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Research Article

Physiological responses and brain oxidative stress in heat exposed growing chickens

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ABSTRACT

This study was designed to elucidate physiological responses and time-course change in hydroxyl radical in brain of chicken exposed to high ambient temperature. Twenty one day old growing chickens were exposed to 34°C for 8 h. The extent of lipid peroxidation and plasma metabolites were investigated before and after 4 and 8 h of heat exposure. Moreover, to study hydroxyl radical (OH) production during heat stress, 4-hydroxybenzoic acid (4-HBA) was perfused as a trapping agent in the lateral ventricle (LV) of chickens. The product of the reaction of 4-HBA with OH, 3, 4-dihydroxybenzoic acid (DHBA) was collected using *in vivo* microdialysis techniques and analyzed by HPLC. Suppression of food intake was observed in heat treatment (HT) group at 3 to until 7 h. Rectal temperature was significantly increased at 2 h by acute heat exposure and the difference between the HT and CT groups was maintained at each time point until the end of experiment. The plasma MDA concentration and corticosterone were intended to increase at 4 and 8 h in HT group but no significant difference was observed. The concentration of uric acid was significantly lower in HT than CT group at 4 h and 8 h ($P<0.05$). Calcium, total cholesterol, total protein and GOT were tended to increase at 4 h and 8 h in HT group but no significant difference observed in comparison with CT group. Local perfusion of 4-HBA in chicken LV increased extracellular levels of 3, 4-DHBA in HT group compared with baseline. The significantly increased level of 3, 4-DHBA was observed at 4 h and continued to increase until the end of experiment. These results indicate that brain oxidative stress should be considered as part of the stress response of growing chickens to heat exposure.

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1. INTRODUCTION

Heat stress that places the birds above their thermo-neutral zone causes serious physiological dysfunction that may decrease animal performance. A number of studies have suggested that heat stress not only acutely but also chronically results in reduced feed intake, body weight gain, and increased mortality (Azad et al., 2010; Mustaq et al., 2005; Mujahid et. al., 2009). Hot climate can also produce what are known as reactive oxygen species (ROS) within the body. These molecules are highly reactive and can cause damage to various tissues and impair the production and reproduction performance of chickens (Mujahid et al., 2005, 2007, 2009). Superfluous ROS induced by heat stress can cause oxidative injury, such as lipid peroxidation (Mujahid et al., 2007). Malondialdehyde (MDA) is a major breakdown product of lipid peroxidases.

Moreover, high environmental temperatures also affect metabolic alterations in chickens. Earlier findings have shown that blood metabolic and hormonal changes occur in chickens due to high ambient temperature. Plasma

corticosterone concentrations are widely used as a measure of the responses of chickens to environmental stressors (Fraisse and Cockrem, 2006; Hull et al., 2007). In that connection some papers showed that heat challenge may elevate corticos-terone (Zulkifli et al., 2009; Mahmoud et al., 2004) or did not affect plasma corticosterone concentrations (Chowdhury et al., 2012).

The heat responses in poultry are mediated mainly by the activation of hypothalamic-pituitary-adrenal (HPA) axis. There has been a lot of research on brain function because the central nervous system is thought to be one of critical centers for stress responses. Ito et al., (2015) reported that hypothalamic neurons can perceive the increase in body temperature and exert an influence on cells that are responsible for controlling food intake. Although production of ROS in some tissue mitochondria of chickens has been reported to increase under high environmental temperatures, there has been no reported work evaluating ROS in the brain. Therefore, it may be interesting to examine if the heat stress responses are related to the brain ROS's production in growing chickens.

In this paper, 4-hydroxybenzoic acid (4-HBA) was perfused centrally as a trapping agent to determine the hydroxyl radical (OH) formation in the brain of chickens subjected to lateral ventricle. The product of the reaction of 4-HBA with OH, 3, 4-dihydroxybenzoic acid (DHBA) was collected using *in vivo* microdialysis techniques and analyzed using HPLC-ECD. It is believe that this is the first study using 4-HBA as an *in vivo* ROS trapping agent in conjunction with microdialysis in heat exposed egg-type growing chickens. In the present study the effect of high environmental temperatures on chicken performance and metabolic responses in growing chickens was addressed using an acute heat stress model. In addition, the functional relationships between brain ROS and the heat stress responses including food intake was investigated.

