

Effects of L-tryptophan on diencephalic tryptophan hydroxylase gene expression in heat-stressed broilers

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Received: 27.11.2023

Accepted: 14.06.2024

Abstract

The brain monoaminergic system is changed during heat stress condition. Research has shown that commercial poultry are very sensitive animals to heat stress. Hence, this study investigated the effects of L-tryptophan on diencephalic tryptophan hydroxylase type 1 and 2 (TH1 and TH2) gene expression in heat-stressed broiler chicks as animal model. Forty eight, seven-day old broiler chicks were divided into three groups. Chicks were intraperitoneally injected L- tryptophan (25 and 50 mg/Kg) and normal saline. Then they were exposed to the heat stress (39°C) for 5 hours. After 5 hours of treatment, the birds were anesthetized with isoflurane before being euthanized. The brain samples were taken for gene expression evaluation. The data showed declined diencephalic gene expression of TH1 and TH2 in heat stress condition. Tryptophan administration at dose of 50 mg/kg significantly increased the expression levels of TH1 and TH2 in heat stress exposed chicks. It can be concluded that diencephalic serotonergic pathway may have an important role in tryptophan ameliorating effect during heat stress condition.

Key words: Broiler, Diencephalon, High ambient temperature, Tryptophan hydroxylase

Introduction

Physical and psychological stressors disrupt homeostasis and can lead to mental and physical illness. The hypothalamic-pituitary-adrenal (HPA) axis and adrenergic pathways are the primary systems involved in processing stress, leading to elevated plasma corticosteroid levels, depletion of bodily reserves, and impaired immune function. Thermal stressors such as heat stress stimulate neuroendocrine responses including activation of the HPA axis, and decline of brain serotonin content. Hence, there exists an interaction between the HPA (Hypothalamic-Pituitary-Adrenal) axis and

monoaminergic neurotransmitter systems in the regulation of stressful conditions (de Lima et al, 2017; Nakagawa et al, 2016).

Serotonergic system is a monoaminergic pathway and hypothalamic content of serotonin is important agent for coping with stress clues. Serotonin is synthesized from amino acid tryptophan by tryptophan hydroxylase (TH) enzyme. Two isoforms of tryptophan hydroxylase enzyme, tryptophan hydroxylase 1 (TH1) and tryptophan hydroxylase 2 (TH2) have been known until now. TH1 is mainly synthesized in enterochromaphin cells of gut

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and pineal gland while TH2 is commonly expressed in central nervous system (Walther et al, 2003).

Serotonin modulates many physiological functions including cardiovascular, respiratory and immune function, and energy balance as well as body temperature. The balance and levels of serotonin in the brain, compared to norepinephrine and dopamine, have demonstrated delayed recovery in rats exposed to both short and long periods of heat. This suggests that brain serotonin levels are more susceptible to damage during heat stress conditions (Nakagawa et al, 2016). In addition, serotonin and dopamine concentration are increased in dorsal and posterior hypothalamic areas during exposure to cold weather condition (Ishiwata et al, 2018). Serotonin-mimicking drugs or tryptophan treatment during both normal and high temperatures reduce body temperature and improve heat stress-induced hyperthermia (Kumar et al, 2018; Mota et al, 2020), increase blood cortisol level and telencephalic 5-hydroxytryptamine concentration (Höglund et al, 2017). Furthermore, heat stress exerts a deleterious effect on brain electrical activity by alteration in brain neurotransmitters levels and administration of parachlorophenylalanine before heat stress exposure significantly elevates body temperature in rats (Sinha, 2008).

It has been demonstrated that Brain-Derived Neurotrophic Factor (BDNF) plays key roles in the development and function of the central nervous system, and stressful conditions have a vulnerable effect on BDNF expression (Li et al, 2016; Tang et al, 2016).

