

The functions of *Patchouli* and *Elsholtzia* in the repair of hen follicular granular cells after heat stress

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ABSTRACT The objective of this experimental study was to examine the effects of the Chinese herbal medicines *Patchouli* and *Elsholtzia* on the follicular granulosa cells of hens undergoing heat stress conditions. In the current investigation, hen follicular granulosa cells were isolated from the prehierarchal follicles of layer hens and then cultured in-vitro. The cells were randomly divided into the 6 groups. Following the completion of this study's experiments using different heat stress and medicinal treatments, the cell activities of each group were measured using an MTT method. The levels of the heat shock protein 70 (**HSP70**) were detected using ELISA. The expressions of the steroidogenic acute regulatory protein (**StAR**) mRNA; cytochrome P450 family 11, subfamily A, member 1 (**CYP11A1**) mRNA; proliferating cell nuclear antigen (**PCNA**) mRNA; and the follicle stimulating hormone receptor (**FSHR**) were detected using the real-time quantitative polymerase chain reactions. The concentration levels of estrogen

and progesterone in the cell supernatant of each group were measured using ELISA. The results showed that cell activity had significantly decreased following the heat stress treatments at 43°C, 44°C, and 45°C ($P < 0.01$), respectively. Meanwhile, cell activities observed in *Patchouli* and *Elsholtzia* were found to be much better than those of heat stress group ($P < 0.05$). In addition, the expression levels of HSP70 in the follicular granulosa cells of *Patchouli* and *Elsholtzia* groups were lower than those of heat stress group. *Patchouli* and *Elsholtzia* can maintain expressions of the receptor at 43°C. This study determined that the estrogen and progesterone in the supernatant fluid of *Patchouli* and *Elsholtzia* were higher than those observed in heat stress. Therefore, the results obtained in this study indicated that the *Patchouli* and *Elsholtzia* treatments administered prior the heat stress experiments had successfully protected the follicular granulosa cells from heat damages while maintaining the normal secretory functions of the granulosa cells.

Key words: *Patchouli*, *Elsholtzia*, follicular granulosa cell, heat stress treatment, secretory function

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INTRODUCTION

It has been found that in the poultry industry, if the ambient environmental temperature exceeded the thermo-neutral zone (16–25°C), thermal injuries may be initiated (Abd El-Hack et al., 2020). Continuous high temperature levels can cause strong stress responses in animals, especially during the summer months on high-density farms or during long-term animal transporting processes in hot weather. These conditions may seriously affect the production performances of the poultry. Previous studies have consistently indicated that the

temperature levels of feeding environments are important factors affecting the production performances of chickens. The bodies of the affected animals will display a series of feedback expressions as external environmental temperatures rise, resulting in various heat stresses (**HS**) responses. That is to say, the defense responses and dysfunctional behaviors of animals undergoing high temperature environmental conditions have been well documented. It has been determined that the optimal ambient temperatures of laying hens range approximately between 20°C and 25°C (Tumova and Gous, 2012). However, when the temperatures increase to more than 30°C, there will be HS reactions (Liu et al., 2020). After undergoing HS, hens not only show losses of appetite and increased water consumption, but harmful effects on their reproductive systems have also been observed, such as follicular development. The quality of the oocytes tends to gradually develop into such problems as reductions in egg production, egg quality, and eggshell quality

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(Mashaly et al., 2004; Xing et al., 2019; Bei et al., 2020). Chronic HS can even cause organ damage, as it eventually leads to a decline in performance, changes in blood chemistry, and increased mortality rates. Heat stress is known to have negative effects on chicken production performances and reproductive abilities. It has been proven that the decreases in chicken production performance are related to damages of the follicular granulosa cells caused by HS (Khan et al., 2011). Therefore, it is important to protect hen follicular granulosa cells from injuries caused by heat stress while maintaining their secretory functions.

Natural products extracted from plants have been widely used in traditional Chinese medicine due to their special properties (Swamy and Sinniah, 2016; Wang et al., 2019). *Patchouli* and *Elsholtzia* have been found to promote sweating and combat rheumatism (Yao et al., 2020). *Patchouli* oil is widely used in aromatherapy to relieve depression and anxiety, and calm nerves (Li, 2013). It also is known to have a variety of pharmacological properties, including antibacterial, analgesic, anti-inflammatory, and antioxidant properties (Dantas et al., 2020). During the hot summer months, *Patchouli* and *Elsholtzia* are often added in the diets of animals to prevent the effects of sweltering conditions (Fang et al., 2003; Su, 2018). However, at the present time, few studies have been conducted regarding the use of traditional Chinese medicine to alleviate the heat stress symptoms of chicken follicular granulosa cells. In this study's experimental tests, follicular granulosa cells from follicles in the developmental stage were subjected to high-temperature treatments in-vitro. Then, the effects of the extracts from *Patchouli* and *Elsholtzia* on the cell growth, hormone secretion, and receptor expressions of follicular granulosa cells were examined after heat stress.

