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Oral Administration of Watermelon Rind Extract to Induce Hypothermia in Chicks

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Oral administration of L-citrulline (L-Cit) caused hypothermia, but L-Cit is not recommended in poultry diets in Japan. Watermelon is a natural source of L-Cit. The objective of this study is to examine the effect of watermelon waste, i.e., watermelon rind (WR) on the body temperature and plasma free amino acids of chicks. In Experiment 1, 14-day-old chicks were subjected to acute oral administration of WR extract (WRE) (2 m*l*) under control thermoneutral temperature (CT). In Experiment 2, 15-day-old chicks were orally administered 1.6 m*l* of either WRE, low-dose L-Cit (7.5 mmol/10 m*l*), or high-dose L-Cit (15 mmol/10 m*l*) under CT. In both experiments, rectal temperature (RT) and plasma free amino acids were analyzed. In Experiment 3, after dual oral administration of (1.6 m*l*) WRE or L-Cit (15 mmol/10 m*l*), 15-day-old chicks were exposed to high ambient temperature (HT; 35±1℃, 2 h) to monitor changes in RT. Acute oral administration of WRE significantly reduced RT under CT. The degree of RT reduction by WRE was similar to that by high L-Cit. Moreover, RT was significantly low at HT owing to the oral administration of WRE. However, the reduced RT was difficult to explain by the content of Cit in WRE alone. In conclusion, WRE could be used as a dietary ingredient to reduce body temperature for imparting thermotolerance in chicks.

Key words: body temperature, chicks, plasma free amino acids, watermelon rind extract

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Introduction

Heat stress induced by high ambient temperature is a serious concern in poultry farming. Heat stress increases body temperature and induces heat stress responses in chicks (Chowdhury *et al*., 2012; Ito *et al*., 2014). High ambient temperature can reduce food intake, food efficiency, and body weight gain in broilers (Howlider and Rose, 1987; Azad *et al*., 2010). Additionally, several free amino acids, including L-citrulline (L-Cit), were found to decline in the plasma of heat-exposed chicks (Chowdhury *et al*., 2014). Recently, it has been further found that oral administration of L-Cit can lower the body temperature of chicks (Chowdhury *et al*., 2015) and impart them with thermotolerance (Chowdhury *et al*., 2017). However, the use of synthetic L-Cit in poultry rations is still not approved in Japan (Food and Agricultural Materials Inspection Center, Japan, 1953).

Watermelon is a rich natural source of L-Cit, and interestingly, watermelon rind (WR), an agricultural waste product, contains a greater amount of L-Cit than its flesh (Rimando and Perkins-Veazie 2005; Tarazona-Díaz *et al*., 2011). In our recent study, dried WR powder was fed as a diet supplement to 3- to 15-day-old chicks to examine its effect on their rectal temperature (RT) and food intake. Although RT did not significantly change under control thermoneutral temperature (CT; 30±1℃), food intake and

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plasma L-Cit increased significantly (Nguyen *et al*., 2019). We attributed these effects to the fact that WR powder contains fiber that dilute the L-Cit concentration in the WR powder. In this study, therefore, we collected the juice of WR, i.e., WR extract (WRE), and examined its effect on the RT and plasma free amino acids of chicks orally administered WRE.

Materials and Methods

Animals

One-day-old male layer chicks (Julia strain; *Gallus gallus domesticus*) were obtained from a local hatchery (Murata Hatchery, Fukuoka, Japan) and housed together in metal cages (50×35×33 cm) in a group (14 birds) at a constant temperature of 30±1℃ with continuous light illumination. Food (adjust diets: metabolizable energy > 12.55 MJ/kg, protein > 23%; Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were provided *ad libitum* during the experimental period. This study was performed in accordance with the guidelines of animal experiments and carried out in the Faculty of Agriculture, Kyushu University, and it adhered to Law No. 105 and Notification No. 6 of the Japanese government.