Serotonin is a trophic agent for brain BDNF production and BDNF expression will be reduced by brain serotonin depletion (Zhou et al, 2008). BDNF expression and synthesis is influenced by environmental temperature. So that, 20 minutes head-out water (with 42 °C temperature) immersion had a significant

increasing and decreasing effect on plasma BDNF and cortisol, respectively (Kojima et al, 2018). It has been shown that exercise at high room temperature elevates body temperature and leads to higher BDNF level than exercise at low room temperature (Goekint et al, 2011). It also has been investigated that exposure to high ambient temperature increases body temperature and plasma glucocorticoids concentration and reduces brain BDNF expression in chicks (Tanizawa et al, 2014).

The aim of the present study was to investigate the potential effect of L-tryptophan treatment during acute heat stress on levels of tryptophan hydroxylase 1 and 2 expression in neonate broiler chickens.

Material and Methods

A total of forty eight one-day-old broiler chicks (*Gallus gallus domesticus*) were prepared from a commercial hatchery (Mahan poultry Farming-Kerman, Kerman Province-Iran). The chicks were kept in a flock (30±1 °C and 50±2% relative humidity (RH)) for 2 days. After 48 hours, they were transferred into individual plexi-glass cages (14×24×21 cm) (width×length×height) for acclimatizing to the individual cages. They had free access to water and commercial starter diet (2850 Kcal/Kg metabolizable energy, 23 % crude protein) and lighting. As the heat stress group, seven days old chicks (N=36) were exposed to high temperature for 5 hours (39±1 °C and 50±2 % RH) and the control group were kept in normal temperature (N=12) (30±1 °C and 50±2% RH).

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the National Institute of Health (USA) and the current laws of Iranian Government for Animal Care (IR.UK.VETMED.REC.1398.037).

L-tryptophan was purchased from Bio-Basic Inc. (20 Konrad Cres, Markham Ontario, Canada). The white crystalline powder was dissolved in 0.9% saline and

0.1 N hydrochloric acid (9:1), 1 h before the intraperitoneal injection. Tryptophan was injected based on BW of chicken. Chicks average BW was 75 g. Thus, at the dose of 25 mg Trp/kg BW, about 1.88 mg for each chick was dissolved in 0.25 ml (volume of intraperitoneal injection) HCl solution (for 24 chicks, 24×1.88 mg in 24×0.25 ml HCl solution). Finally, in primary stock, each 0.25 ml of solution contained 1.88 mg Trp. At the dose of 50 mg Trp/kg BW two fold Trp concentration, according to above instruction primary stock was prepared.

The heat stressed chicks were randomly divided into three equal groups (N=12). Different doses of L-tryptophan (25 and 50 mg/Kg body weight) and normal saline were intraperitoneally injected. The control temperature group received normal saline. All injections were in a volume of 0.25 ml. Immediately the chickens were exposed to the heat stress or control temperature. Rectal temperature was recorded after four hours of heat stress induction. Rectal temperature was measured by inserting thermistor probe (Contact-type digital thermometer- TES-1310-Taiwan) in the cloaca to a depth of 2 cm. At the end of the experiment, all chicks were properly anesthetized with isoflurane (Baxter international Inc, AErrane-isoflurane-USP) before being euthanized. The blood was immediately collected from the jugular vein into tube and centrifuged at 4000 g for 15 minutes to collect the serum. The brain sample (diencephalon) was collected following blood collection and snap frozen

using liquid nitrogen. All the collected samples were stored at -80°C until further analysis.

Total RNA was extracted from each brain tissue sample by RNX-Plus solution (SinaClon BioScience Co). The final RNA pellets were re-suspended in 30 μl diethylpyrocarbonate-treated water (DEPC-treated water). The amount of the purified RNA (A260/A280 ratio was ≥ 1.9) was determined by Nanodrop and the integrity of RNA samples was analyzed on a 1.5% agarose gel (Sigma).