MATERIALS AND METHODS

This study was approved by the Experimental Animal Ethics Committee of Hebei Agricultural University.

Extractions of the Chinese Herbal Medicines

The *Patchouli* and *Elsholtzia* used in this study were purchased from Anguo Oriental Medicine City (Hebei, China). The 2 types of Chinese herbal medicine were crushed into a powder; distilled water was added according to a 1:10 ratio; and the mixtures were stirred evenly. The mixtures were then placed into an ultrasonic extractor (UE) with 200 W power at 50°C for 15 min and extracted 3 times. The extractions were pumped and filtered, respectively, using filter bottles and concentrated to 1 mg/mL using a rotary evaporator at 80°C. The samples were then stored for later use at 4°C (Zhang et al., 2021).

Isolation of the Follicular Granulosa Cells and Treatments

In the present study, follicular granulosa cells were isolated from prehierarchal follicles (6–8 mm in

diameter) of thirty 200-day-old Hy-Line brown layer hens provided by the Dingnong Corporation of Hebei (Baoding, China). The layer hens were killed using a cardio-puncturing method. This study's research experiments on live animals complied with guidelines approved by the Animal Ethics Committee of Hebei Agricultural University. The granulosa cells were separated from the follicular theca in cold phosphate-buffered saline (PBS, HyClone) using sterile needles. The cells were then dispersed with a 0.1% (w/v) collagenase II solution at 37°C for 30 min by gently shaking the samples using a constant temperature shaker. At that point, serum-containing culture fluid was added in order to terminate the digestion process and the solution was filtered using a 200-mesh sieve. Following centrifugation, the granulosa cells were washed twice with a serum-free medium, and then suspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Bethesda, MD) with 10% fetal bovine serum (FBS). The cells were subsequently placed in petri dishes or 96-well plates at a density of 1×10^6 cells/mL. This study divided the follicular granulosa cells into the 6 groups, as detailed in Table 1.

Viability of Follicular Granulosa Cells Following the Heat Stress Treatments in the Different Groups

Each of the 6 examined experimental groups was further divided into 3 heat stress groups which were subjected to temperatures of 43°C, 44°C, and 45°C. Prior to the 8 h heat stress exposure, the EXP1 and EXP3 groups were treated with *Patchouli* and *Elsholtzia* in concentrations of 1×10^{-3} mg/mL. Then, all of the groups were placed in a constant temperature incubator at 43°C, 44°C, and 45°C for a 10 h period, with the exception of the CON2 groups. Following the heat stress treatments, the EXP2 and EXP4 groups were further treated with *Patchouli* and *Elsholtzia* in concentrations 1×10^{-3} mg/mL. All of the groups were then placed in a constant temperature incubator at 37°C and 50% CO₂ for 12 h. At the end of the experiment, the culture medium of each group was collected for estrogen (E2) and progesterone (P4) detection using a radio-immunoassay method. The follicular granulosa cells were collected and each group of cells (1×10^6 cells/mL) was placed into 96-well culture plates, and treated with 10 μL of 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-

Table 1. Follicular granulosa cell grouping table.

Groups	Treatment measures
CON1	heat stress or herbal medicinal treatments
CON2	heat treatments and without drug treatments
EXP1	<i>Patchouli</i> additives prior to heat stress
EXP2	<i>Patchouli</i> treatments following heat stress
EXP3	<i>Elsholtzia</i> additives prior to heat stress
EXP4	<i>Elsholtzia</i> treatments following heat stress

Note: There were 4 repeating groups established in each treatment group of the 6 examined groups.

Table 2. Primers used for detection of proliferating cell nuclear antigen (PCNA), steroidogenic acute regulatory protein (StAR), cytochrome P450 family 11 subfamily A member 1 (CYP11A1), and follicle stimulating hormone receptor (FSHR) gene by real-time quantitative polymerase chain reaction.

