Improving effects of *Epimedium* flavonoids on the selected reproductive features in layer hens after forced molting

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ABSTRACT In the present study, for the purpose of investigating the effects of the total flavonoids of *Epimedium* (**TFE**) in regard to preventing the development of atrophied oviducts and follicles induced by forced molting, 300-day-old Hy-Line Brown layer hens were divided into 3 study groups as follows: the control (**CON**) group was the normal group, without forced molting and TFE treatments; the TFE1 group was treated by adding a 1‰ TFE treatment after forced molting; and the TFE0 group was not treated by TFE after forced molting. During this study's experimental process, the egg production rates were recorded each day. In addition, the hens were randomly chosen to be weighed every 4 D and also randomly selected to be sacrificed every 7 D. Then, sample tissues of albumen-secreting part and uterus from the fallopian tube of the layer hens were collected for PCR and hematoxylin-eosin staining tests. The results showed that the body weights, number of follicles, and weights and sizes of the fallopian tube for the TFE1 and TFE0 groups were significantly reduced when compared with those of the control group on the 15th D of the experiment. Furthermore, at the end of study, it was found that the egg production rates, weights of the fallopian tube, and ovarian follicles of TFE1 had recovered to normal levels. At the same time, the serum estrogen and the expressions of the progesterone receptor and estrogen receptor mRNA in fallopian tube were higher than those observed for the TFE0 group. The results of this study provided valuable evidence that TFE could improve the development of atrophied oviducts and increase the egg laying rates, thereby making it a potential multicomponent natural drug for egg production in the future.

Key words: fallopian tube, forced molting, layer hen, total flavonoid of *Epimedium*

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INTRODUCTION

Molt induction is an important tool used to prolong the production cycles of laying hens by egg producers on commercial poultry farms (Andreatti Filho et al., 2019). Artificially forced molting causes the layer hens to lose body weight and shed feathers due to hunger and light deprivation. The hens go into reduction states resulting in various hormone secretions, which ultimately lead to follicular and reproductive system organ atrophy (Gongruttananun et al., 2017; Socha et al., 2017). Significant regression in ovary and oviduct weights ($P < 0.01$) has been observed in molting hens (Bozkurt et al., 2016). It was found that the oviduct weights and lengths of the late-phase molting layer hens not fully fed with cassava meal for 3 or 4 wk were diminished in comparison with the control group (Gongruttananun et al., 2017). At the present time, forced molting practices are widely used in the poultry farming industry in China.

Egg formation takes place in the oviducts of laying hens over a 24-h period (Khan et al., 2019). The development and normal functioning of the oviducts are very important for the hens' laying abilities. The oviducts, especially the magnum and the uterus, play indispensable roles in the formation of eggs (Yin et al., 2019). The oviductal magnum of laying hens is the organ which is responsible for the synthesis and secretion of egg white proteins. It has been found that environmental stress can differentially modify the expressions of egg white proteins in laying hens (Kim and Choi, 2014). For example, infectious bronchitis virus may cause pathological lesions in the chicken oviducts (Khan et al., 2019; Wibowo et al., 2019). It has been observed that any changes in the development and functions of the oviducts of laying hens will directly affect both egg

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production and egg quality (Alsafy et al., 2019). Therefore, to assure the high egg production and quality performance of eggs in layer hens, it is important to protect the oviduct of layer hens from being injured and improve the atrophied oviduct development especially after forced molting.

