005). It is well-known that L-Cit, a metabolic intermediate

Received: November 26, 2018, Accepted: January 28, 2019

Released Online Advance Publication: March 25, 2019

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in arginine (Arg) metabolism, is produced from L-Arg, an essential amino acid in chicks, by nitric oxide (NO) synthase, along with the production of NO (Palmer *et al.*, 1987). NO is involved in the regulation of body temperature (De Luca *et al.*, 1995; Osaka, 2010). However, L-Cit-dependent hypothermia was not found to be related to the level of plasma NO in chicks, which indicates the involvement of a novel mechanism (Chowdhury *et al.*, 2017). As consumption of crystallized L-Cit has not yet been approved (Food and Agricultural Materials Inspection Center, Japan), its use in poultry ration is a matter of concern. Therefore, studies on the hypothermic functions of non-crystallized L-Cit might provide useful information. Erwan *et al.* (2014) reported that oral administration of D-aspartate (D-Asp), another hypothermic factor, lowered the body temperature of chicks both under a control thermoneutral temperature (CT) and HT. In addition, the administration of live bacteria into an animal's digestive tract has long been reported to exert beneficial effects on the growth and performance of the host. We recently observed that oral administration of a medium containing D-Asp-producing live bacteria reduced body temperature in chicks (Do *et al.*, 2017). In the present study, we used a medium containing L-Cit-producing live bacteria to examine its effect on body temperature regulation under CT.

Materials and Methods

Animals and Drugs

One-day-old male layer chicks (Julia, *Gallus gallus domesticus*) were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan) and housed in groups (15–20 birds per cage) in metal cages (50×35×33 cm) at a constant temperature of 30±1°C under continuous light conditions. Food (Adjust diets; Toyohashi Feed and Mills Co. Ltd., Aichi, Japan; metabolizable energy: > 12.55 MJ/kg, protein > 23%) and water were provided *ad libitum*. This study was performed in accordance with the guidelines for animal experiments in the Faculty of Agriculture at Kyushu University, and complied with Law No. 105 and Notification No. 6 of the Japanese government (experimental protocol number was A26-074-1). The medium (containing live bacteria [*Lactococcus lactis* subsp. *Lactis*; the number of live bacteria in the medium was 6.50E+09 cfu/mL] producing L-Cit, supplemented with L-Cit (277 mM), L-ornithine (12.4 mM), L-Arg (2.6 mM), L-lactic acid, and milk peptide (20.3 mM), was provided by Meiji Co., Ltd., Kanagawa, Japan. The components of the medium were decided by the provider. The concentration of L-Cit and other additives in the medium was determined using high-performance liquid chromatography (HPLC).

Experimental Design

Six-day-old chicks were randomly distributed into treatment and control groups based on their body temperature such that the average body temperature between the groups was as uniform as possible. In Experiment 1, 30 chicks (7 days old) were divided into three groups ($n=10$ /group) for different treatments as follows: 1) acute oral administration of 2 mL of the medium (containing 554 μ mol L-Cit/chick); 2)

acute oral administration of 2 mL solution containing an equimolar amount of crystallized L-Cit/chick; 3) acute oral administration of 2 mL water/chick (control group). Oral administration was performed using an elastic plastic needle on a small syringe. Rectal temperature and body surface temperature were measured 0, 30, 60, and 120 min after oral administration of the medium or the equimolar amount of L-Cit solution.

In Experiment 2, chicks (7 days old) were distributed into three groups ($n=10$ /group) as follows: 1) chronic oral administration of 2 mL of the medium (containing 554 μ mol L-Cit/chick); 2) chronic oral administration of 2 mL solution containing an equimolar amount of crystallized L-Cit/chick; 3) chronic oral administration of 2 mL water/chick (control group). The chicks were orally administered in this manner every day from 7 to 13 days. Rectal and body surface temperatures were recorded daily immediately before the oral administration and 60 min after the oral administration during the period of the experiment. The chicks were euthanized following anesthesia with isoflurane (Mylan Inc., Tokyo, Japan) after completion of the experiments.

Measurement of Body Temperature

The rectal temperature of the chicks was measured using a digital thermometer with an accuracy of $\pm 0.1^\circ\text{C}$ (Thermalert TH-5, Physitemp Instruments Inc., Clifton, NJ, USA). The body surface temperature was measured on the earlobe of each chick using a Thermo Shot F30S camera (NEC Avio Infrared Technologies Co., Ltd, Tokyo, Japan) as shown in Fig. 1. The method of measuring surface temperature has been described elsewhere (Giloh *et al.*, 2012).