Gene	Primer sequences	Amplicon size (bp)	Annealing temperature (°C)	Accession number
GAPDH-F ¹	ACGTCGCACTGGATTTCGAG	82	60	NM_204305
GAPDH-R ²	TGTCAGCAATGCCAGGGTAC			
PCNA-F ³	GCAGATGTTCTCCTCGTTGTGGAG	95	60	NM_204170.2
PCNA-R ⁴	GAGCCTTCCTGCTGGTCTTCAATC			
StAR-F ⁵	CGCTGCCATCTCCTACCAACAC	197	60	NM_204686.2
StAR-R ⁶	AGGACATCTCCATCTCGCTGAAGG			
CYP11A1-F ⁷	CCGCCACCTCAACACCAAGAC	157	60	NM_001001756.1
CYP11A1-R ⁸	CACAAGGAGGCTGAAGAGGATGC			
FSHR-F ⁹	AAGAGCGAGGTCTACATACA	414	52	XM_025148544.1
FSHR-R ¹⁰	GTGGTGTTCACAGTGATAG			

^{1,2}Refers to the forward primer and reverse primer glyceraldehyde phosphate dehydrogenase (GAPDH, a housekeeping gene as control for normalization).

^{3,4}Indicates the forward primer and reverse primer of PCNA.

^{5,6}Indicates the forward primer and reverse primer of StAR.

^{7,8}Indicates the forward primer and reverse primer of CYP11A1.

^{9,10}Indicates the forward primer and reverse primer of FSHR.

diphenyltetrazolium bromide at 37°C for 4 h. This was followed by the addition of 150 μ L of dimethyl sulphoxide (DMSO) in each well. The samples were mixed at 37°C at 200 r/min in a shaker for 30 min. Finally, the absorbance measurements were determined under 630 nm. Each group underwent 3 repetitions.

Expressions of HSP70 of the Follicular Granulosa Cells Under Different Temperature Treatment Conditions

The expressions of HSP70 were measured using an HSP70 assay kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Minhang District, Shanghai) by applying a double antibody one-step sandwich enzyme-linked immunosorbent assay (ELISA). At the end of the culturing process, the cells of each group were made into cell suspensions and centrifuged in a 1,000 r/min centrifuge for 10 min. The supernatant was extracted and handled in accordance with the instructions of the HSP70 assay kit. Finally, the OD values were determined at a wavelength of 450 nm.

Expressions of the PCNA, StAR, CYP11A1, and FSHR mRNA in the Follicular Granulosa Cells

At the conclusion of this study's experiments, the follicular granulosa cells from each group were collected and the total RNA was extracted using the TRzol reagent from a commercial RNA assay kit obtained from Invitrogen (Carlsbad, CA). Following that, reverse transcription was performed using 20 μ L of the reaction mixtures containing 12 μ L of the total RNA extraction solution and RNase-free and 4 μ L of 4 \times g DNA Wipex Mix. The mixture was heated at 42°C for 2 min, and then 4 μ L of 5 \times HiscriptII qRT SuperMix II was added to the mixture. The mixture was heated at 50°C for 15 min and at 85°C for 5 s.

PCR reaction processes were performed using 25 μ L of the reaction mixtures containing 2 μ L cDNA; 0.5 μ L forward and reverse primer (Sangon Biotech [Shanghai] Co., Ltd., Songjiang District, Shanghai) (Table 2); 12.5 μ L of 2 \times M5 Hiper SYBR Premix Es Taq (Mei5 Biotechnology Co. Ltd., Changping District, Beijing); and 9.5 μ L ddH₂O. In the current study, melting curves were used to confirm the specificity of each product, which allowed for the use of a $2^{-\Delta\Delta C_t}$ method for the calculations of the relative gene expression levels. All samples were amplified in triplicate, and the data were normalized to glyceraldehyde phosphate dehydrogenase expressions.

Patchouli and Elsholtzia in the Secretions of E2 and P4 by Follicular Granulosa Cells After Heat Stress Treatments

By the end of the culturing process, the cell-culture medium of each group was collected for E2 and P4 detections using E2 and P4 assay kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.). The cell-culture medium of each group, along with the standard blank diluent samples, was added to the ELISA Kit. All procedures were conducted according to the manufacturer's protocol. The absorbance was measured at 600 nm. A standard curve was established and the hormone content levels of each sample were calculated.

Data Analysis Results

In the present study, all of the experimental processes were repeated at least 3 times, and the results were expressed as means \pm SEM. Statistical analysis was performed using the SPSS software (Version 10.0, SPSS, Chicago, IL). All the data were analyzed using a one-way analysis of variance (ANOVA) method to determine the differences among the groups. The groups were considered to be significantly different if $P < 0.05$ (indicated by the different small case letters a, b, c, and d,

respectively). In addition, the groups were considered to be very significantly different if $P < 0.01$ (indicated by the different capital letters A, B, C, and D, respectively).