Preparation of WRE

Fresh watermelon was procured from Suika-no-Meisan (Kumamoto, Japan). The rind was separated from the flesh, and its juice was extracted with a commercial juicer (MJ-H600 T; Panasonic, Kadoma, Japan). The juice was passed through four-layer gauze (FC Gauze, China), filtered, and dried in an oven (Matsui MFG Co., Ltd., Osaka, Japan) at 60℃ for 120 h. After complete drying, the WRE was stored in airtight plastic bags at room temperature until use.

Experimental Design

We gradually isolated (1st day, 2 chicks/cage; 2nd day, 1 chick/cage; and 3rd day, adaptation) the chicks individually before the experiment to reduce isolation stress. In Experiment 1, 14-day-old chicks were orally administered a single dose (2 m*l*) of either double-distilled water (DDW) or WRE prepared by dissolving dried WRE in DDW at a ratio of 1:2 (wt/wt). In Experiment 2, 15-day-old chicks were orally administered 1.6 m*l* of the following: 1) control (0.25 % methylcellulose (MC)); 2) WRE prepared by dissolving WRE in MC solution at a ratio of 1:2 (wt/wt); 3) low-dose L-Cit (7.5 mmol L-Cit/10 m*l*); and 4) high-dose L-Cit (15 mmol L-Cit/10 m*l*). Experiment 3 was conducted to examine the effect of WRE and L-Cit (15 mmol L-Cit /10 m*l*) on the thermotolerance of chicks under heat stress condition (35±1℃) (HT). After the first oral administration of WRE or L-Cit, chicks were allowed to remain at room temperature for 60 min. This was followed by the second administration, and the chicks were then kept at room temperature for 30 min or in another HT chamber (MIR-254, Panasonic, Japan) for 120 min. In Experiments 1 and 2, plasma was collected for analysis of free amino acids. RT was measured with a digital thermometer with an accuracy of±0.1℃ (Thermalert TH-5; Physitemp Instruments Inc., USA) by inserting the thermistor probe into the rectum through the cloaca to a depth of approximately 2 cm from the anus, at 0, 30, 60, and 120 min after oral administration of the treatment. In Experiment 3, RT was measured at −90, −30, 0, 60, and 120 min.

Analysis of Free Amino Acids

Because the system used for amino acid analysis could not separate the L- and D-forms of amino acids, the results of the amino acid analysis described only the name of the amino acids.

Free Amino Acids in WRE

Free amino acid concentrations in WRE were analyzed by high-performance liquid chromatography (HPLC) based on the method of Boogers *et al*. (2008) and Furudate and Meguro (2002) with some modifications. WRE (40 mg) was homogenized in 200 μl of 99% ethanol for 30 s. After that, the WRE sample was stored for 30 min at room temperature. The homogenized WRE was then filtered through a 70-mm filter paper (Advantec; Toyo Roshi Kaisha, Ltd., Japan). The remaining WRE homogenate in the centrifuge tube was washed with 1200 μl of 80% ethanol and filtered as mentioned above. Next, the filtrate was dried under reduced pressure at −100 kPa (Centrifugal Vaporizer, CVE-200D, Eyela, Japan). The dried residue was dissolved in 400 μl of DDW, and then filtered again through a 0.20-μm filter (Millipore, Bedford, MA, USA). Twenty μl of the supernatants were adjusted to pH=7 with 1 M sodium hydroxide. Next, the WRE sample and 10 μl standard were dried under reduced pressure. The dried residues were dissolved in 10 μl of 1 M sodium acetate-methanol-triethylamine (2:2:1), dried under reduced pressure, and then converted to their phenylthiocarbamoyl derivatives by dissolving in 20 μl of methanol-distilled water-triethylamine-phenylisothiocyanate (7:1:1:1) and allowed to react for 20 min at room temperature. The sample and standard solutions were dried again and dissolved in 200 μl of Pico-Tag Diluent (Waters, Milford, CT, USA). These diluted samples were filtered through a 0.20-μm filter (Millipore). The same procedure was carried out on the standard solution, which was prepared by diluting a commercially available L-amino acid solution (type ANII, type B, L-asparagine (L-Asn), L-glutamine (L-Gln), and L-tryptophan; Wako, Osaka, Japan) in distilled water. The solution containing the derivatives was applied to a Waters HPLC system. They were equilibrated with buffer A (70 mM sodium acetate adjusted to pH 6.45 with 10% acetic acid-acetonitrile, ratio 975:25) and eluted with a linear gradient of buffer B (water-acetonitrile-methanol (40:45:15) 0, 3, 6, 9, 40, and 100%) at a flow rate of 1 m*l*/min at 46℃. The concentrations of free amino acids and dipeptides were determined by the absorbance at a wavelength of 254 nm. The concentrations of WRE amino acids are expressed as pmol/mg.