In poultry, estrogen can promote follicle development and activation involving its increased receptor (Brady et al., 2019), progesterone can stimulate the development of reproductive system and make hens reach the laying period and the high expression of progesterone receptor (**PR**) in uterus part is very important for eggshell formation (González-Morán, 2016). Although hormones possess protective properties, the use of hormonal drugs in the production of food-providing animals is not permitted in China. *Epimedium* is a genus of approximately 52 species in the family Berberidaceae (Ma et al., 2011). Epimedium, also referred to as Yin Yang Huo in Chinese, is a type of herbal medicine used in China, Japan, and Korea (Ma et al., 2011) for curing reproductive diseases in both male and female animals (Zhu et al., 2012; Zhang et al., 2015). Many of the plants of the *Epimedium* species have been proven to possess efficacy for sexual dysfunction and osteoporosis and more than 260 compounds have been isolated. Among those compounds, total flavonoids are the major constituents known to possess wide pharmacological actions. In particular, total flavonoids are used for strengthening kidney yang (enhancing reproductive function); hormone regulation; anti-osteoporosis; immunological function modulation; anti-oxidation; antitumor; antiaging; antiatherosclerosis; and antidepressant activities (Ma et al., 2011). The total flavonoids of *Epimedium* (**TFE**) were found to play a role similar to estrogen in ovariectomized mice by improving the coefficients of uterine weight and promoting endometrium thickening (Wang et al., 2012). It has also been determined that TFE had displayed the ability to effectively reduce oxidative DNA damage in the testis of aging rats (Zhao et al., 2017). In addition, previous research results demonstrated that TFE exerted beneficially protective effects on the structural and functional damage of reproductive systems of male mice and reduced apoptosis in spermatogenic cells (Yuan et al., 2014). It has been found that anhydroicaritin and genistein have the ability to enhance human breast cancer MCF-7 cell proliferation by estrogen receptor (**ER**) α (Zhu et al., 2017). Previous studies have revealed that TFE can decrease ovarian androgen secretion, raise serum estradiol ($\mathbf{E_2}$) level, and improve ovary functions in polycystic ovary syndrome rats (Xu et al., 2013). However, it is not yet clear whether or not TFE can positively affect the development of atrophied oviducts and improve the laying abilities of hens.

In this study, an atrophied oviduct model was established for hens that experienced forced molting. Then, the effects of TFE on the development of the hens' fallopian tube and follicles, estrogen concentration, and expressions of the PR and ER mRNA in the fallopian tube of the layer hens were observed.

MATERIALS AND METHODS

Extraction and Determination of the TFE

The *Epimedium* used in this study were purchased from the Anguo Oriental Medicine City (Hebei, China). First, the *Edimedium* were crushed into powder, and then 75% ethanol aqueous solution was added in a 1:30 ratio. The solution was allowed to sit undisturbed for 30 min at room temperature (Wang et al., 2017). The active components of the *Epimedium* were extracted using an ultrasonic method in an ultrasonic purifying device, with a power of 200 W at 50°C for 45 min (Zhang et al., 2008). The solution was then filtered using a filter paper and concentrated to 1 mg/mL using a rotary evaporator at 78°C. The evaporated liquid was then stored at 4°C.

The quantitative determination of the TFE was measured by colorimetry at 496 nm, using rutin as the reference and aluminum nitrate as the chromogenic agent (Guo and Zhang, 2019). This study acquired 2 mg of rutin standard products from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), which were added to 25 mL of 95% ethanol solution, and then diluted to 0.04 mg/mL, 0.02 mg/mL, 0.01 mg/mL, and 0.005 mg/mL,. The total flavonoids content was determined using a spectrophotometric method, with rutin as the standard sample. Then, 1.0 mL of 5% sodium nitrite solution was added to all of the samples. The samples were then shaken and allowed to sit undisturbed for 6 min at room temperature. After which, 1.0 mL of 10% of aluminum nitrate solution was added to all of the samples, which were once again shaken and allowed to sit for 6 min. Finally, 10.0 mL of 4% sodium hydroxide solution was added to each sample. The samples were then shaken and allowed to sit for a 12-min period. A solution without samples was used as a blank reference. The absorbance measurement was determined to be 496 nm.

Experimental Animals and Treatment Method

In the present study, 300-day-old Hy-Line Brown layer hens were provided by Ding Nong Corporation of Hebei (Baoding, China). The hens were divided into 3 groups (normal group without forced molting [**CON**], 1‰ TFE treatment after forced molting [**TFE1**], and no TFE treatment after forced molting [**TFE0**]) with 50 layer hens in each group and 5 repeating groups in each treatment group. All of the hens were raised in the poultry breeding farm of Hebei Agricultural University. The room temperature was maintained between 22°C and 24°C. Water for the hens was supplied *ad libitum*, and daily rations were supplied as per the nutrient suggestions for layer hens (Yin et al., 2014). The hens of 3 groups were raised for 5 D on the same condition before beginning of the experiment. Then, during the first 15 D of the experimental trial period, the hens in the CON

