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RESEARCH ARTICLE

Protective Effect of Huangteng Soluble Powder on Endogenous Pyrogen and Central Mediators of Fever Induced by Lipopolysaccharide in Chicken

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ABSTRACT

The objective of this study was to understand the effect of Huangteng Soluble Powder (HTSP) on endogenous pyrogen and central mediators of fever induced by Lipopolysaccharide (LPS) in chicken. The model was established, and 90 chickens were randomly assigned into 6 groups, including Group I (control), Group II (injected with LPS), Group III (administered with Banqing Granules once a day for 3 days after 5 h post injection of LPS), Group IV, V and VI (administered with HTSP once a day for 3 days after 5 h post injection of LPS). The temperature was measured at different time periods, and samples were collected at 5 h post injection of LPS and 12 h, 24 h after the administration of HTSP. The results showed that HTSP significantly decreased the temperature of fever chicken as compared to Group II. The levels of IL-1, IL-6, TNF-α in serum and Prostaglandin E2 (PGE2), cyclic adenosine monophosphate (cAMP) in hypothalamus in Group II significantly increased at 5 h post injection of LPS and the mRNA expression levels of PGE2 and cAMP were also increased significantly in Group II as compared to control respectively. Furthermore as compared with Group II, the levels of IL-1, IL-6 in serum and PGE_2, cAMP in the hypothalamus in Groups III, IV, V and VI decreased after 12 h and 24 h, the levels of TNF-α in serum in Group III and V decreased after 12 h and 24 h, and the mRNA expression levels of PGE2 and cAMP of Group V were significantly decreased at the end of the administration. In conclusion, HTSP can attenuate the LPS-induced fever by diminishing the levels of IL-1, IL-6 and TNF-α in serum and the levels of PGE2 and cAMP in the hypothalamus and inhibiting the mRNA expression of PGE2 and cAMP.

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INTRODUCTION

Increased stocking density and intensive selection pressure reported to influence the performance of the chicken and supply inadequate air circulation inside the poultry house. The poultry birds seems to very sensitive to suddenly changing in housing temperature or heat stress; the negative climate change would result in birds will be more vulnerable to pathogens and disease incidence. Cold stress is primarily mediator of the fever in poultry; it lowered the feed-intake, depression on growth performance, weight loss and alters overall immunity of the chicken causing serious economic losses.

The pyrogen acts on the body to induce fever and thermoregulatory center of the body dysfunction (Roth and Blatteis, 2014; Zhang *et al.*, 2017). Pyrogens can be classified into exogenous and endogenous pyrogen (Jia *et al.*, 2013). Generally, some exogenous pyrogens (e.g., Lipopolysaccharide) do not act directly on the brain to cause fever, however by activating the mononuclear macrophages *in vivo* to produce and release endogenous pyrogen (EP) and then interact with both the thermoregulatory center of the body and central mediators of fever (PGE_2, cAMP, etc.) to cause fever (Bastos-Pereira *et al.*, 2015; Zampronio *et al.*, 2015). The well-accepted EPs include interleukin-1 (IL-1) and IL-1

receptor antagonist protein (IL-1ra), tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interferons and so on (Dinarello *et al.*, 1987; Zampronio *et al.*, 2015). These cytokines banded their receptors, stimulated the fever reaction, mediated the inflammatory process, participated in the immune regulation and affected tissue metabolism (Schneiders *et al.*, 2015; Ye *et al.*, 2015).

HTSP, composed of two traditional Chinese herbs, *Scutellaria baicalensis* and *Ren Dong Teng* (*Honeysuckle* Stem), have the function in heat-clearing and damp-drying, purging fire and detoxifying toxicosis, expel the wind (China, 2015). Reportedly, *Scutellaria baicalensis* has been used for the treatment of inflammation, fever, dysentery, diarrhea, influenza, pneumonia, and have potential anticancer activities (Nhoek *et al.*, 2018; Xu *et al.*, 2018). For instance, the effective constituent of *baicalin* in *Scutellaria baicalensis* can effectively treat the fever induced by LPS in rats and rabbits (Tsai *et al.*, 2006; Ye *et al.*, 2015). *Ren Dong Teng* has good antibacterial, antipyretic, anti-inflammatory and antiviral effects (Xiong *et al.*, 2013; Fan *et al.*, 2015). Thus, to date, there is little information regarding the mechanisms of HTSP in curing the poultry fever. Therefore current study was designed to evaluate the protective effects and mechanism of HTSP on fever induced by LPS in chicken.