2. MATERIALS AND METHODS

2.1. Animals and diets

Newly hatched male egg-type chickens (Boris Brown) were purchased from a local hatchery (Ghen Corporation, Gifu, Japan) and reared in heated batteries that were kept in a temperature-controlled (32°C) room with a 12:12h light/dark cycle (lights on 09:00-21:00) until 6 d of age. They were given free access to a commercial starter diet (CP: 210 g/kg; ME: 12.3 MJ/kg) and *ad- libitum* access to water until the experiment. The feed was manufactured according to requirements all essential amino acids be present as recommended in the Japanese Feeding Standard for Poultry (JFSP, 2004). At 7 d of age, chickens were weighed and selected so that the average body weight was as uniform as possible and they were housed in individual cages (16 × 22 × 25 cm, W × D × H) in an experimental room at a temperature of 28°C. Each cage was equipped with an individual feeder and waterer. The experimental protocols were approved by the Animal Experimentation Committee of Utsunomiya University.

2.2. Experimental design and heat treatment

Experiment 1 studied the effect of acute high environmental temperatures on chicken performance and physiological responses in growing chickens. On each experimental day, a pair of chickens was used for a heat trial; one was the control (CT; 24°C) and the other was the heat treatment group (HT; 34°C). The HT chickens were then placed in their cages into a temperature-controlled chamber (Eyela Electric Co. Ltd., Japan), whilst CT chicks were put in their cages on similar racks. The day before the experiments, each chicken was placed in individual cages to acclimatize them. Body weights, rectal temperatures, and blood samplings were done with both groups. Food intake was individually recorded every h for 8 h from 9:30 through 17:30 for both groups. Water intake was measured as water volume remaining in the water cup. The total water intake (TWI, mL) was calculated as the difference between the initial (0 h) and final water volume (8 h). The heat treatment and blood sampling were done at 21 d of age. Chickens had free access to water and feed during exposure to HT or CT.

On the other hand, experiment 2 examined the functional relationships between brain ROS and the heat stress responses including food intake. The heat treatment procedure was same as above. Body weights and rectal temperatures were recorded initially. Each experimental day, at about 9:30 am, the microdialysis probes were inserted into the lateral ventricle via the implanted guide cannula. The temperature for the HT group was changed 34°C until the basal level of 3, 4-DHBA became stable and this did not occur until at least 3 h after probe insertion. 3, 4-DHBA is a product of reaction of ROS and 4-hydroxybenzoic acid (4-HBA), and has been proved to be useful to trap hydroxyl radical in the dialysate from the brain (Liu et al., 2002). Food intake was measured at every hour for 5 or 6 h of the heat treatment trial and the dialysate was collected simultaneously.

2.3. Body mass and rectal temperature

Body mass was recorded before starting and at 4 and 8 h after heat exposure. Meanwhile, rectal temperature of chickens were simultaneously measured using a digital thermometer with an accuracy of ±0.1°C (Thermalert TH-5, Physitemp Instruments Inc., USA), by inserting the thermistor probe in the cloaca to a depth of 2 cm. Rectal temperature were taken for both CT and HT groups at 0, 2, 4, 6, and 8 h.

2.4. Blood sampling

A total of eighteen pairs chickens were used for blood sampling. Blood samples were taken from the wing vein of each chicken at 0, 4, and 8 h and transferred into heparinized eppendorf tubes. Immediately after blood collection, plasma was obtained following centrifugation at 15,000 g for 10 min at 4°C and was stored at -20°C until the analyses were done. The plasma samples were divided into three sets in which one set was used for plasma corticosterone (n=6 in each group) while the other set was used for thiobarbituric acid reactive substance (TBARS) (n=6 in each group) determinations. On the other hand, the third set was used to measure some metabolite concentrations (n=6 in each group) in experimental chickens.

2.5. Analytical procedures with the blood plasma

The corticosterone concentrations were determined using an enzyme immunoassy kit (Assaypro LLC, Catalog No. EC3001-1) and expressed as ng per ml plasma. A TBARS assay kit (Cayman Chemical item no. 10009055) was used for assaying lipid peroxidation in the plasma. The TBARS content was expressed as nmol of MDA. The plasma metabolites (glucose, total cholesterol, total protein, glutamic oxaloacetic transaminase (GOT), uric acid, and calcium) were determined using an automatic analyzer (SPOTCHEM EZ SP-4430, Arkray, Japan).