group were fed ad libitum on a regular layer hen diet and not subjected to molting with normal illumination (16L: 8D). At the same time, the hens of TFE1 and TFE0 groups were fasted and light shaded by a dark nylon cover that allowed no light transfer to isolate molted hens from that of the unmolted hens (Bozkurt et al., 2016). The molted period was 15 D; hens of 3 groups drank water *ad libitum* throughout the experiment. From the 16th D to the 30th D of the experiment, the hens in the TFE1 group were fed with a daily diet containing 1‰ TFE, and the hens in TFE0 were fed with a daily diet that did not contain TFE, but the amount of diet was given one-fifth of the normal amount on the 16th D and added gradually to the normal diet on the 20th D, maintaining until the end of the experiment. Both TFE1 and TFE0 groups were maintained in a normal light–dark cycle (16L: 8D) from the 16th D to the 30th D. As the normal control group, the CON group hens were fed with a layer diet with normal illumination throughout the experiment. The egg production rates during the experiment were recorded each day, and the average egg production rate and egg productive ratio were calculated. During this study's experiment, 20 layers were randomly selected to be weighed on different days (day 1, day 4, day 7, day 11, day 15, day 19, day 22, day 26, and day 30). In addition, 10 of the experimental animals were randomly selected to be killed by cardiopuncturing on day 1, day 7, day 15, day 22, and day 30. Blood was collected from the heart in each group, and serum was obtained with separating gel vacuum tubes for the measurement of E_2. The fallopian tube was weighed, and the relative weights were calculated using the absolute fallopian tube weights/body weights. Then, samples of the albumen-secreting part and uterus were extracted from the fallopian tube for polymerase chain reaction (**PCR**) and hematoxylin-eosin (**HE**) staining.

Histomorphology of the Albumen-Secreting Part and Uterus From the Fallopian Tube

The harvested tissues of the albumen-secreting part and uterus from the laying hens' fallopian tube were fixed in 4% paraformaldehyde solution buffered by phosphate buffer saline with pH 7.4 for more than a 12-h period. The tissue samples were then embedded in paraffin. Histological sections of 5-μm thickness were sliced using an automatic slicer and subsequently used for HE staining.

Expressions of ER and PR mRNA in the Fallopian Tube

In this study, the total RNA was extracted from the uterus and albumen-secreting part of the laying hens' fallopian tube on the 15th D and 30th D of the experimental process using the TRIzol reagent of a commercial RNA assay kit obtained from the Invitrogen Co. of Carlsbad, California (US) as per the manufacturer's instructions. Then, reverse transcription was performed using 25 μL of the reaction mixtures containing 10 μL of the total RNA extraction solution, 2 μL Olig (dT),2 μL RNase inhibitor, 5 μL dNTPs, 5 μL 5× M-MLV buffer, and 1 μL M-MLV reverse transcriptase. Before the addition of 1 μL of the M-MLV (100 U) reverse transcriptase, the other components were incubated at 65°C for 5 min and then placed on ice for 5 min. After 1 μL of the M-MLV (100 U) reverse transcriptase was added, reverse transcription was conducted under 42°C conditions for 1 h.

The PCR reaction process was performed using 25 μL of the reaction mixtures containing 2 μL cDNA; 0.5 μL forward and reverse primer (Sangon Biotech (Shanghai) Co., Shanghai, China) (see Table 1); 10 × 2.5 μL of buffer; 2 μL of dNTP; 0.5 μL of taqase; and 17 μL of H_2O. The PCR reaction process was conducted under the following cycling conditions: predenaturation at 94°C for 2 min; amplification for 35 cycles with denaturation at 94°C for 1 min; annealing at the same temperature for 1 min; extension at 65°C for 1 min; followed by a final extension at 72°C for 10 min (Joensuu, 1990; Mowa and Iwanaga, 2000). Then, in accordance with the conventional method, the PCR products were electrophoresed and photographed using a gelatin imager, and the results were observed and calculated to determine the relative value of the target band to the glyceraldehyde phosphate dehydrogenase (**GAPDH**) using an AlphaImager 2200 device acquired from the Alpha Innotech Corporation of San Leandro, California (US).