Free Amino Acids in Plasma

Amino acid concentrations in plasma samples were analyzed by HPLC. The concentrations of free amino acids were analyzed according to the method of Boogers *et al*. (2008) with some modifications. Plasma was deproteinized by filtration through a 10,000 Da molecular weight cut-off filter (Millipore) via centrifugation at 12,000×g for 10 min at 4℃ (MX-307; Tommy, Tokyo, Japan). Ten μl of plasma

samples and standard solution were dried under reduced pressure. The dried residues were dissolved in 10 μl of 1 M sodium acetate-methanol-triethylamine (2:2:1) and then analyzed by HPLC as described above for analysis of WRE. Plasma amino acid concentrations are presented as pmol/μl.

Statistical Analysis

Plasma free amino acids were analyzed by Student's *t*-tests in Experiment 1 and by one-way ANOVA followed by Tukey-Kramer *post-hoc* test in Experiment 2. Changes in RT were analyzed by two-way ANOVA. When a significant interaction was detected, the *t*-test and Tukey-Kramer test were applied as a *post-hoc* test at each time point in Experiment 1 and in Experiments 2 and 3, respectively. Statistical analyses were performed using the Stat View Version 5.0 software (SAS Institute, Cary, NC, USA, 1998). Values are presented as mean±S.E.M.

Results

Free amino acid contents in WRE are shown in Table 1. Cit was the most abundant free amino acid present in WRE (6638 pmol/mg). In addition, the second most abundant free amino acid was arginine (Arg) at 1175 pmol/mg. The concentrations of other amino acids (β-alanine, valine, isoleucine, proline (Pro), gamma-aminobutyric acid (GABA), alanine (Ala), Asn, Gln, leucine, aspartic acid (Asp), and glycine (Gly)) were lower than 1000 pmol/mg.

Experiment 1. Effects of Single Oral Administration of 2 ml of WRE or DDW on RT and Plasma Free Amino Acids in 14-day-old Chicks

Changes in RT following oral administration of WRE are shown in Fig. 1. Initial RT at 0 min in the control and WRE groups was 41.1±0.2℃ and 41.1±0.2℃, respectively. WRE decreased the RT significantly at 30 and 60 min ($P<0.05$) following administration. Table 2 shows the effect of WRE on plasma free amino acids. Only the concentration of Cit increased significantly ($P<0.001$), whereas the concentrations of all other free amino acids (Asn, Ala, Gln, Gly, histidine, Pro, serine (Ser), and tyrosine) decreased significantly ($P<0.05$, $P<0.01$ or $P<0.001$).

Table 1. **Free amino acid contents in watermelon rind extract**

Amino acids	Content
Citrulline	6638±571
Arginine	1175±57
Valine	692±31
Isoleucine	582±55
Proline	486±55
GABA	410±33
Alanine	329±16
Asparagine	271±12
Leucine	236±25
Aspartic acid	229±16
Glycine	204±15
Glutamine	193±14

Thirty-three samples were analyzed. GABA, gamma-aminobutyric acid. Values are mean± S.E.M. in pmol/mg.