2.6. Surgical procedures for implantation of the guide cannula

Seventeen- or 18-day-old chickens were fasted for at least 2 h before surgery. At 17 or 18 days of age, the chickens were stereotaxically implanted with a guide cannula aimed at one side of the lateral ventricle (LV). In preparation for cannulae implantation, chickens were anaesthetized with intraperitoneal administration of sodium pentobarbital (4 mg/100 g body weight). They were then placed in a stereotaxic frame (David Kopf Instruments, CA, USA) and a small incision with a scalpel was made to expose the skull. A high speed microdrill was then used to drill a hole in each chicken skull overlying the LV (LV, anterior to the interaural line by 7.4 mm; lateral to the midline by 1.0 mm, and 4.0 mm below the skull surface (Kuenzel and Masson, 1988) and the guide cannula (0.5 × 8 mm, diameter and length, respectively, Eicom, Kyoto, Japan) was lowered below the skull surface. Two additional holes were drilled in the skull for anchoring of the stainless-steel support screws (2 × 6 mm, diameter × length, respectively). The guide cannula was fixed in place with dental cement (Shofu Inc., Tokyo, Japan). A dummy cannula was then inserted to prevent obstruction. The chickens were individually housed and allowed at least 4 days for post-surgical recovery before the start of the microdialysis experiment (Khalil et al., 2010). During this period, the chickens were given *ad-libitum* feed and water.

2.7. Preparation of solutions

The composition of normal Ringer's solution was 147 mMNaCl, 4 mMKCl, and 4 mM $CaCl_2$. The 4-HBA was obtained from Sigma Chemicals (St. Louis, MO, USA). The 30 µM 4-HBA was dissolved in Ringer's solution. The 4-HBA containing Ringer's solution was then filtered through a 0.20 µm membrane filter (Dismic-25AS, Advantec, Kyoto, Japan) before perfusion into the chickens. Freshly prepared Ringer's solution was used each experimental day. HPLC-grade methanol and phosphoric acid were obtained from Wako Chemical Co. Ltd. (Osaka, Japan).

2.8. In vivo brain microdialysis experiment

The microdialysis experiment was done on 21 or 22 days of age. At about 9:30 am each day the microdialysis probes were inserted into the VMH via the implanted guide cannulae while gently restraining the fully conscious chickens. The animal was then placed individually into the microdialysis cages (cages 1 and 2). Microdialysis was carried out in accordance with a previously reported method (Tachibana et al., 2000; Alam et. al., 2012). Briefly, the probes (3 mm long, 0.22 mm wide, molecular weight cut-off of approximately 50,000 Da according to the manufacturer, Eicom, Kyoto, Japan) were inserted into the LV via the implanted guide cannulae. Using a syringe pump (EP 60, Eicom) with a 2.5ml gas-tight syringe, the probe was perfused with a Ringer's solution at a flow rate of 1 µl/min. The collection time interval for each dialysis sample was 30 min and the dialysate was collected for 8 or 9 h. During the microdialysis experiment, the chickens were given free access to water and a commercial diet.

2.9. Determination of 3, 4-DHBA usinghigh performance liquid chromatography (HPLC) with electrochemical detection (ECD)

Dialysates were collected from the freely moving chicken. Dialysate samples were analyzed using HPLC-ECD. Details of the dialysate detection by HPLC-ECD are given elsewhere (Tachibana et al., 2000; Alam et al., 2011). Briefly, 3, 4-DHBA in the dialysate was analyzed using a HPLC system with a reversed phase ion-exchange column SC-50 DS 3 x 150 mm (Eicom). The mobile phase contained 100mM phosphate buffer,

5 mg/ml Na_2EDTA, and 8% methanol at pH 2.8. This solution was pumped through the system at 0.5 ml/min using a pump (EP-300, Eicom). The 3, 4-DHBA was detected using an electrochemical detector (ECD-300, Eicom) with an applied potential of +600 mV. Chromatograms were integrated and compared with standards and analyzed using a computer-based data acquisition system (eDAQ Power Chrome 280, NSW, Australia). The concentration of 3, 4-DHBA was determined by calculating peak areas and comparing with standard solutions.