Detection of the Serum E_2

The E_2 concentrations were assayed by radioimmunoassay using commercially available kits from the Northern Biotechnology Co. (Beijing, China). The sensitivity was 2 pg/mL; intra-assay coefficient of variation was less than 9.5%; and the interassay coefficient of variation was determined to be less than 10.3%.

Results of the Statistical Analyses

All the experiments were repeated at least 3 times, and the results were expressed as means ± SEM (n = 10 per group). In addition, all of the data were analyzed using a one-way analysis of variance method to determine the

Table 1. Primers used for detection of PR and ERα gene.

Gene primer sequences product size (bp) annealing temp. (°C)
PR-F[1] 5′-CTTCTGGTTGCCCATCGG-3′ 141 56
PR-R[2] 5′-ACTTCCTCAAGCGACACG-3′
ERα-F[3] 5′-GGGTCTGGTCTTGTGAGG-3′ 273 55
ERα-R[4] 5′-GACTTGTCCAAAGGGTTG-3′
GAPDH-F[5] 5′-TCACAAGTTTCCCGTTCTCA-3′ 226 54
GAPDH-R[6] 5′-GGAACACTATAAAGGCGAGAT-3′

[1,2]The forward primer and reverse primer of progesterone receptor (PR).
[3,4]The forward primer and reverse primer of estrogen receptor α (ERα).
[5,6]The forward primer and reverse primer of glyceraldehyde phosphate dehydrogenase (GAPDH, a housekeeping gene as control for normalization).

differences among the 3 groups using SPSS software (version 10.0, SPSS. Chicago, IL). In the present study, the groups were considered to be significantly different if $P < 0.05$.

RESULTS

TFE Concentrations of Epimedium *Extract*

In accordance with the relationship curves of the optical density and the TFE concentrations of rutin (Figure 1), the linear regression equation of the absorbance Y and concentration X was determined to be y = 14.061x + 0.0046, R^2 = 0.9991. The average OD value of the *Epimedium* extract solution was brought into the linear regression equation to calculate the concentration. The results showed that the TFE concentration of *Epimedium* extract was 16.5 ± 0.32 mg/mL, and the weight content of TFE powder had reached 45%.

Effects of the TFE on the Body Weight Recovery Rates

According to the results shown in Figure 2, it can be seen that the body weights of the hens in the TFE1 and TFE0 groups were reduced by one-third when compared with the CON group on the 15th D of the experiment ($P < 0.01$). However, the body weights gradually began to recover after a basal diet feeding program and normal illumination conditions had commenced. The body weights of the hens in the TFE1 group were found to have recovered back to normal on the 30th D and were significantly higher than those of the hens in the TFE0 group ($P < 0.05$) on the 30th D. These results indicated that TFE could effectively improve the body weights of the laying hens.

Effects of the TFE on the Egg Production Rates

As detailed in Table 2, the egg production ratios of the TFE1 and TFE0 groups were reduced to 11.14% and 13.69% on the 15th D of the experiment, respectively. Then, after the TFE treatments, it was observed that the laying hens in the TFE1 group had produced eggs earlier than those of the TFE0 group. The egg production ratio of the TFE1 group had increased to 91.34% on the 30th D of the experiment, which was significantly higher ($P < 0.01$) than the egg production ratio observed for the TFE0 group at only 77.36%. Therefore, the results obtained in this study's experiments indicated that TFE could increase egg production.

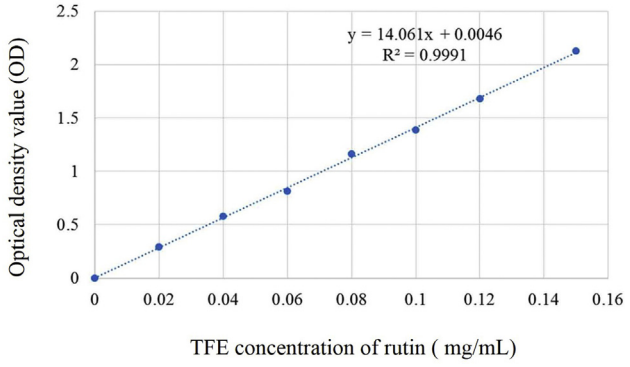

Figure 1. Relationship curves of the OD values and TFE concentrations of rutin. The drafting of TFE standard curves was using rutin as the standard sample, the absorbance measurement was determined at 496 nm. Abbreviations: OD, optical density; TFE, total flavonoids of *Epimedium*.