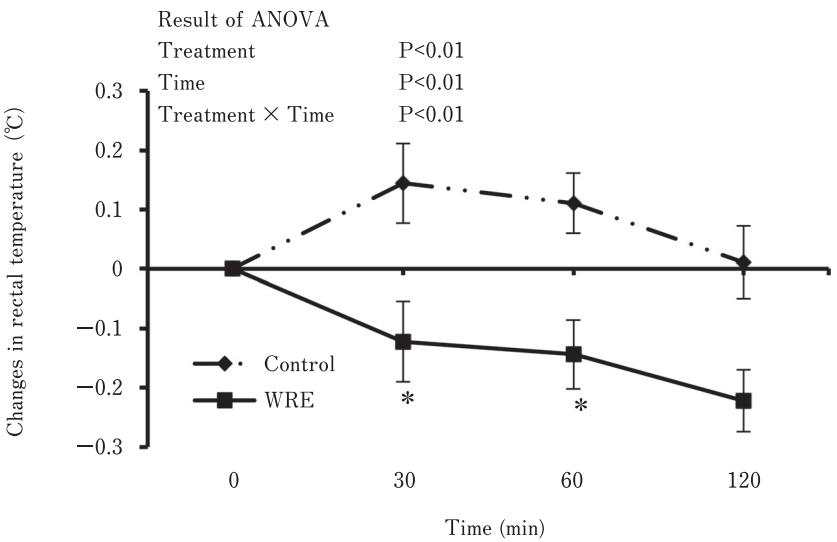

Fig. 1. **Effects of single oral administration of 2 m*l* of WRE on changes in rectal temperature during 120 min in 14-day-old chicks.** The number of chicks used in each group was 9. Values are mean± S.E.M. *, $P<0.05$. WRE, watermelon rind extract was prepared by dissolving dried watermelon rind juice in distilled deionized water at a ratio of 1:2 (wt/wt).

Table 2. **Effect of oral administration (2 m*l*) of WRE on plasma free amino acids in 14-day-old chicks**

Amino acid	Control	WRE	P value
Citrulline	52±6	231±18	$P<0.001$
Alanine	407±25	302±17	$P<0.01$
Asparagine	213±14	121±6	$P<0.001$
Glycine	234±15	180±11	$P<0.05$
Glutamine	875±44	533±10	$P<0.001$
Histidine	143±10	111±8	$P<0.05$
Proline	511±40	322±18	$P<0.001$
Serine	381±13	262±11	$P<0.001$
Tyrosine	209±13	159±9	$P<0.01$

The number of samples in each group was eight to nine. WRE, watermelon rind extract dissolved in deionized distilled water at a ratio of 1:2 (wt/wt). Values are mean±S.E.M. in pmol/μl.