RESULTS

Effects of the Patchouli and Elsholtzia Treatments on the Viability of the Follicular Granulosa Cells After Different Heat Stress Treatments

According to the results in Table 3, compared with the CON1 groups, the activity of follicular granulosa cells in CON2 groups were extremely significantly different at 43°C, 44°C and 45°C heat treatment temperatures ($P < 0.01$), indicating that high temperature would affect the proliferation ability of cells. There was no significant difference between the pharmacotherapy and protection groups compared to the CON2 groups at the 45°C heat treatment level ($P > 0.05$), giving us the possibility that the damage caused by heat treatment at 45°C is irreversible. After 44°C heat stress treatment, compared with the CON2 groups, the EXP1 groups were extremely significantly different ($P < 0.01$), and the EXP4 groups were significantly different ($P < 0.05$). This study found that under the heat treatment level of 43°C, compared with the CON2 groups, there were differences in the EXP1, EXP3 and EXP4 groups ($P < 0.05$). The value of follicular granulosa cells viability in the EXP1 groups reached 1.82 ± 0.22 , and the EXP3 groups were 1.59 ± 0.24 . These results also indicate that high temperature levels lead to severe damage to the viability of granulosa cells. However, the value of cell viability obtained by dressings before heat treatment was higher than that obtained by dressings after heat treatment, indicating that *Patchouli* and *Elsholtzia* had the potential to improve the heat stress resistance of follicular granulosa cells before heat stress treatment. The

Table 3. Viability (OD) of follicular granulosa cells of each group under different stress temperature.

Groups	Stress temperature		
	43°C	44°C	45°C
CON1 ¹	1.73 ± 0.14^{Aa}	1.77 ± 0.20^{Aa}	1.81 ± 0.22^{Aa}
CON2 ²	1.10 ± 0.13^{Bb}	1.21 ± 0.12^{Bb}	1.37 ± 0.18^{Bb}
EXP1 ³	1.82 ± 0.22^{Aa}	1.68 ± 0.04^{Aa}	1.55 ± 0.16^{Bc}
EXP2 ⁴	1.36 ± 0.16^{Bc}	1.36 ± 0.08^{Bb}	1.37 ± 0.20^{Bb}
EXP3 ⁵	1.59 ± 0.24^{Bc}	1.58 ± 0.07^{Bc}	1.42 ± 0.09^{Bb}
EXP4 ⁶	1.43 ± 0.10^{Bc}	1.38 ± 0.07^{Bb}	1.39 ± 0.15^{Bb}

No ^{a,b,c} Indicates significant differences ($P < 0.05$), ^{A,B,C} means extremely significant differences ($P < 0.01$).

¹Control group without heat stress or medicinal treatments.

²Control group with heat treatments and without drug treatments.

³Experimental group with *Patchouli* additives prior to heat stress.

⁴Experimental group with *Patchouli* treatments following heat stress.

⁵Experimental group with *Elsholtzia* additives prior to heat stress.

⁶Experimental group with *Elsholtzia* treatments following heat stress.

data of the EXP1 groups were higher than that of the EXP3 groups, indicating that the effect of *Patchouli* additives was better than *Elsholtzia* additive.

Effects of the Patchouli and Elsholtzia Treatments on the HSP70 of Follicular Granulosa Cells Undergoing Heat Treatment Experiments In-Vitro (43°C)

As shown in Figure 1, according to the relationship curves of the concentrations of the standard and optical densities of the HSP70, the linear regression equation of absorbance x and concentration y was $y = 998.15x - 51.411$, $R^2 = 0.9996$. The relative expression of the different groups was calculated using the aforementioned linear regression equation in this study. The results showed that the expression of HSP70 in the CON2 groups was the highest, close to 300 pg/mL. There was no significant difference in HSP70 expression between EXP1 groups and CON1 groups ($P > 0.05$), and the