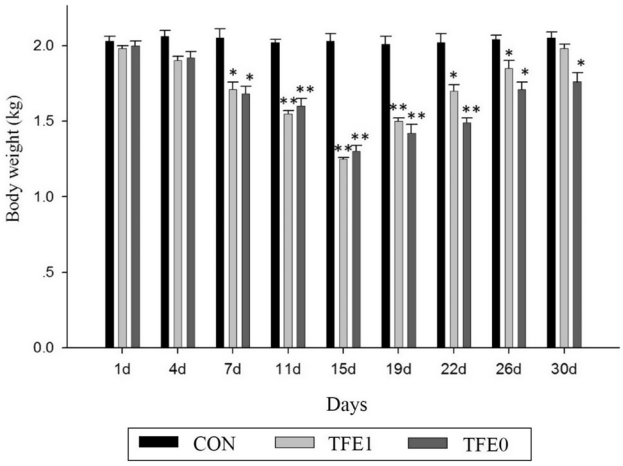

Figure 2. Body weights of the laying hens in the CON, TFE1, and TFE0 group at different times during the experimental process. *$P < 0.05$; **$P < 0.01$. Abbreviations: CON, control; TFE, total flavonoids of *Epimedium*; TFE1, 1‰ TFE treatment after forced molting; TFE0, no TFE treatment after forced molting.

Effects of TFE on Follicular Growth and Development of the Fallopian Tube

It was found in this study that the follicle numbers of the TFE1 and TFE0 group were obviously decreased on the 15th D of experiment, as illustrated in images B

Table 2. The egg productive ratios of each group at different times during the experiment (n = 50).

Groups	Egg productive ratio (%)		
	1st D[1]	15th D[2]	30th D[3]
CON [4]	89.32 ± 3.02	88.90 ± 2.84	89.09 ± 4.12
TFE1[5]	88.96 ± 4.64	11.14 ± 4.62**	91.34 ± 2.52
TFE0 [6]	87.92 ± 4.60	13.66 ± 3.10**	77.36 ± 3.96**

Abbreviations: CON, control; TFE, total flavonoids of *Epimedium*; TFE1, 1‰ TFE treatment after forced molting; TFE0, no TFE treatment after forced molting.

**Extremely significant differences comparing the TFE1 and TFE0 group with the CON group ($P < 0.01$).

[1,2]Egg productive ratios were calculated on the first day and 15th D of forced molting.

[3]Egg productive ratios were calculated on the 30th D of the experiment (the last day of TFE treatment from 16th D to 30th D).

[4]The control group without forced molting and TFE treatment.

[5]The group with forced molting from first day to 15th D, and then with 1‰ TFE treatment from 16th D to 30th D of the experiment.

[6]The group forced molting from first day to 15th D, and then without TFE treatment from 16th D to 30th D.

Table 3. Levels of estradiol in the serum of each group.

Groups	Concentrations of estradiol (pmol/L)	
	15th D[1]	30th D[2]
CON[3]	664.6 ± 12.3	627.7 ± 25.4
TFE1[4]	201.2 ± 10.5**	674.7 ± 32.5
TFE0[5]	216.5 ± 19.2**	557.0 ± 19.8*

Abbreviations: CON, control; TFE, total flavonoids of *Epimedium*; TFE1, 1‰ TFE treatment after forced molting; TFE0, no TFE treatment after forced molting.

*Indicates significant differences comparing the TFE1 and TFE0 group with the CON group ($P < 0.05$).

**Extremely significant differences comparing the TFE1 and TFE0 group with the CON group ($P < 0.01$).

[1]Egg productive ratios were calculated on the 15th D of forced molting (the last day of molting).

[2]Egg productive ratios were calculated on the 30th D of the experiment (the last day of TFE treatment from 15th D to 30th D).