Statistical Analysis

Body temperature data were analyzed using a repeated-measure two-way analysis of variance (ANOVA), and the Tukey-Kramer test was performed as a post-hoc test. $P < 0.05$ indicated statistically significant differences. Values were presented as means \pm S.E.M. Statistical analysis was performed using the commercially available package Stat View (version 5, SAS Institute, Cary, USA 1998).

Results

Changes in Body Temperature Following Acute Oral Administration of the Medium in Chicks

Changes in the rectal and surface temperatures of the chicks after acute oral administration of the medium containing L-Cit or an equimolar amount of crystallized L-Cit are shown in Fig. 2. The medium significantly ($P < 0.05$) reduced both rectal and surface temperatures in chicks under CT. However, oral administration of crystallized L-Cit at the equimolar dose (554 μ mol/2 mL) did not affect the body temperature of the chicks. Time after administration was also significant ($P < 0.05$) in terms of the reduction in rectal temperature (Fig. 2a). Treatment and time interacted significantly ($P < 0.05$) in the reduction of rectal (Fig. 2a) and surface temperatures (Fig. 2b), with pronounced reduction in body temperature occurring 60 min after administration of the medium.

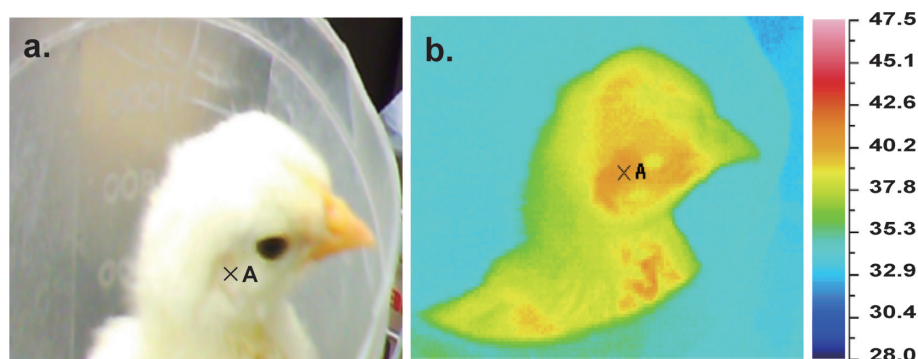


Fig. 1. Measurement of body surface temperature using a normal camera (a) and a thermal camera (b). X A indicates the point of the earlobe where measurement of the surface temperature was performed. The temperature range was determined by the color palette as shown in panel (b).

Changes in Body Temperature Following Chronic Oral Administration of the Medium in Chicks

Figure 3 shows the changes in body temperature of chicks after chronic oral administration of the medium containing L-Cit or an equimolar amount of crystallized L-Cit. Chronic oral administration of the medium, but not of the equimolar amount of crystallized L-Cit, significantly ($P < 0.05$) reduced both the rectal (Fig. 3a) and surface temperature (Fig. 3b) of the chicks. Time significantly affected the reduction in rectal temperature ($P < 0.05$).

Discussion

The objective of the current study was to examine whether oral administration of a medium containing L-Cit-producing live bacteria can reduce the body temperature of chicks under CT. Interestingly, we observed that both acute and chronic oral administration of a medium containing L-Cit-producing live bacteria reduced the body temperature of chicks. However, oral administration of an equimolar amount of crystallized L-Cit alone was not able to reduce the body temperature of the chicks. Chowdhury *et al.* (2015) reported that oral administration of 15 mmol/kg body weight, but not of 3.75 or 7.5 mmol L-Cit /kg body weight, reduced the body temperature of 6-day-old chicks. In the present study, the 7-day-old chicks (average body weight 65 g) were administered L-Cit at a dose of 8.52 mmol/kg body weight/day/2 mL and the 13-day-old chicks (average body weight 125 g) were administered L-Cit at a dose of 4.43 mmol/kg body weight/day/2 mL when calculated based on a basal concentration of 277 mM L-Cit in the medium. Therefore, the amount of L-Cit administered to the chicks in this study was lower than the effective dose (15 mmol/kg body weight) required for reducing body temperature as described elsewhere (Chowdhury *et al.*, 2015, 2017). The involvement of several factors should be considered for understanding the hypothermic effect of the treated medium in the present study. First, lactic acid, milk peptide, and other amino acids, the other components of the medium, may contribute to the induction of

the main effect of the medium. Second, the survival rate of live bacteria might reduce with the time of preservation and the number of dead and/or damaged bacteria might increase. Dead bacteria have been reported to exert bioactive function in the digestive tract of the host (Yuen and Ausubel, 2018). However, the results of the present study and those of our previous study on the effect of oral administration of a medium containing D-Asp-producing live bacteria (Do *et al.*, 2017) confirm that the introduction of L-Cit-producing live bacteria mainly induced the hypothermic effect of the medium. Further studies should clarify the thermoregulation effect of the medium with and without the presence of other subcomponents and the effect of dead and/or damaged bacteria on the body temperature of neonatal chicks.