2.10. Histological verification of the position of the dialysis probe

After completion of each microdialysis experiment, chickens were deeply anaesthetized using an intra-peritoneal injection of sodium pentobarbital (8 mg/100 g b.w.) and perfused via cardiac puncture with Ringer's solution (0.9% NaCl) followed by Zamboni fixative solution (10% paraformaldehyde). Then the chickens were decapitated and their brains were fixed in the same fixative. Serial coronal sections were cut, mounted, and stained with cresyl violet and analyzed using a light microscope and features identified using the chicken brain atlas of Kuenzel and Masson (1988).

2.11. Statistical analysis

All results were expressed as mean±SEM. Data on rectal temperature and plasma metabolites were analyzed using a two-way ANOVA. To compare the results of food intake, the t-test was performed. The 3, 4-DHBA in the dialysate was expressed as a percentage of the baseline for each individual chicken. The average 3, 4-DHBA level in the three samples immediately preceding the heat treatment was defined as the baseline (100%). The dialysis data were analyzed using a two-way ANOVA with the Dunnett's test at each time point between CT and HT groups. A probability level of $P < 0.05$ was considered to be significant.

3. RESULT AND DISCUSSION

Effect of HT on body weight, rectal temperature, food and water intake in chickens

Body weight was not significantly affected ($P>0.05$) by the heat treatment in both groups. However, the rate of body weight was gradually suppressed in HT group than CT (data not shown). Rectal temperature quickly increased ($P<0.05$) at 2 h exposure to HT, and the difference between the HT and CT groups was maintained at each time point until the end of experiment (Fig. 1A). Heat treatment showed strong suppression of food intake in HT group at 3 to until 7 h (Fig. 1B). No significant difference of water intake was observed between heat exposed and control chicks (data not shown). These responses of rectal temperature and food intake are partly similar to previous studies (Chowdhury et al., 2012), which shows the temperature during heat exposure was enough to affect the body temperature and food intake.

Heat stress is of major concern in the poultry industry in particular the tropical regions of the world. Chickens have a greater challenge in maintaining body temperature during exposure to HT than other animals due to poultry lack sweat glands and relatively high body temperature, relaying on evaporative cooling to keep themselves cool (Ensminger et al., 1990). In the present study, rectal temperature was significantly increased by HT, suggesting that the experimental temperature acted on thermoregulatory mechanisms to increase sensible heat loss in the chicks.

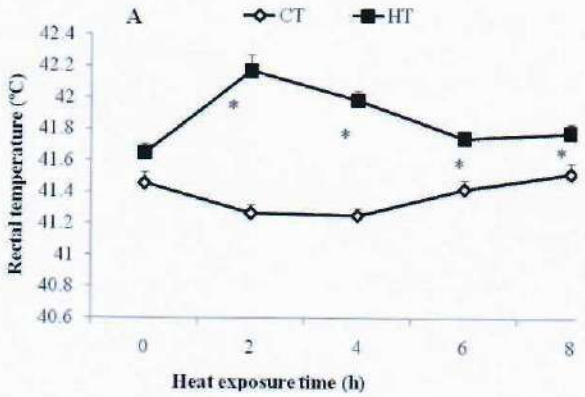


Fig. 1 (A). Rectal temperature in growing chickens exposed to a control temperature (CT; 24°C) or a high ambient temperature (HT; 34°C) for 8 h. Values are means ± SEM (n= 16 in each group). *Significantly different from the CT group ($P < 0.05$).

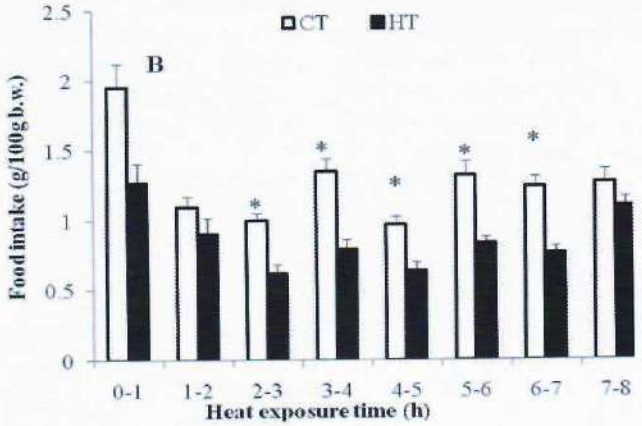
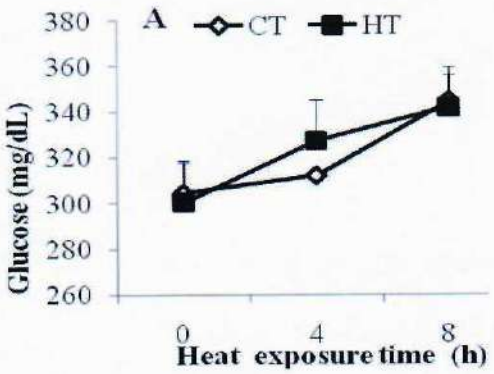
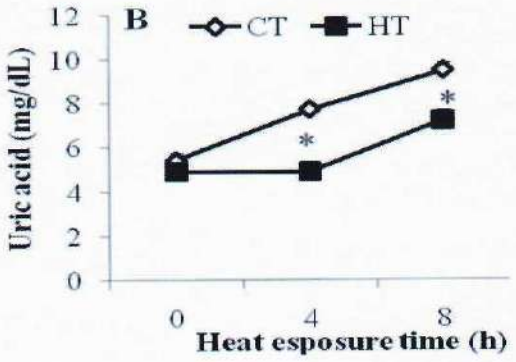