[3]The control group without forced molting and TFE treatment.

[4]The group with forced molting from first day to 15th D, and then with 1‰ TFE treatment from 16th D to 30th D of the experiment.

[5]The group with forced molting from first day to 15th D, and then without TFE treatment from 16th D to 30th D.

and C of Figure 3. In addition, only prehierarchical follicles were found, with preovulatory follicles undetectable in the ovaries of the laying hens by the 15th D of the experiment. The fallopian tube of the hens in the TFE1 and TFE0 groups were found to be atrophied. It was further observed that the relative weights of the fallopian tube of the hens from the TFE1 and TFE0 groups were lower than those of the CON group on the seventh day ($P < 0.05$) and remarkably lower than those of the CON group on the 15th D ($P < 0.01$), as shown in Figure 4. In the present study, after the restoration of light and normal feeding conditions, it was determined that the number of follicles of the TFE1 group (with TFE treatments) had recovered to normal, and the preovulatory follicles were much more abundant than those observed in the TFE0 group (without TFE treatments). In addition, the relative weights of the fallopian tube of the TFE1 hens were higher than those of the TFE0 hens on the 22th D of the experiment and had recovered to a normal level on the 30th D of experiment, as shown in Figure 4. It was observed that the relative weights of the fallopian tube from the TFE0 group had slowly increased and were significantly lower than those of TFE1 and CON on the 30th D ($P < 0.05$). Therefore, the results indicated that TFE had displayed the ability to improve the development of follicles and fallopian tube in the examined laying hens.

Histomorphology of the Fallopian Tube

On the 30th D of the experiment, it was found that in the uterus and albumen-secreting part of the fallopian tube of the laying hens, the ciliated columnar epithelial cells appeared to be normal and arranged in order in the TFE1 group, with intact serosa observed, as shown in images C and D of Figure 5. A small number of mucosal lesions and sparse cilia were observed in the TFE0 group in the same time period. In addition, there was minor atrophy of the uterus and albumen-secreting part observed in the TFE0 laying hens, as illustrated in images A and B of Figure 5. Therefore, the results indicated that TFE had the ability to stimulate the development of the uterus and albumen-secreting part of the hens' fallopian tube.

Effects of TFE on the Expressions of PR mRNA and ERα mRNA in the Fallopian Tube of the Laying Hens

As detailed in Figures 6 and 7, the results of the analyses of the PR mRNA and ERα mRNA expressions in the fallopian tube of the laying hens on the 15th and 30th D of the experiment, respectively, were as follows: The PR mRNA of the uterus and albumen-secreting part from TFE1 were not expressed on the 15th D, and the relative value to GAPDH of the PR in the uterus and albumen-secreting part from the TFE1 was found to be extremely lower than those of the CON group ($P < 0.01$). The ERα mRNA of the uterus and albumen-secreting part from the TFE1 were found to be weakly expressed, and the relative value to GAPDH of the ERα mRNA in the uterus and albumen-secreting part of TFE1 was determined to be significantly lower than those of CON ($P < 0.05$). These results showed that the forced molting had not only caused body weight and fallopian tube weight reductions but had also inhibited the PR mRNA and ERα mRNA expressions.

On the 30th D of the experiment, the PR mRNA of the uterus and albumen-secreting part from TFE1 were expressed normally and similar to those of CON. In addition, the relative value to GAPDH wase observed to be significantly higher than those of TFE0 ($P < 0.01$). The ERα mRNA of the uterus sections from the TFE1 and TFE0 groups were found to have expressions similar to the CON group, and no significant differences were observed in the relative value to GAPDH of the ERα mRNA in the uterus sections of TFE1 and TFE0 when compared with CON ($P > 0.05$). The relative values to GAPDH of the ERα mRNA in the albumen-secreting part of TFE1 and TFE0 were observed to be lower than those of CON. These results indicated that the TFE could potentially improve the expressions of PR mRNA in the fallopian tube of the laying hens but had no effect on the expressions of the ERα mRNA.