Briefly, the reaction was performed using Oligo-dT primer and M-MuLV reverse transcriptase (Thermo Fisher Scientific, Germany) based on the manufacturer's protocol. The reproducibility of single results was determined with two strategies: two-time measurement of cDNA aliquots; analysis of two different cDNA prepared from the same RNA extract.

Quantification of relative RNA expression was followed by an established method using qPCR with the SYBR green reporter dye and protocol. The 2X universal master mix (Biofact-DQ383-40H without ROX, South Korea) was used in the PCR reactions. Thermal cycling utilized LightCycler® 96 Instrument (Roche-Germany).

A final melting curve of fluorescence versus temperature was generated to screen the primer dimers and to document a single product formation. Primer sequences, RT-PCR fragment lengths and NCBI accession numbers are reported in Table 1.

Table 1: Primer sequences

Primer name	length	sequence	NCBI code
BDNF	109	F:GAAGGCTGCAGGGGCATAGA	NM_001031616.1
		R: ACCGCCAGCCAACTCTCTTT	
TH2	200	F:GACCTCCGCAGTGATCTAAACA	NM_001001301.1
		R: CACAATGACACAAGCCGCAG	
TH1	180	F: AAGAGCATTGCCAGTGTGGT	NM_204956.1
		R:AGTCCTGCATACAGACGTTACA	
GAPDH	162	F:TGACCACTGTCCATGCCATC	NM_204305.1
		R:TAAGCTTCCCATTCAGCTCAGG	

All primer pairs produced a single band on agarose gel electrophoresis corresponding to the predicted size. The amount of PCR products were normalized with housekeeping (GAPDH) primers in separate reactions. All samples were assayed in triplicate. The relative mRNA levels were calculated by the expression $2^{-\Delta\Delta CT}$ equation.

Serum corticosterone level was measured by using a solid phase sandwich ELISA method (chicken ELISA Kits; MyBioSource.com, USA). The sensitivity of corticosterone kit was 1.0 ng/ml. The intra-assay precision (precision within an assay) and the inter-assay precision (precision between assays) were CV=4.2% and CV=6.5%, respectively.

Values were expressed as mean \pm SEM (standard error of mean). Statistical evaluation of significant difference between means, for multiple comparisons of all groups, was performed with one-way analysis of variance (ANOVA) followed by the Tukey's multiple range test, using the SPSS16 program. The significance level was considered $P<0.05$.

Results

Rectal temperature and corticosterone level

The data showed that heat stress significantly caused to increase in rectal temperature at 4th h of the experiment compared with the control group ($P<0.05$). Tryptophan treatment (25 and 50 mg/kg) decreased heat stress-induced hyperthermia ($P<0.05$) (Table 2).

Table 2: Effect of tryptophan and heat stress on rectal temperature

Groups	Rectal temperature (°C) (Means \pm SEM)	Minimum-Maximum Values (°C)
Control Temperature	39.66 ^a \pm 0.088	39.3-40.2
Heat stress	41.2 ^b \pm 0.057	40.9-41.4
Heat stress +Tryptophan 25 (mg/kg)	40.35 ^c \pm 0.076	39.8-40.6
Heat stress +Tryptophan 50 (mg/kg)	40.2 ^c \pm 0.055	40-40.5

“a, b, c At each column, different superscript letters show significant difference between the groups ($P<0.05$).”

Analysis of serum corticosterone data showed that corticosterone levels were significantly increased in heat stress exposed chicks in comparison to the control

group ($P<0.05$); While in heat stress tryptophan treated groups corticosterone was decreased ($P<0.05$) (Table 3).

Table 3: Effect of tryptophan and heat stress on serum corticosterone level

Groups	Corticosterone (ng/ml) (Means \pm SEM)	Minimum-Maximum Values (ng/ml)
Control Temperature	49.98 ^a \pm 0.73	49.5-51.1
Heat stress	59.77 ^b \pm 1.33	58.2-61.4
Heat stress +Tryptophan 25 (mg/kg)	49.83 ^a \pm 1.88	48.5-52.4
Heat stress +Tryptophan 50 (mg/kg)	50.07 ^a \pm 1.85	48.7-52.2

“a, b, c At each column, different superscript letters show significant difference between the groups ($P<0.05$).”