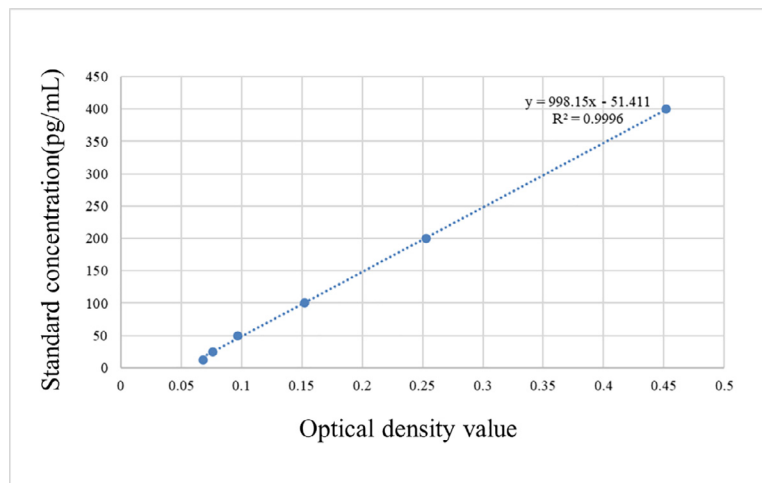


Figure 1. The relation curves of heat shock protein 70 (HSP70) concentration and optical density in follicular granulosa cells. The standard curves of HSP70 in follicular granulosa cells were drawn with different concentrations of HSP70 as standard substances, and the absorbance of HSP70 was determined at 450 nm.

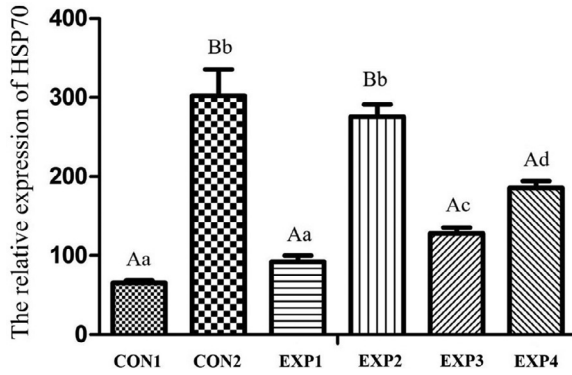


Figure 2. Heat shock protein 70 (HSP70) of follicular granulosa cells in different groups after heat treatment at 43°C. No ^{a, b, c} indicates significant differences ($P < 0.05$), ^{A, B, C} means extremely significant differences ($P < 0.01$). Control Group 1 (CON1) without heat stress or herbal medicinal treatments; Control Group 2 (CON2) with heat treatments and without drug treatments; Experimental Group 1 (EXP1) with *Patchouli* additives prior to heat stress; Experimental Group 2 (EXP2) with *Patchouli* treatments following heat stress; Experimental Group 3 (EXP3) with *Elsholtzia* additives prior to heat stress; and Experimental Group 4 (EXP4) with *Elsholtzia* treatments following heat stress.

values were both below 100 pg/mL. The data of EXP3 and EXP4 groups were significantly different ($P < 0.05$), and the expression levels of HSP70 ranged from 100 pg/mL to 200 pg/mL. It was also found that HSP70 in the EXP2 groups displayed no differences compared with the CON2 groups, as detailed in Figure 2. Therefore, the results indicated that the *Patchouli* and *Elsholtzia* had achieved effective protective effects on the follicular granulosa cells against heat stress in this study's experiments.

Effects of the Patchouli and Elsholtzia Treatments on the Expressions of PCNA, StAR, and CYP11A1 mRNA in the Follicular Granulosa Cells Under the Conditions of In-Vitro Heat Stress Treatments (43°C)

In this study's experimental investigations, the expressions of PCNA, StAR, and CYP11A1 mRNA in the CON2 groups were found to be significantly lower than the other groups ($P < 0.01$). In addition, the expression levels of PCNA, StAR, and CYP11A1 mRNA in the EXP1 groups were extremely significantly different from those of the other groups ($P < 0.01$). The expression of PCNA mRNA was about 3.36 ± 0.02 , and StAR mRNA was about 2.67 ± 0.26 . The expression levels of CYP11A1 mRNA were about 2.47 ± 0.24 , as detailed in Table 4. Compared with the CON2 groups, the expression levels of PCNA mRNA, StAR mRNA and CYP11A1 mRNA in the EXP2, EXP3 and EXP4 groups were different. The results confirmed that additions of *Patchouli* and *Elsholtzia* could effectively protect follicular granulosa cells while maintaining normal proliferation and achieving the recovery of the synthesis

Table 4. Expression of proliferating cell nuclear antigen (PCNA), steroidogenic acute regulatory protein (StAR), and cytochrome P450 family 11 subfamily A member 1 (CYP11A1) mRNA in each group after heat treatment at 43°C.