Effects of TFE on the Levels of Serum E_2 Among the Different Groups

On the 15th D of this study's experiment, it was found that the levels of serum E_2 were significantly lower in TFE1 and TFE0 ($P < 0.01$). However, the levels of serum E_2 of TFE1 had improved to normal by the 30th D of the experiment, and the levels of serum E_2 of TFE0 were determined to be lower than those of TFE1 and CON ($P < 0.05$). These results indicated that the

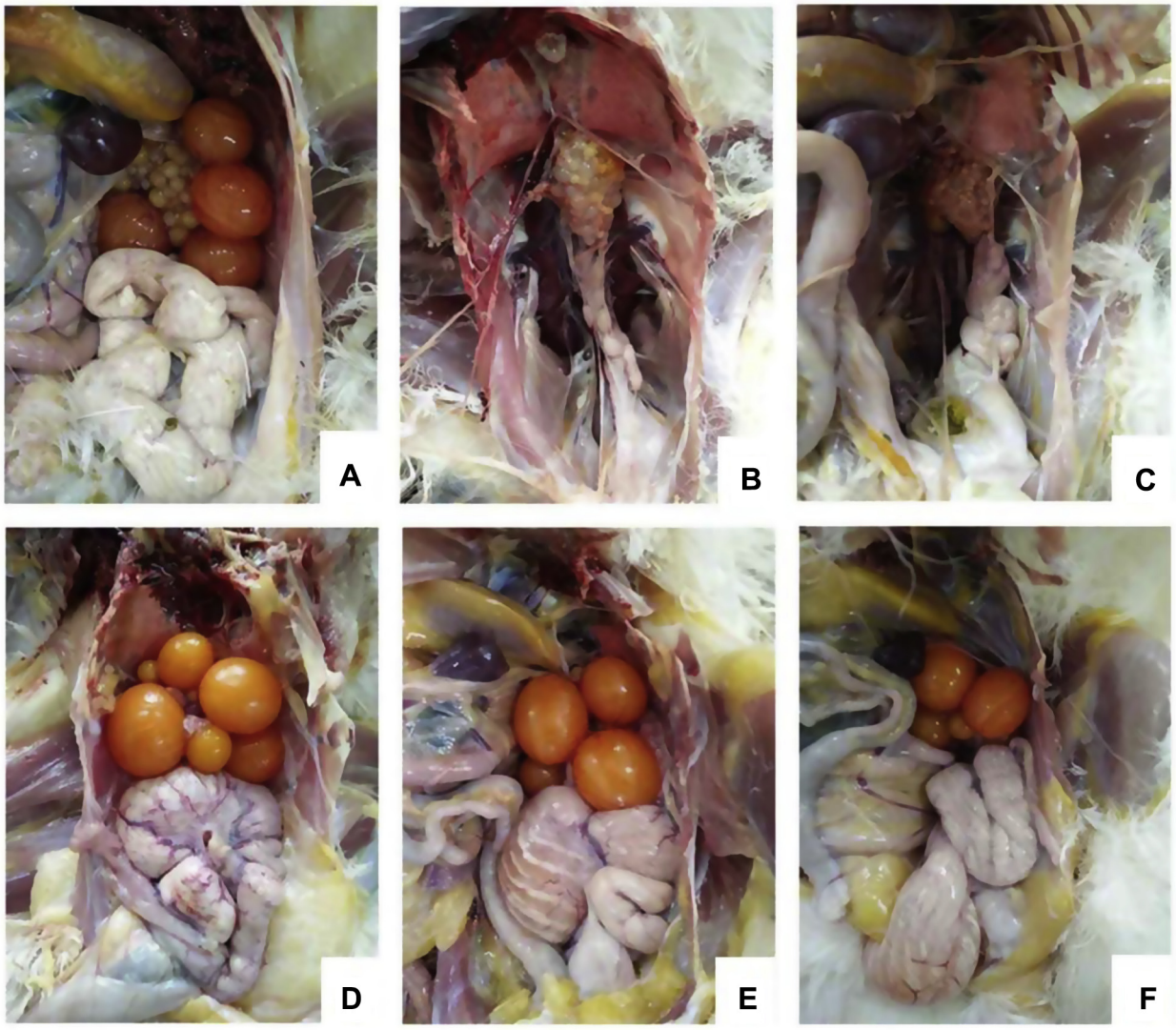

Figure 3. Ovarian follicles and fallopian tube of the 3 groups on the 15th D and 30th D of the experiment. (A–C) The ovarian follicles and fallopian tube of the CON, TFE1 and TFE0 group on the 15th D of the experiment. (D–F) The ovarian follicles and fallopian tube of the CON, TFE1 and TFE0 group on the 30th D of the experiment. Abbreviations: CON, control; TFE, total flavonoids of *Epimedium*; TFE1, 1‰ TFE treatment after forced molting; TFE0, no TFE treatment after forced molting.