MATERIALS AND METHODS

Animals and Treatment: One hundred and thirty 30-day-old healthy three-yellow chickens (weighing 550±50g), 50% females and 50% males, were purchased from Rongchang Guili poultry farm, Chongqing China. All chickens were maintained in a standard sterilized chicken coop, with standard controlled temperature (20~25°C) and relative humidity (56%~65%), fed with standard laboratory ration and water. All the experiments were conducted the national legislation concerning the protection of bird welfare and approval following the institutional animal welfare and committee guidelines of Southwest University, China. (Approval number SYXK20110001)

Drugs: Huangteng Soluble Powder (HTSP): 50g/bag, drinking water administration, once a day for 3 consecutive days, and produced by Luoyang Ruihua Animal Health Products Co., Ltd, No. 20150227; Banqing Granules: 100g/bag, 0.5g/100ml in water once a day for 3 consecutive days, produced by Jiangxi Chinese Patent Medicine Raw Materials Co., Ltd, No. 2015072201.

FT-IR characteristics of HTSP: The effective active components of HTSP were analyzed by the Fourier transform infrared (FT-IR) spectroscopy. Briefly, take 0.2 mg (0.201 mg) HTSP and 2 mg (2.001 mg) Potassium bromide into an agate mortar, mixed, tablet, and then placed in the Fourier transform infrared spectrometer for testing. Select 2000 and 400 cm^{-1} as the starting and ending points of the spectral baseline and calculate the height ratio of the characteristic peaks (Zhang *et al.*, 2010; Liu *et al.*, 2012).

Fever model establishment: According to recent reports (De Boever *et al.*, 2008; Lieboldt *et al.*, 2017), using different doses of LPS combined with body temperature detection was select for best LPS dose for fever model. Finally, according to the optimal LPS dose (150 μg/kg) has been screened, and every chicken in the experimental group received the LPS 150 μg/kg with intraperitoneal route injection. The chickens were fasted 6 h and then provided water normally before the attack. The positive chickens in fever model were used for further experiment.

Animal grouping and treatment: The 90 chickens were randomly divided into 6 groups (15 chickens in each group): Group I (control group without any treatments), Group II (only challenge without drugs), Group III (After challenged 5h, drinking water administration of Banqing Granules 0.5g/100ml once a day for three consecutive days), Group IV, Group V, Group VI (After challenged 5h, drinking water administration of HTSP 1.0g/100ml, 0.5g/100ml, 0.25g/100ml once a day for three consecutive days). During the experiment, the body temperature of each group was measured at 5h after challenged, 2, 7, 12, 24, 36, 48 and 72h respectively after drug administration, and recorded in detail. Blood samples were collected to obtain the serum samples for the analysis before the experiment and 5h after challenged, 12h and 24h after administration (drugs). Hypothalamus was collected at the end of the third day, and then frozen immediately in liquid nitrogen, stored at -80°C for further analysis.

IL-1, IL-6, TNF-α, PGE_2 and cAMP levels: The concentration of IL-1, IL-6, and TNF-α in the chicken serum, cAMP and PGE_2 in the chicken hypothalamus were detected by using ELISA kit and calculated by standard curve, and the absorbance (OD) was measured at 450 nm using a Microplate Reader according to the ELISA kit instructions.

Analysis of relative mRNA expression of PGE_2 and cAMP: Total RNA was extracted with Trizol reagents according to manufacturer's instruction (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China). Then cDNA was prepared with Primer-Script™ reagent kit (TakaRa Biotechnology Co. Ltd, Dalian, China). The gene-specific primers of cAMP, PGE_2 and GAPDH were synthesized by TakaRa Biotechnology Co. Ltd (Dalian, China) (Table 1). All the reactions were run with following cycling conditions were 95°C for 5min, then 40 cycles of following: 95°C for 30s, 63°C for 30s. The $2^{-\Delta\Delta Ct}$ method was used to analyze Quantitative Real-time PCR data.

Statistical analysis: SPSS22.0 statistics software was used for data processing. All data were analyzed by the SPSS statistical software, using a one-way ANOVA and expressed as mean±standard error (SE). $P<0.05$ was considered as significant statistical difference.