Fig. 1 (B). Food intake in growing chickens exposed to a control temperature (CT; 24°C) or a high ambient temperature (HT; 34°C) for 8 h. Values are means ± SEM (n= 16 in each group). Food intake expressed as g/100 g body weight. *Significantly different from the CT group ($P < 0.05$).

Effect of HT on plasma corticosterone, MDA and plasma metabolites

Neither plasma MDA nor corticosterone was affected by heat exposure (data not shown). Plasma metabolites except for uric acid did not differ between the control and HT groups. To investigate whether heat stress changes in plasma metabolites, the concentrations of glucose, total protein, total cholesterol, uric acid, calcium and GOT were measured in HT and CT chickens at 0, 4 and 8 h (Figure 2A, B, C, D, E, and F). The concentration of uric acid was significantly lower in HT than control group at 4 h (4.9 and 7.7 mg/dl) and 8 h (7.2 and 9.5 mg/dl) ($P<0.05$). Total cholesterol, total protein and GOT were tended to increase at 4 h and 8 h in HT group but no significant difference observed in comparison with CT group. The concentration of plasma metabolites varied with studies previously reported (Chowdhury et al., 2012, Del Vesco et al., 2014). It is reported that stress stimulates the body to release glucose and other energetic metabolite into the blood stream in physiological preparation for the fight or flight response. Denbow (1994) suggested that higher plasma glucose in the HT group may contribute to reduce food intake. However, regulation of food intake is complicated in animals including domestic birds (Furuse, 2002); several hypothalamic factors are suggested as regulating factors for food intake in chickens (Furuse, 2007). The present study observed a decreased uric acid level in plasma of chicks kept under HT compared to control birds. As body mass was not suppressed significantly there was no possibility of protein catabolism to increase plasma uric acid concentration in this study.