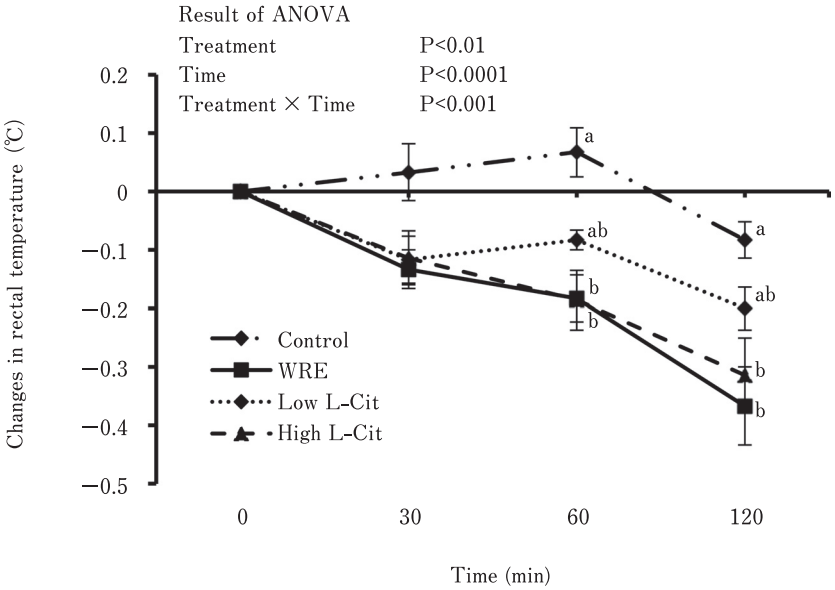

Fig. 2. **Effects of single oral administration of 1.6 m*l* of WRE, low-dose L-Cit (7.5 mmol L-Cit/10 m*l*), or high-dose L-Cit (15 mmol L-Cit/10 m*l*) on changes in rectal temperature during 120 min in 15-day-old chicks.** The number of chicks used in each group was 7. Values are mean±S.E.M., with different letters at each time point indicating significant difference at $P<0.05$. Control, 0.25% methylcellulose solution; WRE, watermelon rind extract was dissolved in 0.25% methylcellulose solution at a ratio of 1:2 (wt/wt).

Experiment 2. Effects of Single Oral Administration of 1.6 ml of WRE, or Low- or High-dose L-Cit on RT and Plasma Free Amino Acids Under CT in 15-day-old Chicks

Fig. 2 shows changes in RT after oral administration of either WRE, low-dose L-Cit, or high-dose L-Cit. RT at the beginning of experiment in the control, WRE, low-dose L-Cit, and high-dose L-Cit groups was 41.3±0.1℃, 41.3±0.1℃, 41.2±0.1℃, and 41.2±0.2℃, respectively. There was a significant ($P<0.01$) decline in RT after oral administration of WRE, low-dose L-Cit, and high-dose L-Cit. We observed significant effects of time ($P<0.0001$) and significant interaction between treatment and time ($P<0.001$), indicating that WRE and high-dose L-Cit consistently reduced RT as the experimental time progressed. Table 3 reveals the effect of orally administered WRE, low-dose L-Cit, and high-dose L-Cit on free amino acids. L-Cit significantly ($P<0.001$) increased the concentrations of Cit, Arg, ornithine (Orn), methionine, Ser, taurine, Asp, and GABA compared to those in the control and WRE groups. In contrast, WRE significantly ($P<0.01$ or $P<0.001$) decreased the amounts of almost all amino acids, including Cit, compared to those in the control and L-Cit groups.

Table 3. **Effects of oral administration of 1.6 m*l* of WRE, low L-Cit, or high L-Cit on plasma free amino acid concentrations in 15-day-old chicks**

Amino acid	Control	WRE	Low L-Cit	High L-Cit	P value
Citrulline	347±33[a]	263±40[a]	450±48[b]	562±54[b]	$P<0.001$
Alanine	130±4[a]	96±10[b]	141±3[a]	141±11[a]	$P<0.01$
Arginine	344±49[a,b]	283±35[b]	492±34[a,c]	530±46[c]	$P<0.001$
Asparagine	228±12[a]	141±6[b]	227±14[a]	200±21[a]	$P<0.001$
Aspartic acid	33.0±2[a,b]	26.6±3[a]	37.1±2[b]	40.9±4[b]	$P<0.01$
GABA	20±1[a]	71±4[a]	2280±75[c]	4629±427[b]	$P<0.001$
Glutamine	848±36[a]	506±21[b]	734±31[a,c]	655±41[c]	$P<0.001$
Glycine	248±16[a]	173±8[b]	270±15[a]	282±27[a]	$P<0.01$
Histidine	107±8[a]	66±3[b]	103±6[a]	89±6[a,b]	$P<0.001$
Methionine	105±12[a]	67±4[a]	273±11[c]	471±55[b]	$P<0.001$
Ornithine	57±7[a]	367±37[a]	124±7[b]	133±15[b]	$P<0.001$
Proline	613±38[a]	357±33[b]	563±13[a]	549±62[a]	$P<0.001$
Serine	487±24[a]	325±22[b]	497±33[a]	455±22[a]	$P<0.001$
Taurine	55±8[a]	61±7[a]	93±15[a,b]	133±18[b]	$P<0.01$
Threonine	668±33[a]	513±28[b]	662±13[a]	701±561[a]	$P<0.01$

Different superscripts indicate mean values that are significantly different at $P<0.05$. WRE, watermelon rind extract dissolved in a 0.25% methylcellulose solution at a ratio of 1:2 (wt/wt). Low L-Cit, 7.5 mmol/10 m*l*. High L-Cit, 15 mmol/10 m*l*. The number of samples used for analysis was 6 to 7. Values are mean±S.E.M. in pmol/μ*l*.