Heat stress significantly reduced the brain mRNA level of tryptophan hydroxylase 1, 2 and bdnf (Figure 1, 2 and 3) ($P<0.05$). While treatment with tryptophan (50 mg/Kg BW) in this condition significantly increased gene expression of TH 1 and TH 2. Gene

expression of BDNF mostly incremented by tryptophan administration especially in chickens that received 50 mg/Kg body weight tryptophan.

Agarose gel electrophoresis of cDNA amplification products was presented in Figure 4.

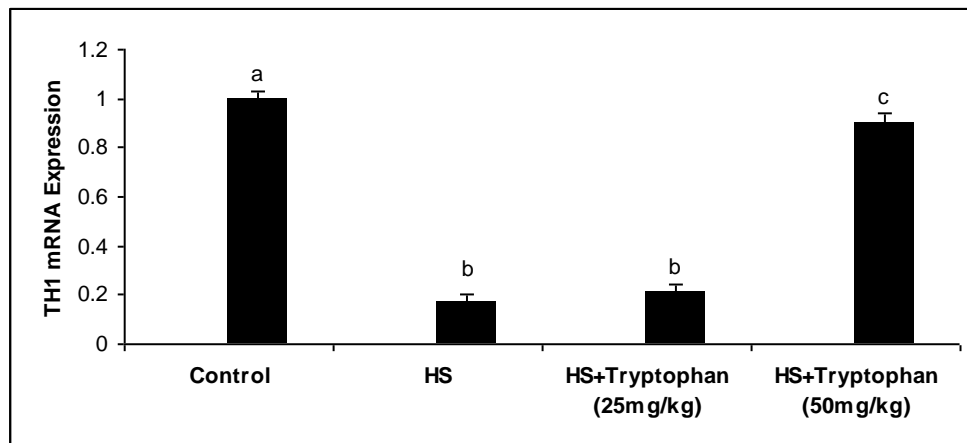


Figure 1: Effect of tryptophan and heat stress on diencephalic TH1 expression relative to GAPDH (Means±SEM).

“Different superscript letters show significant difference between the groups (P<0.05).”

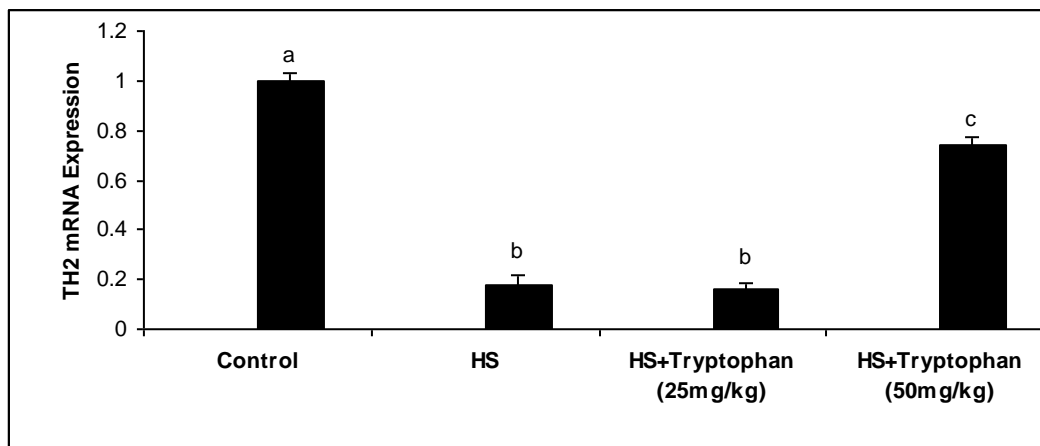


Figure 2: Effect of tryptophan and heat stress on diencephalic TH2 expression relative to GAPDH (Means±SEM).