Table 1: Primer sequences used in quantitative real time PCR assays

Gene	Forward primer (5'-3')	Reverse primer (3' -5')	Size (bp)	Accession No.
cAMP	GATCCCGTTCTGGTCCAAC	GCCCCATTTATTCATTCAGC	123	NM_001024830
PGE_2	TGCTGGATGAGAAGGAGACA	GGGAGATGAGGTGAACAAGC	108	XM_015302168
GAPDH	GCACGCCATCACTATCTT	GGACTCCACAACATACTCAG	82	NM_001289746.1

RESULTS

FT-IR characteristics of HTSP: HTSP is composed of two Chinese herbal medicines-*Scutellaria baicalensis* and *Ren Dong Teng*. Flavonoids are the main active ingredients of *Scutellaria baicalensis*, and *chlorogenic acid* and *galuteolin* are the main active ingredients of *Ren Dong Teng*. Fig. 1 showed that the absorption peaks were around 1735.87, 1619.83, 1509.75, 1453.22, 1402.64, 1316.36 and 1247.93 cm^{-1} respectively which were mainly absorption peaks of flavonoids. The absorption peaks around 3363.31, 2922.98, 1619.83, 1111.07 and 1054.55 cm^{-1} were mainly absorption peaks of *chlorogenic acid* and *galuteolin*. In conclusion, it is showed that HTSP contains qualified *Scutellaria baicalensis* and *Ren Dong Teng*.

Validation of fever chicken model: The change of body temperature in different doses of LPS in chicken is shown in Fig. 2. Obviously, the group with the fastest onset of action and the longest duration of maintenance was the LPS dose 3 (150 µg/kg LPS). As a consequence, the fever model dose of 150 µg/kg LPS was selected.

Effect of HTSP on body temperature and fever of chicken induced by LPS: The results showed that the basal body temperature of the chicken was in the normal range (40 to 42°C) before the test, and there was no significant difference between each group (P>0.05) as shown in Fig. 3. After 5h of LPS injection, as compared with the Group I, the body temperature of all the experimental groups increased significantly (P<0.01). HTSP administration started after 5h of LPS injection. After 2h of administration, as compared with the Group II, the body temperature in Group III and Group IV decreased after 7h of the administration (P<0.05); the body temperature in Group V decreased significantly after 7h of administration (P<0.01); and the body temperature in Group VI decreased significantly after 12h of administration (P<0.01). (Fig. 3)These results showed that the fever model was reliable and that both of Groups IV and V could effectively protect the chicken from the LPS-induced fever.

Effects of HTSP on the levels of IL-1, IL-6 and TNF-α in the chickens' serum: The results shows that the concentration of IL-1 in the chickens' serum of each experimental Group (Group II to VI) increased significantly (P<0.05) at the period 2 as compared to control group I. As compared to the Group II, the concentration of IL-1 in the chickens' serum of Group III, IV, V and VI was decreased at the period 3 and decreased (P<0.05) at the period 4 significantly (Fig. 4).

The content of IL-6 in the chickens' serum of each experimental Group (Group II to VI) increased significantly (P<0.05) at the period 2 as compared to control group I. As compared with the Group II, the levels of IL-6 in the chickens' serum of Group IV, V and VI decreased significantly (P<0.05) at the period 3, as well as there was no significant difference (P>0.05) at the period 4. (Fig. 5).

The level of TNF-α in the chickens' serum of each test group (Group II to VI) increased significantly (P<0.05) at the period 2 in comparison to Group I. However as compared with the Group II, the levels of TNF-α in the chickens' serum of Group III and VI decreased at the period 3, and the Group III and V decreased at the period 4, whereas there was no significant difference (P>0.05). (Fig. 6).

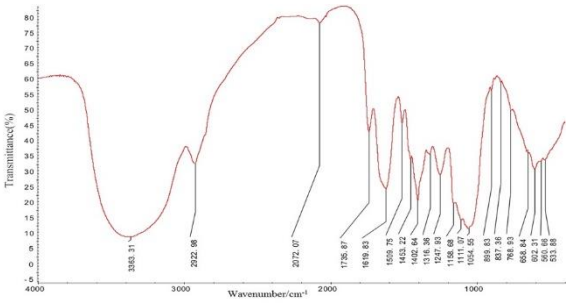

Fig. 1: Fourier Transform Infrared Spectroscopy spectrum of HTSP.