Experiment 3: Dual Oral Administration of 1.6 ml of WRE and L-Cit Altered RT in 15-day-old Chicks Under CT and HT

Fig. 3 shows changes in RT in response to WRE under CT and HT. RT at the start of experiment in the control, WRE, and L-Cit groups was 41.3±0.2℃, 41.3±0.3℃, and 41.2±0.1℃, respectively. WRE significantly ($P<0.001$) reduced RT; significant ($P<0.0001$) effect of time and significant interaction between treatment and time ($P<0.05$) was observed, which indicated that the effect of treatment progressed with time, although RT is dependent on the environmental temperature.

Discussion

In WRE, Cit content was higher than that of other free amino acids, in accordance with the reports by Rimando and Perkins-Veazie (2005) and Tarazona-Díaz *et al*. (2011). In a previous study, we found that WRE and WR powder contain Cit at 8.61 nmol/mg (1.51 mg/g) and 6.64 nmol/mg (1.12 mg/g), respectively. Other studies have reported higher concentration of Cit in WR than that reported by us; however, these previous studies analyzed other parts of watermelon and processed the watermelon differently. For instance, Rimando and Perkins-Veazie (2005) reported 24.7 mg Cit/g dry weight for WR processed by freeze-drying. Tarazona-Díaz *et al*. (2013) found 2.33 g Cit/l in fresh flesh juice after pasteurization at 80℃ for 40 s followed by immediate cooling on ice to 8℃ until use. Furthermore, the species and growth stages of the watermelon used may be different among the different studies, and it was difficult to determine these aspects based on the information reported in the papers.

The present study was based on our previous study of WR powder in chick diet (Nguyen *et al*., 2019) and WR juice administration (unpublished data). However, RT was not found to change significantly during the period. We assumed that because WR powder contained high fiber content of 14.5% and WR powder mash diet contained 0.002 mmol of Cit/mg of food, chicks consumed 0.03 and 0.04 mmol of L-Cit when they were 6- and 15-day-old, respectively (Nguyen *et al*., 2019). Further, a 2-m*l* dose of WR juice was calculated to contain high water content of 82.8−88.2% (Pansy and Thin, 2011) and low Cit content of 0.002 mmol. In addition, an effective L-Cit dose of 1.03 mmol has been reported to reduce RT in 6-day-old chicks upon acute oral administration (Chowdhury *et al*., 2015). Thus, in the present study, WRE was used owing to its low fiber content and high Cit concentration for examining the effect of natural Cit on the chick body.

In Experiment 1, RT decreased significantly ($P<0.05$) after oral administration of 2 m*l* of WRE. Oral administration of 2 m*l* was selected in accordance with a previous study (Do *et al*., 2017). Although 2 m*l* of WRE contained 0.004 mmol of L-Cit, which is lower than the effective L-Cit dose of 1.03 mmol (Chowdhury *et al*., 2015), administration of pure synthetic L-Cit (Chowdhury *et al*., 2015) and concomitant administration of Cit with other ingredients in the present study may have different effect on RT. In fact, acute or chronic administration of a medium containing L-Cit-producing live bacteria reduced RT and surface body temperature in chicks even though the L-Cit level was low (Tran *et al*., 2019). WRE contains several free amino acids, as shown in Table 1. These amino acids may interact with each other to decrease RT. The significant increase in plasma Cit concentration ($P<0.05$) after administration of 2 m*l* WRE may be caused by the presence of Cit in WRE. Other free amino acids were also reduced significantly ($P<0.05$) after oral administration of WRE. Under short-term