Heat loss is a process via which heat is transferred from the body to the surrounding environment. In humans, the sweat glands of the skin are triggered to produce additional moisture to remove heat as a vapor from the surface of the body when the core body temperature increases (Wilmore *et al.*, 2008). However, chicks cannot dissipate heat via cutaneous evaporation as they lack sweat glands (Marder and Arad., 1989). Therefore, other means of reducing surface temperature is critical for maintaining body temperature homeostasis under heat stress. In the current study, both the core body temperature (rectal temperature) and surface temperature were reduced by the administration of the medium under CT. Reports show that chronic oral administration of a medium containing D-Asp-producing live bacteria has a hypothermic effect in chicks under heat stress (Do *et al.*, 2017). Whether oral administration of the medium containing L-Cit-producing live bacteria can provide thermotolerance in young chicks under heat stress should be investigated in future.

Experiment 1 shows that acute oral administration of the medium containing L-Cit-producing live bacteria reduced the body temperature of chicks. Therefore, L-Cit-producing live bacteria are immediately effective in reducing body temperature. The reduction in body temperature was pronounced 60

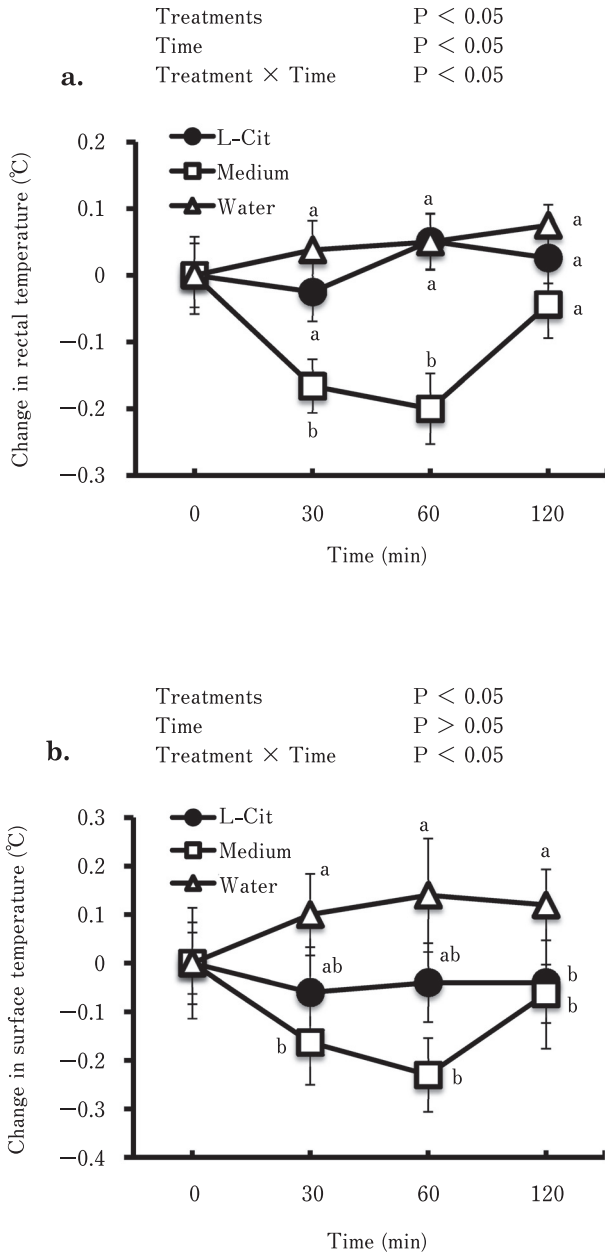


Fig. 2. Effects of acute oral administration of the medium containing L-Cit-producing live bacteria on rectal temperature (a) and body surface temperature (b) of chicks. The number of chicks used in each group ranged between 8 and 9. Data are expressed as means ± S.E.M. L-Cit, L-citrulline.

min after acute oral administration of the medium, and the effect diminished 120 min after administration. Hence, changes in plasma L-Cit level should be determined in chicks following acute oral administration of the medium compared to that of the control group in future.