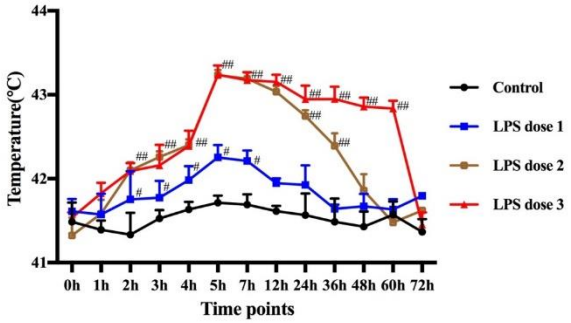

Fig. 2: Temperature change of chicken in each group after injection with different does of LPS. ##P<0.01 vs Control group, #P<0.05 vs Control group.

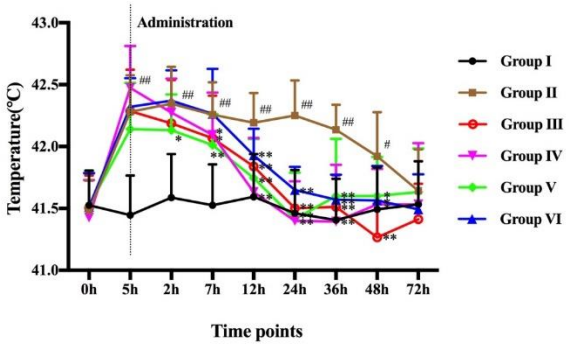

Fig. 3: Effect of HTSP on body temperature of LPS-induced fever in Chickens. ##P<0.01 vs group I, #P<0.05 vs group I, **P<0.01 vs group II, *P<0.05 vs group II.

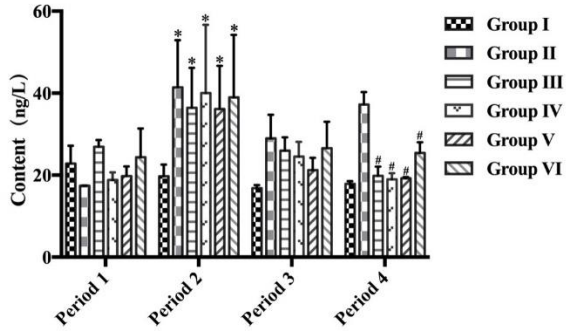

Fig. 4: Changes of the IL-1 content in chickens' serum. Period 1: before the test, Period 2: after 5 h of LPS injection, Period 3: after 12 h of administration, Period 4: after 24 h of administration. *P<0.05 vs group I, #P<0.05 vs group II. The same as below.

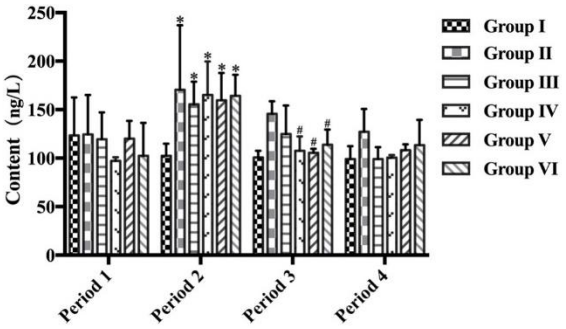

Fig. 5: Changes of the IL-6 content in chickens' serum.

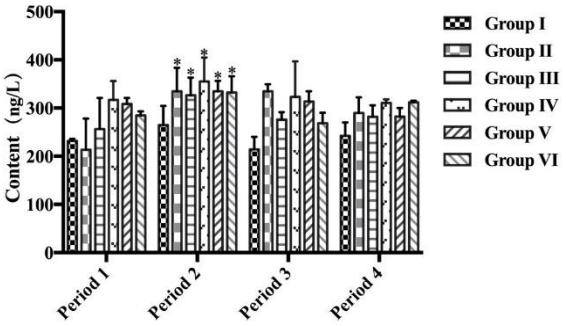

Fig. 6: Changes of the TNF-α content in chickens' serum.

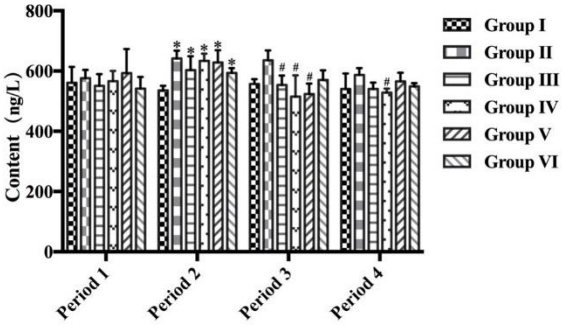

Fig. 7: Changes of the PGE_2 content in chickens' serum.