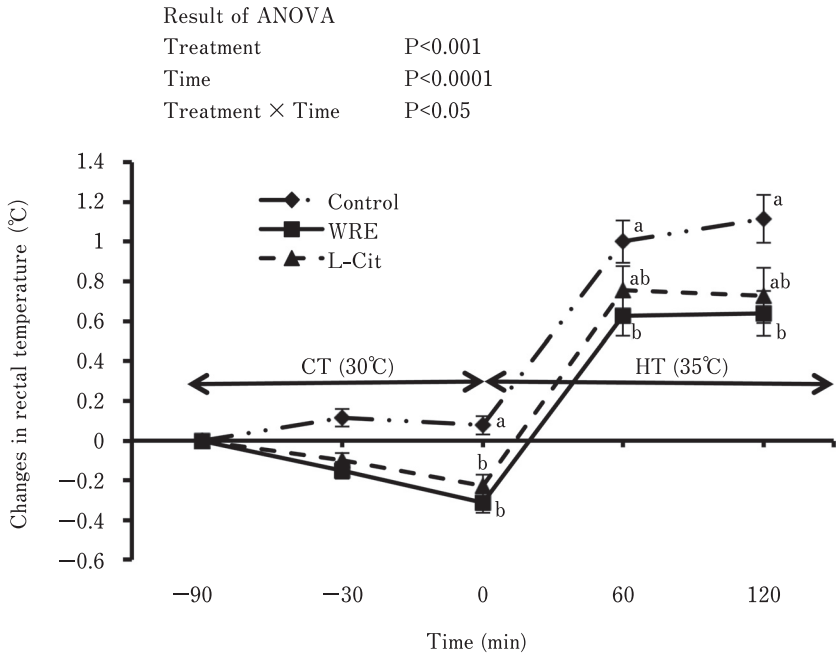

Fig. 3. **Effects of dual oral administrations of 1.6 m*l* of WRE or L-Cit (15 mmol L-Cit/10 m*l*) on changes in the rectal temperature of 15-day-old chicks following oral administration under control and heat stress.** The number of chicks used in each group was 7−16. Values are mean±S.E.M., with different letters at each time point indicating significant difference at $P<0.05$ at the same time point. WRE, watermelon rind extract was dissolved in 0.25% methylcellulose solution at a ratio of 1:2 (wt/wt).

heat stress, RT increases as the blood concentrations of several free amino acids increase (Ito *et al*., 2014). Conversely, some free amino acids in the brain of neonatal chicks subjected to either restraint with isolation-induced or fasting stress were found to decrease (Hamasu *et al*., 2009). In these cases, plasma amino acids are correlated with brain amino acids (Hamasu *et al*., unpublished data).

In Experiment 2, there was a similar effect of decreasing RT due to both WRE and high L-Cit under CT (Fig. 2). Because it is difficult to dissolve synthetic L-Cit in water, MC was used to prepare the suspension. Thus, MC was used as a control and for dissolving WRE in this experiment. High-dose L-Cit (15 mmol/10 m*l*) was used as an effective dose for regulating RT in chicks, in accordance with Chowdhury *et al*. (2015). The 1.6 m*l* dose was based on 10 m*l*/kg body weight of 15-day-old chicks. This L-Cit dose acted as a potential hypothermic agent to impart thermotolerance in the chicks (Chowdhury *et al*., 2017). Although L-Cit concentration in the high-dose L-Cit group (2.40 mmol) was approximately 470-fold higher than that in the WRE group (0.004 mmol), plasma Cit level in the WRE group was nearly half that in the high-dose L-Cit group (263±40 and 562±54 pmol/μl, respectively). Both groups showed the same effect in reducing RT even at 120 min after oral administration, implying that the nutrients in WRE and the high-dose L-Cit could be easily absorbed and were active, as mentioned above. In contrast, changes in plasma free amino acids after WRE injection showed an opposite trend to those after high-dose L-Cit injection (Table 3). High-dose L-Cit significantly ($P<0.05$) increased plasma Cit concentration and led to a significant increase in the concentrations of Arg and Orn due to the urea cycle; Cit can bypass the liver and then be converted to Arg in the kidney (Windmueller and Spaeth, 1981). Birds lack carbamoyl phosphate synthetase, an enzyme of the urea cycle necessary for synthesizing L-Cit from L-Orn in the liver and kidney (Tamir and Ratner 1963). Therefore, birds cannot synthesize Cit or Arg, but can synthesize Orn from Arg (Suenaga *et al*., 2008). Additionally, oral administration of high-dose L-Cit may not induce toxic effects in chicks (Chowdhury *et al*., 2017). The content of other free amino acids would significantly increase owing to high L-Cit dose. Conversely, there was a significant decrease in the concentration of almost all plasma free amino acids in the WRE group, compared to that in the L-Cit and control groups. Moreover, Cit content in plasma did not significantly increase in the WRE groups compared to that in the control group, which may be partially due to the lower L-Cit in the 1.6 m*l* dose.