Synthesis of endogenous L-Cit, together with the production of NO, has been demonstrated to be a modulator of

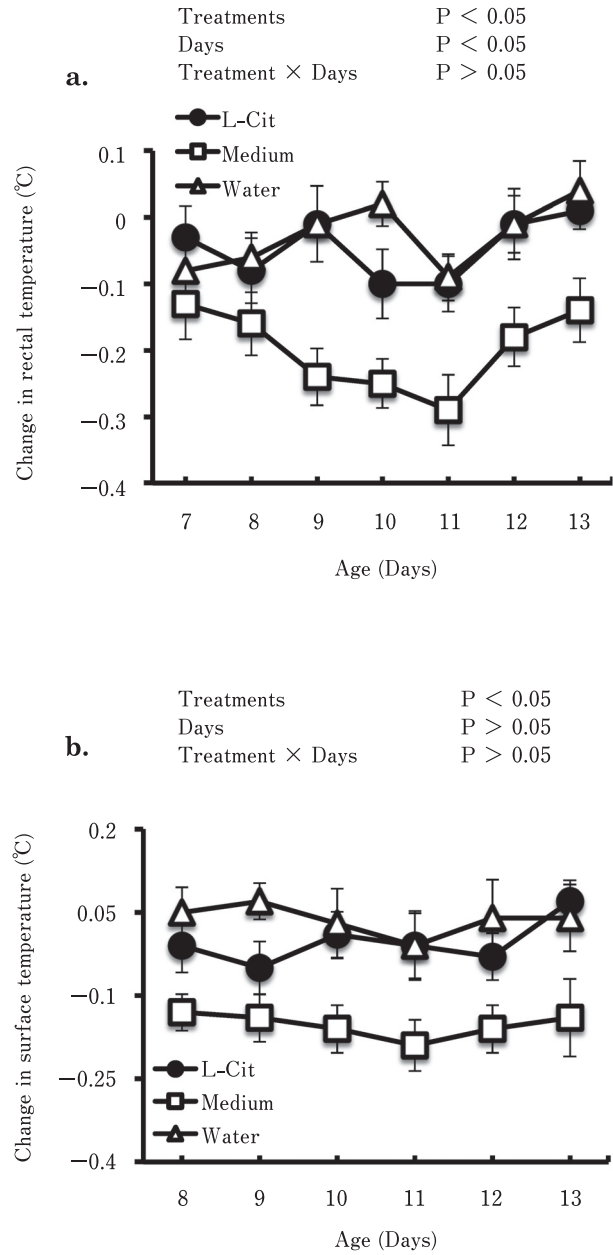


Fig. 3. Effects of chronic oral administration of the medium containing L-Cit-producing live bacteria on rectal temperature (a) and body surface temperature (b) of chicks. The number of chicks used in each group ranged between 8 and 9. Data are expressed as means ± S.E.M. L-Cit, L-citrulline.

various behaviors, including thermoregulation (De Luca *et al.*, 1995; Szabo, 1996). However, L-Cit-dependent hypothermia has been reported to be related to hypoglycemia rather than to plasma NO level in chicks (Chowdhury *et al.*, 2017). Reduction in plasma glucose level results in decrease in body temperature in mammals (Branco, 1997). Studies investigating the relationship between hypoglycemia and the

hypothermic action of the medium might be useful in this regard.

Nevertheless, chicks can adjust to environmental conditions via a process of non-shivering thermogenesis, which involves the function of mitochondrial uncoupling protein (avUCP) and adenine nucleotide translocator (avANT) (Mozo *et al.*, 2005). Importantly, reduction in body temperature following oral administration of a medium containing D-Asp-producing live bacteria has been observed, together with downregulation of avUCP and upregulation of avANT in the skeletal muscle of the medium-treated chicks (Do *et al.*, 2017). Therefore, avUCP or avANT might have been involved in reducing body temperature in the present study. The involvement of genes associated with mitochondrial biogenesis in the reduction of body temperature following oral administration of medium containing L-Cit-producing live bacteria should be investigated in future.

In conclusion, acute and chronic oral administration of a medium containing L-Cit-producing live bacteria exerted hypothermic effect in young chicks. This medium may be a potential novel feed supplement for inducing hypothermia.

Acknowledgment

This work was partially supported by JSPS KAKENHI Grant Numbers JP15K07694 and JP18K19271 to VSC as well as JP17H01503 to MF.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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