Groups	Gene		
	PCNA	StAR	CYP11A1
CON1 ¹	1.81 ± 0.17^{Aa}	1.90 ± 0.32^{Aa}	1.85 ± 0.28^{Aa}
CON2 ²	1.00 ± 0.20^{Bb}	1.25 ± 0.22^B	0.81 ± 0.09^B
EXP1 ³	3.36 ± 0.02^C	2.67 ± 0.26^C	2.47 ± 0.24^C
EXP2 ⁴	1.53 ± 0.11^{Bc}	1.63 ± 0.16^{Aa}	1.37 ± 0.29^{Ab}
EXP3 ⁵	2.30 ± 0.17^{Ab}	2.26 ± 0.13^{Ac}	1.96 ± 0.04^{Aa}
EXP4 ⁶	1.74 ± 0.25^{Aa}	2.04 ± 0.03^{Aa}	1.63 ± 0.28^{Ac}

No ^{a, b, c} indicates significant differences ($P < 0.05$), ^{A, B, C} means extremely significant differences ($P < 0.01$).

¹Control group without heat stress or medicinal treatments.

²Control group with heat treatments and without drug treatments.

³Experimental group with *Patchouli* additives prior to heat stress.

⁴Experimental group with *Patchouli* treatments following heat stress.

⁵Experimental group with *Elsholtzia* additives prior to heat stress.

⁶Experimental group with *Elsholtzia* treatments following heat stress.

functions of key enzymes of hormones following heat stress exposure.

Effects of the Patchouli and Elsholtzia Treatments on the Expressions of the FSHR mRNA of the Follicular Granulosa Cells Undergoing Heat Stress Conditions In-Vitro (43°C)

The expression levels of FSHR mRNA in the follicular granulosa cells undergoing 43°C heat treatments were reviewed in this study. Though the difference analysis, the CON2 groups were extremely significantly different from the other groups ($P < 0.01$), and the expression of FSHR mRNA was less than 1. In addition, it was found that the expression of FSHR mRNA in the EXP1 groups

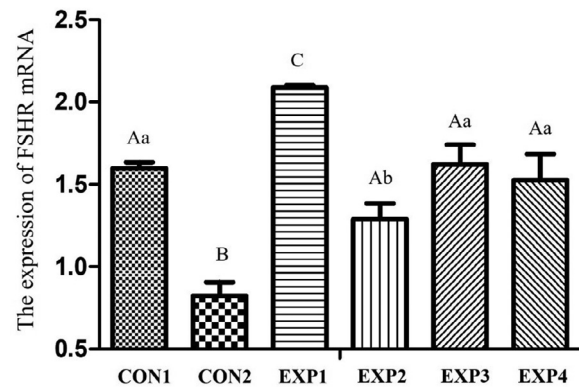


Figure 3. Expression of follicle stimulating hormone receptor (FSHR) mRNA in each group after heat treatment at 43°C. No ^{a, b, c} indicates significant differences ($P < 0.05$), ^{A, B, C} means extremely significant differences ($P < 0.01$). Control Group 1 (CON1) without heat stress or herbal medicinal treatments; Control Group 2 (CON2) with heat treatments and without drug treatments; Experimental Group 1 (EXP1) with *Patchouli* additives prior to heat stress; Experimental Group 2 (EXP2) with *Patchouli* treatments following heat stress; Experimental Group 3 (EXP3) with *Elsholtzia* additives prior to heat stress; and Experimental Group 4 (EXP4) with *Elsholtzia* treatments following heat stress.

Table 5. Concentration of estrogen (E2) and progesterone (P4) in supernatant fluid of each group after heat treatment at 43°C.

Groups	Hormone	
	E2	P4
CON1 ¹	193.5 ± 29.2 ^{Aa}	481.4 ± 19.1 ^{Aa}
CON2 ²	67.5 ± 16.7 ^{Bb}	280.1 ± 26.5 ^{Bb}
EXP1 ³	132.4 ± 11.1 ^{Ac}	347.7 ± 27.0 ^{Bc}
EXP2 ⁴	68.9 ± 0.8 ^{Bb}	290.3 ± 17.7 ^{Bb}
EXP3 ⁵	90.2 ± 0.3 ^{Bb}	319.8 ± 19.9 ^{Bc}
EXP4 ⁶	69.7 ± 5.9 ^{Bb}	295.2 ± 23.3 ^{Bb}

No ^{a,b,c} Indicates significant differences ($P < 0.05$), ^{A,B,C} means extremely significant differences ($P < 0.01$).

¹Control group without heat stress or medicinal treatments.

²Control group with heat treatments and without drug treatments.

³Experimental group with *Patchouli* additives prior to heat stress.

⁴Experimental group with *Patchouli* treatments following heat stress.

⁵Experimental group with *Elsholtzia* additives prior to heat stress.