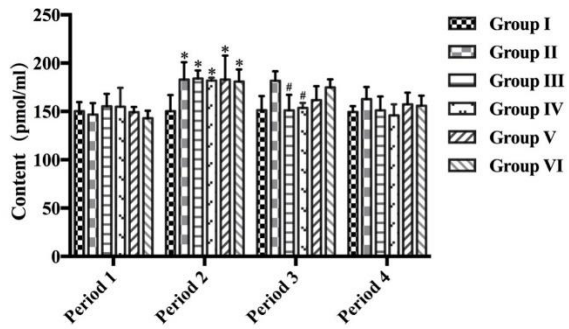

Fig. 8: Changes of the cAMP content in chickens' serum.

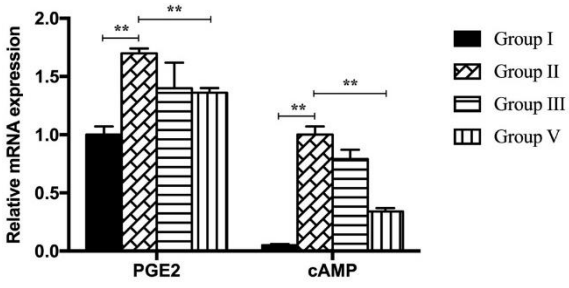

Fig. 9: The effect of HTSP on PGE_2, cAMP mRNA expression in chicken's hypothalamus **P<0.01.

Effects of HTSP on the levels of PGE_2 and cAMP in the chickens' Hypothalamus: As shown in Fig. 7, as compared with the group I, the levels of PGE_2 in the chickens' hypothalamus of each experimental Group (Group II to VI) significantly increased (P<0.05) at the period 2. The levels of PGE_2 in the chickens' hypothalamus of Group III, IV and V decreased significantly (P<0.05) at the period 3 as compared with the group II. At the period 4, the levels of PGE_2 in the chickens' hypothalamus of Group IV, V and VI were decreased, in addition, Group V decreased significantly (P<0.05) as compared with group II.

The levels of cAMP in the chickens' hypothalamus of each experimental group (Group II to VI) significantly increased (P<0.05) at the period 2 in comparison to Group I. Compared to Group II, the levels of cAMP in the chickens' hypothalamus of Group IV and V decreased at the period 3 and 4 respectively, moreover, there was significantly decreased (P<0.05) at the period 3 (Fig. 8).

Effects of HTSP on mRNA expression of PGE_2 and cAMP in the chickens' hypothalamus: As shown in Fig. 9, as compared with the Group I, PGE_2 and cAMP mRNA expression of Group II were significantly increased (P<0.01). PGE_2 and cAMP mRNA expression of Group V were significantly decreased (P<0.01) as compared to group II at the end of the administration.

DISCUSSION

According to the theory of Traditional Chinese Veterinary Medicine, fever is the main clinical symptom of all kinds of epidemic febrile disease. It is mainly caused by cold evil invasion, the decline of body resistance, or climate change. It is a systemic reaction of the body to the disease. And it is the reaction of the between vital qi of the body and disease. In addition, the therapeutic principle is based on relieve heat and save the Yin. In this study, the characters of *Scutellaria baicalensis* with curative effect on heat dampness, cooling blood miscarriage, and detoxifying are bitter and cold, and it converges to heart, lung, gallbladder, large intestine channel (Muluye *et al.*, 2014). Furthermore, the characters of *Ren Dong Teng* with curative effect on clears heat and toxin, dispels wind damp, unblocks the channels and cools the blood are sweet and cold, and it converges to heart and lung channel. The compatibility of *Scutellaria baicalensis* and *Ren Dong Teng* has good effects of heat-clearing and detoxifying, dispelling wind and relieving the exterior (Xie and Preast, 2013). Therefore, it is reasonable for HTSP to treat chicken fever caused by LPS, which is consistent with the theory of Traditional Chinese Veterinary Medicine.