“Different superscript letters show significant difference between the groups (P<0.05).”

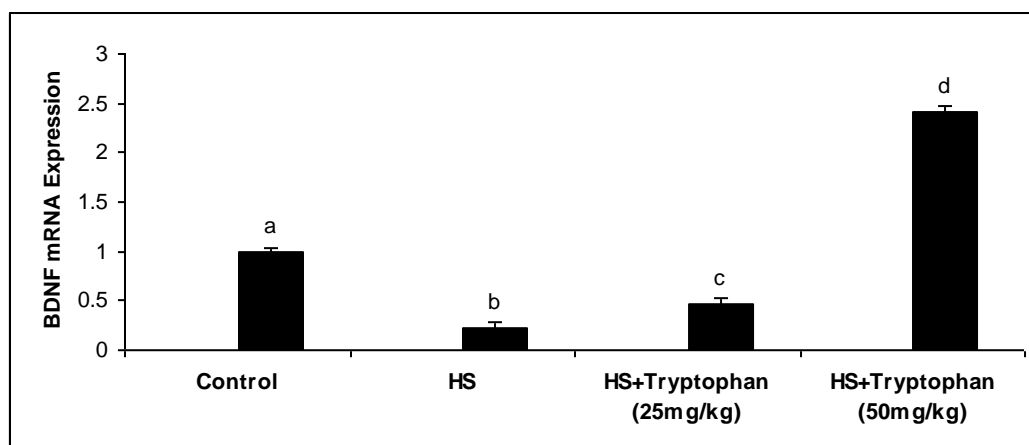


Figure 3: Effect of tryptophan and heat stress on diencephalic BDNF expression relative to GAPDH (Means±SEM).

“Different superscript letters show significant difference between the groups (P<0.05).”

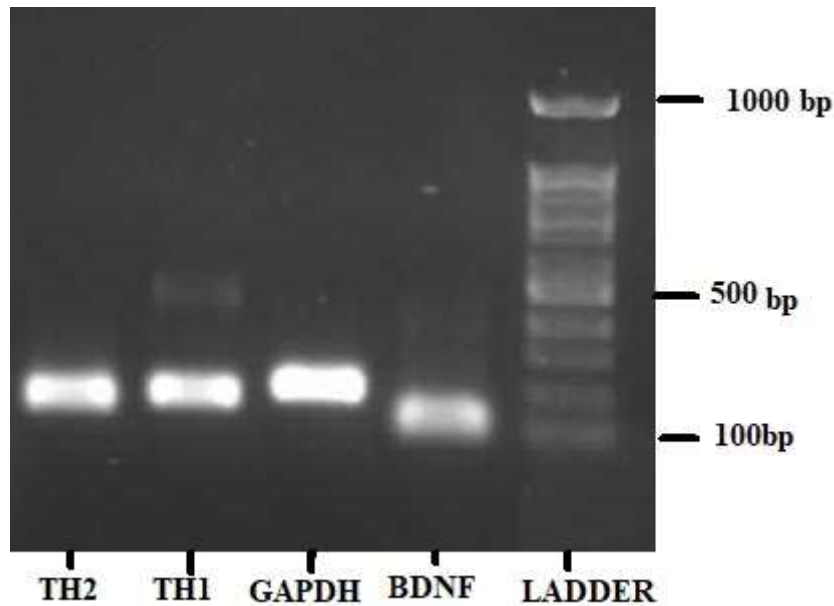


Figure 4: Agarose gel electrophoresis of TH1 (180 bp), TH2 (200 bp), GAPDH (162 bp) and BDNF (109 bp) cDNA amplification products under UV light.