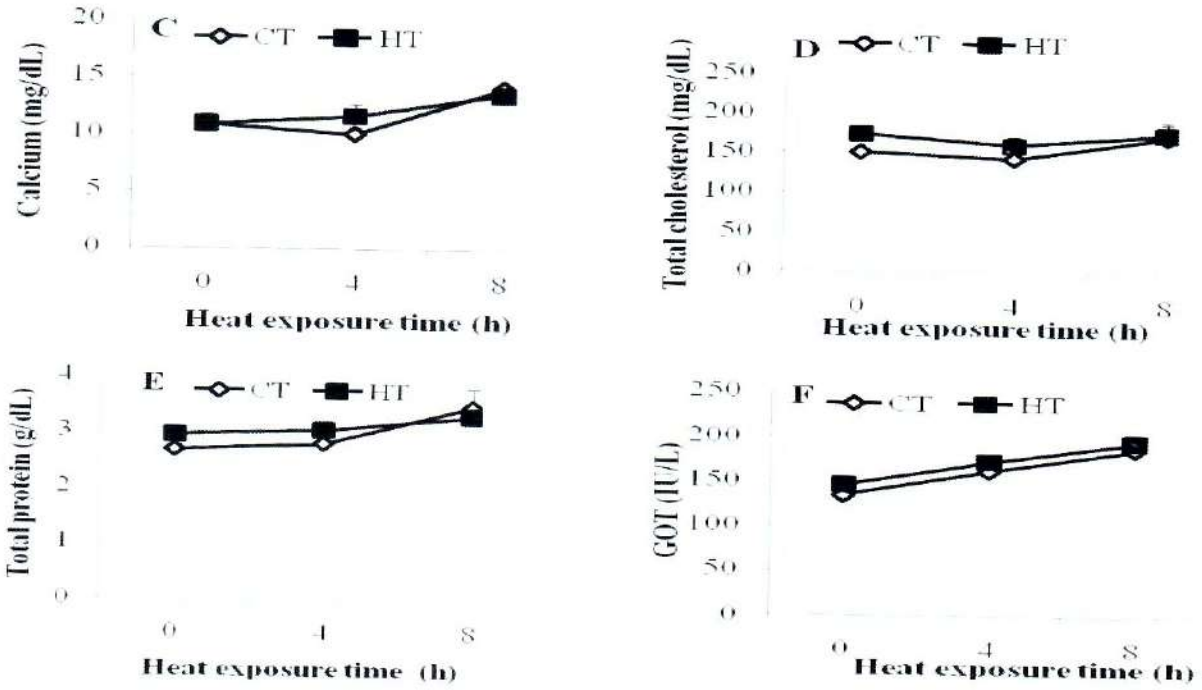


Fig. 2: Plasma concentrations of glucose (A), uric acid (B), calcium (C), total cholesterol (D), total protein (E) and GOT (F) in 21 d old chickens exposed to either CT or HT. Data are expressed as means ± SEM (n = 6 in each group). *Significantly different from the control group (P < 0.05).

Changes in 3, 4-DHBA in chickens exposed to high environment temperature

Fig. 3 shows the time course concentration of 3, 4-DHBA in the dialysate collected from a microdialysis probe implanted in the lateral ventricle of HT and CT chickens. Microscopic observations showed that microdialysis probes were located in the lateral ventricle in chicks for CT and HT groups. Local perfusion of 4-HBA significantly increased the 3, 4-DHBA in HT group compared with baseline. No significant difference of 3, 4-DHBA was observed at first half of the experiment (1-4 h) between CT and HT groups. However, the 3, 4-DHBA significantly increased at the later half of the experiment (4-6 h). In this connection, MDA in the diencephalon of chickens exposed to the temperature of 35°C for 48 hours increased (Chowdhury et al., 2014). The effect of high environmental temperature on the brain oxidative condition likely depends on the duration of exposure. It is not clear what made similarly progressive increase of 3, 4-DHBA of the dialysate from the two groups.

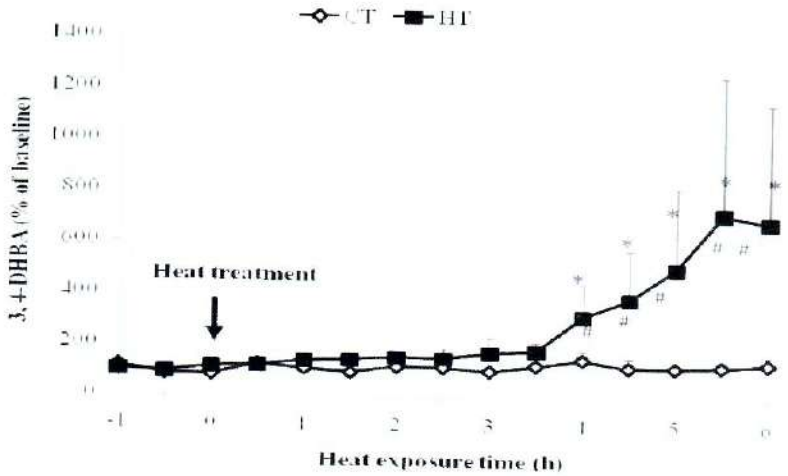


Fig. 3: Dialysate in 3, 4-DHBA concentrations in response to heat treatment in chickens with centrally located microdialysis probes using 4-HBA trapping. The arrow indicates the heat treatment. Data are expressed as means±SEM (n = 9 in each group). * Significantly different from control group (P < 0.05) # Significantly different from baseline.

4. CONCLUSION

Rectal temperature increased and food intake reduced in growing chickens within several hours when they were individually housed under the temperature of 34°C. HT increased hydroxyl radical in lateral ventricle and alter some plasma metabolite. It is likely that the alterations of physiological, biochemical and thermoregulatory response may contribute to the induction of oxidative damage in the chicken's brain.

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