The gram-negative bacterial lipopolysaccharide (LPS), composed of cell-specific polysaccharide, lung-specific core polysaccharide and lipid A3, is a component contained in the outer membrane of gram-negative bacterial cell wall and a representative bacterial pyrogen and also a classic model for inducing fever in experimental animals (Akhtar *et al.*, 2012). According to De Boever's research, the endotoxin inflammation fever model of broiler chickens characterized by fever and cytokine expression has been successfully established in

the laboratory (De Boever *et al.*, 2008). Moreover, the fever is that the body stimulated by the heat activator such as LPS after a series of signal transduction, such as TLR4 signal pathway (Guo *et al.*, 2016), generates endogenous pyrogens, acts on the thermoregulatory center of the body of the preoptic area in the anterior hypothalamus (AH/POA) and releases the central mediators of fever. Thus, the set-point of body temperature are moved up. It has been proposed that LPS can effectively stimulate the organism to produce endogenous pyrogens IL-1β, IL-6 and PGE_2, as well as Baihu Decoction can effectively reduce the levels of IL-1 and TNF-α in rabbit serum and hypothalamus (de Boever *et al.*, 2010; Jia *et al.*, 2013). More recently, Emílio-Silva *et al*. (2017) demonstrated that LPS could increase the levels of IL-1β, IL-6, TNF-α and PGE_2 in plasma of rats. The proinflammatory cytokine, IL-1, is a key medium for local and systemic infection and inflammatory response. Intraperitoneal, intraventricular, intraventricular and hypothalamic injection of recombinant IL-1 can induce a variety of animals to cause fever (Zampronio *et al.*, 2015). The formation of proinflammatory mediums, IL-1 and TNF-α, is increased in infected and injured tissues which stimulate the hypothalamus to cause the increase of body temperature by enhancing the synthesis of PGE_2 in the preoptic area/anterior hypothalamus (Saper and Breder, 1994). In the process of animal fever caused by exogenous pyrogens such as LPS, the expression of PGE_2 mRNA and the levels of PGE_2 in the preoptic area (POA) are increased, and a large number of cAMP-positive cells could be observed (Ivanov *et al.*, 2002; Steiner and Branco, 2003). In 2015, Liu *et al*. (2015) showed that the antipyretic effect is usually a characteristic function of drugs or compounds that have inhibitory effects on the biosynthesis or release of prostaglandins, that the shell and soft tissue of the *Thais luteostoma* can effectively reduce the levels of IL-1, IL-2, IL-6, TNF-α, PGE_2 and cAMP, and that the mechanism of antipyretic effect may be through inhibition the release of the levels of PGE_2 and cAMP.

In this study, the antipyretic effect of HTSP was evaluated for the first time. The results showed that the body temperature of the chickens in each experimental Group (Groups II to VI) increased and peaked after 5h of LPS injection, and the body temperature of chickens in Group III, IV, V and VI was close to Group I (control) after 12h and 24h of administration, furthermore, the body temperature of chickens in Group IV and V decreased the fastest. This shows that HTSP has a great antipyretic effect on chicken fever induced by LPS. The levels of IL-1, IL-6 and TNF-α in chickens' serum as well as the levels and the mRNA expression of PGE_2 and cAMP in chickens' hypothalamus in each experimental group (Group II to VI) were significantly higher than Group I after 5h of LPS injection. It is suggested that LPS can effectively stimulate the organism to produce endogenous pyrogens, IL-1, IL-6 and TNF-α, so that the levels are increased, which directly or indirectly stimulates the release of central mediators of fever (PGE_2 and cAMP) in the thermoregulatory center of the body, leading to the change of the set-point of body temperature of the thermoregulatory center of the body. This is consistent with the reports of de Boever *et al.* (2010) and Jia *et al.* (2013). Nevertheless, after the administration of HTSP, the levels of IL-1, IL-6 and TNF-α in chickens' serum as well as the levels and the expression of mRNA of PGE_2 and cAMP in chickens' hypothalamus in Groups IV and V significantly decreased. Additionally, it has been reported that IL-1 itself is known to be crucial inducers of PGE_2, and at the same time, PGE_2 and cAMP can promote the transcription of IL-1 (Zasłona *et al*., 2017). Therefore, it is presumed that the mechanism may be by inhibiting the stimulation of exogenous pyrogens (IL-1, IL-6 and TNF-α) to the animal body and inhibiting the mRNA expression and the levels of PGE_2 and cAMP in the hypothalamus, so that the set-point of body temperature is lowered and thus play an antipyretic effect.

Conclusions: In conclusion, HTSP can decrease the levels of IL-1, IL-6 and TNF-α in chicken's serum as well as the mRNA expression levels of PGE2 and cAMP in chicken's hypothalamus and has good antipyretic effects on chicken fever induced by LPS.

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Authors contribution: JL conceived and designed the research. BLL, TTY, CFL and ZQW did the experiment and analyzed the data. JL, BLL and TTY wrote this paper, JL finally approved the version.

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