In Experiment 3, we investigated the effect of dual oral administration of WRE and L-Cit on the RT of chicks under HT after its thermotolerance effect was shown in normal condition. Under stress conditions, dual oral administration

of WRE significantly declined RT. Before this experiment, we examined the effect of single oral administration of WRE and L-Cit under HT, and did not observe any change in RT (result not shown). Chowdhury *et al*. (2017) found that dual, but not single, oral administration of L-Cit resulted in thermotolerance; thus, the chicks in this study were subjected to dual oral administration of WRE and L-Cit before being exposed to HT. Although Cit concentration in the WRE group was lower than that in the L-Cit group, RT declined significantly ($P<0.05$) in the WRE group compared to that in the control group. These data suggested that unrevealed substances in WRE may be easily absorbed, and then may produce a strong effect on RT.

It is suggested that thermoregulation is caused by nitric oxide (NO) (De Luca *et al*., 1995; Gourine, 1995), which is synthesized from L-Arg (Wu *et al*., 1998). L-Cit is an endogenous precursor of L-Arg (Wu *et al*., 1998) and synthesis of NO is increased by supplementation of L-Cit (Sureda *et al*., 2009). However, Chowdhury *et al*. (2017) indicated that the RT of chicks was significantly reduced under CT and HT by dual high L-Cit administration under CT or HT condition. Because plasma NO_x (NO_2+NO_3) concentration did not change significantly ($P>0.05$), NO production may not be the main factor of thermotolerance in chicks owing to L-Cit-mediated hypothermia. This implies that the thermotolerance of chicks was not influenced by production of NO following L-Cit oral supplementation. WRE decreased RT not only because of L-Cit but also because of other chemicals present in addition to the amino acids mentioned above. First, we focused on L-Cit in WRE, but the body temperature-lowering effect of WRE could not be explained by L-Cit level in WRE. Tran *et al*. (2019) also indicated that even when L-Cit was present at a low concentration in a medium containing L-Cit-producing live bacteria, the medium reduced the RT and surface body temperature of chicks upon acute oral or chronic administration. Tarazona-Díaz *et al*. (2011) found that WR contains phenolic acids. Furthermore, WR contains significantly greater amounts of chemicals with free radical-scavenging activity, such as β-carotene, 4-hydroxybenzoic acid, vanillin, and coumaric acid (Hanan *et al*., 2013). Moreover, other substances in WRE, which act as antioxidants, may collaborate to impart thermotolerance in chicks after oral administration. For instance, lycopene, which is an antioxidant, could increase the animal parameter under HT (Sivakuma *et al*., 2010). WR in the present study contained lycopene at the level of 1.1 mg/100 g.

In conclusion, WRE induced immediate hypothermia not only under CT upon single administration but also under HT condition upon dual oral administration. WRE could combine with unknown substances that facilitate/replace L-Cit synthesis and decrease body temperature in chicks by regulating plasma free amino acids. Further research is required to elucidate which unknown substances contribute to the effects of WRE in imparting heat tolerance in young chicks under HT.

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