⁶Experimental group with *Elsholtzia* treatments following heat stress.

reached 2, which was extremely significantly higher than that of the other groups ($P < 0.01$), as illustrated in [Figure 3](#). The expression levels of FSHR mRNA in the EXP3 and EXP4 groups were observed to have no obvious differences when compared with the CON1 groups ($P > 0.05$).

Effects of the *Patchouli* and *Elsholtzia* Treatments on the Secretion of E2 and P4 in the Follicular Granulosa Cells Under In-Vitro Heat Treatment Conditions (43°C)

The effects of the *Patchouli* and *Elsholtzia* additions on the secretions of E2 and P4 in the follicular granulosa cells undergoing in-vitro heat treatment conditions at 43°C are detailed in [Table 5](#). The levels of E2 and P4 in the CON2 groups were found to be extremely lower than those of the CON1 groups ($P < 0.01$). The level of E2 in the EXP1 groups were extremely higher than that of the CON2 groups ($P < 0.01$), and the level of P4 was higher than the CON2 groups ($P < 0.05$). The levels of E2 and P4 in the EXP3 groups were both higher than those observed in the CON2 groups ($P < 0.05$), and E2 and P4 in the EXP2 and EXP4 groups had no displayed differences when compared with the CON2 groups. These results inferred that the additions of *Patchouli* and *Elsholtzia* prior to the heat stress treatments were beneficial for the protection of the follicular granulosa cells' secretory functions.

DISCUSSION

This research study successfully determined that the additions of *Patchouli* and *Elsholtzia* prior to heat stress treatments could effectively protect the follicular granulosa cells of hens from heat stress injury, while normal cell viability, cell proliferation, and hormone secretion functions were maintained.

It has been determined that follicular granulosa cells play a key role in the growth and selection of follicles ([Zhu et al., 2019](#)). The development period of follicular

granulosa cells in small white follicles is very important for hormone secretion function. In this study, small white follicles were selected for granular cell separation and culturing processes. The effects of the 2 tested traditional Chinese medicines *Patchouli* and *Elsholtzia* on the activities of primary granular cells undergoing heat stress treatments were then analyzed. The damages to the follicular granulosa cells which had occurred under the heat treatment temperatures of 43°C, 44°C, and 45°C were compared. It could be seen from the results of the viability of that follicular granulosa cells that the addition of *Patchouli* prior to heat stress treatments at 43°C and 44°C had resulted in the follicular granulosa cells maintaining normal viability. There were no differences observed when compared with the CON1 groups. Also, the protective effects of the *Elsholtzia* were found to be lower than those of the *Patchouli*. However, when the temperature level rose to 45°C, regardless of being added before or after the heat stress treatments, it was found that neither the *Patchouli* nor *Elsholtzia* could positively protect the cells from heat stress damage. These results indicated that since the heat stress injuries were irreversible ([Ippolito et al., 2014](#)), the additions of *Patchouli* and *Elsholtzia* after heat stress had occurred were not effective in repairing the viability of the follicular granulosa cells.

In this experimental investigation, it was found that adding *Patchouli* and *Elsholtzia* prior to the heat treatments had significant effects on the HSP70 production. It is known that heat stress can activate the expressions of the heat shock 70kDa protein in GCs. HSP70 is known as the main heat shock protein and is highly conserved. It is located in the cytoplasm. Under normal conditions, its expression levels are very low. However, under the condition of heat stress, and the synthesis of HSP70 becomes significantly increased in order to maintain the antiheat stress abilities of biological bodies ([Rimoldi et al., 2015](#)). In this study, the HSP70 in the CON2 groups were observed to be significantly increased following heat treatments at 43°C, 44°C, and 45°C. It has been confirmed that HSP70 plays a major role in enhancing cell tolerance and resisting injuries caused by stress ([Zuo et al., 2016](#)). However, in the EXP1 and EXP3 groups, it was found that the additions of *Patchouli* and *Elsholtzia* prior to the occurrence of heat stress had effectively protected the follicular granulosa cells from high temperature damage. At the same time, the normal expressions of HSP70 were maintained, and no differences were observed when compared with the CON1 groups. HSP70 is a protein that is known to be responsible for repairing damaged cells, and has the ability to improve cell tolerance to the environmental conditions while maintaining stable internal environments ([Kamboh et al., 2013](#); [Jiang et al., 2020](#)). The results obtained in this study indicated that the additions of *Patchouli* and *Elsholtzia* prior to the heat treatments had successfully maintained stable internal environmental conditions under high temperatures, thereby maintaining the HSP70 at normal levels. The reason may have been that the main components of the *Patchouli*

and *Elsholtzia* had acted on the granulosa cells. The main active components of the *Patchouli* and *Elsholtzia splendens* are volatile oil, inorganic elements, flavonoids, and so on, which are known to possess antiviral, antibacterial, antioxidant, and anti-inflammatory effects (Pudziuvyte et al., 2017). Alternatively, the applied volatile oils may have resulted in antiheat stress effects (Liu et al., 2016; Bai et al., 2018). However, the mechanisms of *Patchouli* and *Elsholtzia* with regard to the granulosa cells' antiheat stress processes require further study.