Discussion

In the present study, the influence of L-tryptophan administration before heat stress exposure was investigated on body temperature, serum corticosterone and diencephalic mRNA level of tryptophan hydroxylase 1, 2 and BDNF. The data indicated that heat stress could elevate body temperature, serum corticosterone concentration and reduce the gene expression of tryptophan hydroxylase 1, 2 and BDNF. Tryptophan treatment during heat stress significantly compensated the changes associated to the heat stress exposure (Nakagawa et al, 2016).

In the present study, administration of tryptophan reduced the increased rectal temperature after thermal stress. Similar changes were seen in the case of corticosterone. Of course, changes in the amount of corticosterone with tryptophan intake are more effective and its amount is closer to the control group.

It has been reported that the disturbance in body temperature regulation is related to the hypothalamic serotonin reduction during rodent-heat stress exposure. Tryptophan hydroxylase is first rate-limiting enzyme of serotonin production. In

depression rat models, serotonin decline is due to the reduction of brain tryptophan hydroxylase 1 and 2 and their dysfunction (Chen et al, 2017). In line with our findings, heat stress exposure reduced diencephalic tryptophan hydroxylase 1 and 2 genes expression. Over-activation of HPA axis and monoaminergic system and elevation of blood corticosterone and catecholamines levels are the most common feature of physical, mental and thermal stress during heat stress condition (K Ito et al, 2014).

It has been shown that the central injection of tryptophan (400 and 800 nmol) in 5-6 day-old chicks significantly counteracts with CRH-augmented social isolation (Yoshida et al, 2015). In addition, Zhang et al (2004) reported that the central injection of serotonin acts as a CRH-induced behaviors modulator. Accordingly, in the current study, tryptophan, as a precursor of serotonin, has influenced the modulation of the CRH axis and prevented the increase of corticosterone.

In the recent experiment, tryptophan administration before heat stress exposure leads to reduce rectal temperature and serum corticosterone level. Glucocorticoids are

known as catabolic hormones and body heat increment is a result of increased catabolism. In agreement with our results, oral supplementation of tryptophan (20, 40, and 60 mg/kg, for 3 days) causes a significant decrease in blood cortisol and heart rate horses (Davis et al, 2017).

Also it has been reported that increase in the amount of plasma corticosterone, decrease in plasma serotonin and enhancement in sensitivity to the stress in tryptophan-deficient diet in rats have occurred (Tanke et al, 2008).

It has been demonstrated that there is a reciprocal relationship between BDNF and serotonergic system so that serotonin depletion promotes BDNF down-regulation and synthesis (Zhou et al, 2008) and central injection of BDNF increases tryptophan hydroxylase mRNA expression and serotonin production (Siuciak et al, 1998). Therefore, it seems logical that in our study, heat stress exposure reduced chicken brain BDNF mRNA level and tryptophan supplementation had a compensatory effect and increased brain BDNF gene expression.

Negative effects of elevated glucocorticoids during stress on brain BDNF production have also been reported (Schaaf et al, 1998). Heat stress exposed chicken in our research had high serum corticosterone and low BDNF gene expression. In contrast, Tanizawa et al (2014) reported a reduced corticosterone level and no changes in brain BDNF gene expression following 3 hours heat stress conditioning repeated 15 minutes every day for 4 consecutive days. Based on the previous reports, at least 5 hours exposure to high temperature is required to induce heat stress in chicken (K Ito et al, 2014). It has been shown that the diencephalic and plasma tryptophan levels are decreased during acute heat stress (K Ito et al, 2014; Kentaro Ito et al, 2015). This might be a conservative and protective mechanism to overcome heat stress. During heat stress gluconeogenesis occurs by amino acids

breakdown and decreases in their reserve pool. This might lead to the reduction of blood and brain tryptophan concentration. Therefore, a tryptophan supplementation will help to prevent heat stress side-effects. It has been reported that peripheral serotonin can control thermogenesis by inhibiting the beta-adrenergic effects on adipose tissue (Crane et al, 2015). In agreement to our results, dietary tryptophan increases blood serotonin and decreases corticosterone levels and improves gut micro-flora (U. Bello et al, 2018). In contrast, using a selective serotonin agonist for TH1A receptors reduces body temperature but increases corticosterone in rats (Newman-Tancredi et al, 2018).