PCNA is a nuclear protein of DNA, and plays an important role in DNA replication, cell cycle regulation, and epigenetics (Boehm et al., 2016). In the present study, the heat stress conditions had reduced the expressions of PCNA mRNA in the primary granule cells. However, it was found that the additions of *Patchouli* and *Elsholtzia* prior to the heat treatments increased the expressions of PCNA mRNA. Therefore, the results indicated that *Patchouli* and *Elsholtzia* could maintain the normal proliferation of primary granule cells. However, the mechanisms of those actions require further research and verification.

It was confirmed in this study that *Patchouli* and *Elsholtzia* increased the expressions of FSHR, StAR, and CYP11A1 mRNA after heat stress. The most important feature of chicken follicle selection is the increased expression of FSHR mRNA in granulosa cells (Johnson, 2012). In mammals, follicular development is a selective and continuous process, and the activities and hormone levels of granulosa cells during the different stages of follicle develop will vary (Onagbesan et al., 2009; Johnson., 2015). In poultry, the growth rates of adult follicles are also connected with follicle stimulating hormones (FSH) and luteinizing hormones (LH). During the different development stages of follicles, the expressions of FSHR and luteinizing hormone receptors (LHR) may differ. In prehierarchical follicles (phGCs), the transcriptional expressions of FSHR mRNA tend to be higher than those of LHR mRNA, with FSHR inducing the expressions of LHR (O'Shaughnessy et al., 1997). It has been found that when LH is secreted in large quantities and specifically binds to LHR, it can promote the expressions of StAR. StAR is a limiting regulator of all steroid synthesis and can transport cholesterol into the cytoplasm. After cholesterol has been transported, pregnenolone is synthesized by CYP11A1, and pregnenolone (P5) can be converted into progesterone (Sechman, 2013). Therefore, the expressions of StAR and CYP11A1 are considered to be very important since they are related to the synthesis of progesterone (Johnson and Bridgham, 2001). The results of this study showed that *Patchouli* and *Elsholtzia* could potentially protect the granulosa cells while maintaining their normal development into adult cells. Therefore, the granulosa cells in primordial follicles, in which FSHR is beginning to be expressed, are very important for the primordial follicles initiations into the primary stages, during which cell proliferation and differentiation are promoted through multiple signal pathways

(Sechman et al., 2014). The expressions of StAR and CYP11A1 transcription levels can be enhanced for the purpose of promoting the synthesis of progesterone by granulosa cells (Das and Kumar, 2018). In the present study, the additions of *Patchouli* and *Elsholtzia* prior to the heat treatments were found to alleviate the damages to pregrading granular cells caused by the high temperatures. It was found that the high temperature levels reduced the estrogen and progesterone secretions of the follicular granulosa cells. It was observed that when compared with the heat treatment group, additions of *Patchouli* and *Elsholtzia* increased the secretions of cellular estrogen and progesterone. It was speculated that the reasons for the observed results were that the *Patchouli* and *Elsholtzia* had increased the expressions of FSHR, StAR, and CYP11A1. It has been reported that 3 β -hydroxysteroid dehydrogenase (3 β -HSD) is the key enzyme of P4, and that the inhibition of 3 β -HSD can significantly reduce P4. Due to the fact that phGCs are undifferentiated, the expressions of StAR and CYP11A1 are low. Therefore, the synthesis of P4 tends to be less. However, membrane cells still have the ability to catalyze the synthesis of estradiol through aromatase (Goodman et al., 1998; Johnson and Bridgham, 2001).

In conclusion, this study determined that the additions of *Patchouli* and *Elsholtzia* prior to heat treatments of the cells can effectively protect the cell viability; promote the proliferation and differentiation of follicular granulosa cells; and reduce the damage to the cells caused by high temperatures. This study provided a method for protecting against damage to hen follicular granulosa cells caused by heat stress, and provided an experimental basis for the future application of *Patchouli* and *Elsholtzia* for the prevention of heat stress in hens during the hot summer months.

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DISCLOSURES

The authors declare there were no conflicts of interest regarding the publication of this manuscript.

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