Although serotonin is a product of tryptophan metabolism, kynurenine is the main product of tryptophan and about 95 percent of tryptophan is converted to kynurenine (Mellor et al, 1999). A key product of kynurenine pathway is kynurenic acid that acts as a glutamate receptor antagonist and thereby antagonizes dopamine release (Amori et al, 2009; Fujigaki et al, 2017). It has been reported that a significant elevation in the blood glutamate, norepinephrine and dopamine is found in heat stress exposed rats (Chauhan et al, 2017). Chronic heat exposure for 4 days activates blood kynurenine metabolites pathway in chickens (Tomonaga et al, 2018).

The above implications show a significant relationship between heat stress and kynurenine metabolite and emphasize the role of tryptophan metabolite for buffering the heat stress consequence.

Based on our results, it can be concluded that heat stress disturbs brain serotonergic system by reducing tryptophan hydroxylase enzymes gene expression. Furthermore, high blood corticosterone levels and body temperature elevation as well as decrease in brain BDNF gene expression are other consequences of acute heat stress in chicks.

Acknowledgment

The authors would like to express their gratitude to Vice-Chancellor for Research of Shahid Bahonar University of Kerman, for approving and funding this research.

Conflict of Interest

The authors declare that there is no conflict of interest.

Funding

This work was supported financially by a Grant for Scientific Research from Vice Chancellor for Research of Shahid Bahonar University of Kerman, Iran.

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Received: 27.11.2023

Accepted: 14.06.2024

اثرات ال-تریپتوفان بر بیان ژن تریپتوفان هیدروکسیلاز دینسفالیک در جوجه‌های گوشتی تحت استرس گرمایی

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تاریخ دریافت: ۱۴۰۲/۹/۶

تاریخ پذیرش: ۱۴۰۳/۳/۲۵

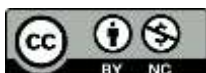
چکیده

سیستم مونوآمینرژیک مغز در شرایط استرس گرمایی تغییر می‌کند. تحقیقات نشان داده است که طیور تجاری حیواناتی بسیار حساس به استرس گرمایی هستند. از این رو، این مطالعه به بررسی اثرات ال-تریپتوفان بر بیان ژن تریپتوفان هیدروکسیلاز ۱ و ۲ (TH1, TH2) دینسفالیک در جوجه گوشتی تحت استرس گرمایی به عنوان مدل حیوانی پرداخت. چهل و هشت جوجه گوشتی ۷ روزه به سه گروه تقسیم شدند. به جوجه‌ها ال-تریپتوفان (۲۵ و ۵۰ میلی‌گرم بر کیلوگرم) و نرمال سالین به صورت داخل صفاقی تزریق شد. سپس به مدت ۵ ساعت در معرض استرس حرارتی (۳۹ درجه سانتی‌گراد) قرار گرفتند. پس از ۵ ساعت درمان، پرندگان قبل از مرگ با ایزوفلوران بیهوش شدند. نمونه‌های مغز جهت ارزیابی بیان ژن گرفته شد. داده‌ها نشان داد که بیان ژن دینسفالیک TH1 و TH2 در شرایط تنش گرمایی کاهش یافته است. تجویز تریپتوفان با دوز ۵۰ میلی‌گرم بر کیلوگرم به طور قابل توجهی باعث افزایش سطح بیان TH1 و TH2 در جوجه‌های در معرض استرس گرمایی شد. می‌توان نتیجه گرفت که مسیر سروتونرژیک دینسفالیک ممکن است نقش مهمی در بهبود اثر تریپتوفان در شرایط استرس گرمایی داشته باشد.

کلمات کلیدی: تریپتوفان هیدروکسیلاز، جوجه گوشتی، دمای محیطی بالا، دینسفال

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