TFE had improved the secretion of endogenous estrogen in the laying hens.

DISCUSSION

The fallopian tube is an important reproductive organ of the laying hens, which not only delivers eggs and sperm but also plays an important role in egg production. Therefore, the health conditions of the fallopian tube will directly affect egg production performances (Madekurozwa and Mpango, 2018). It has been found that infections resulting from bacteria, viruses, or mycoplasmas are common among laying chicken flocks and mainly lead to lowered egg production owing to the atrophic fallopian tube (Andreatti Filho et al., 2019). In addition, the egg laying rates of older hens decrease significantly owing to reductions in follicle numbers. The results of this study confirmed that TFE had the ability to improve the development of atrophied oviducts, promote follicle development, and increase egg laying rates. Therefore, TFE could be potentially used in the treatment of reproductive diseases in laying hens.

To investigate how the TFE exerts protection against injuries to the fallopian tube and follicles, this study detected the hormone levels and expressions of its receptors. TFE are the main effective components of herbal *Epimedium*. The methods of ultrasonic and ethanol extractions adopted in this study determined that the weight content of TFE had reached 45%. Then, the products of TFE were used throughout the entire experimental process.

It was recorded in Tu Jing Ben Cao (Illustrated Classics of Materia Medica) that *Epimedium* has a long medicinal history and contains various medical properties, such as wind-dispelling, dehumidification, kidney-tonifying, and yang-invigorating properties. It has been found to have both androgen-like and estrogen-like

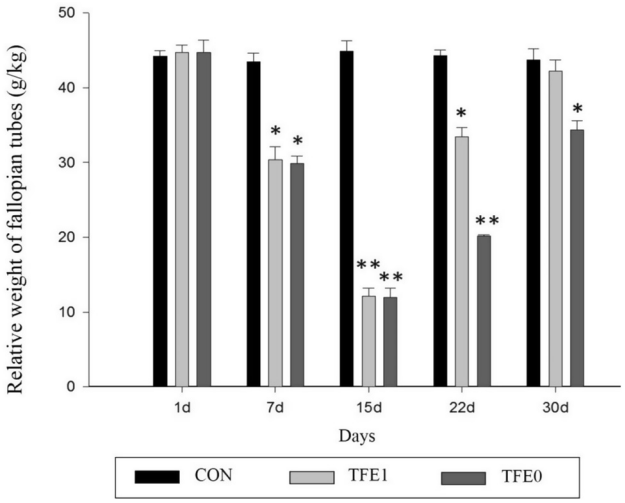

Figure 4. Effects of TFE on the relative weight of the fallopian tube to body weight. $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations: CON, control; TFE, total flavonoids of *Epimedium*; TFE1, 1‰ TFE treatment after forced molting; TFE0, no TFE treatment after forced molting.

effects (Zhang and Yang, 2006; Wang et al., 2009). Some of the ingredients of TFE, such as hyperin and icariin, were confirmed to promote the secretion of E_2 and progesterone through the upregulation of CYP17 and CYP19 in the ovary granulosa cells of rats in vitro (Nie et al., 2019). The results obtained in this study showed that TFE had the ability to improve the secretion of endogenous E_2, which in turn promoted the development of the fallopian tube via ER receptors. In addition, another possibility was that TFE promoted the secretory activities of the hypothalamus–pituitary glands. However, this speculation requires further confirmation.

It is known that forced molting practices will lead to follicular and reproductive system organ atrophy (Gongruttananun et al., 2017). This study's results showed that the egg productive ratios dropped to 11.14%, while the fallopian tube became atrophied and only prehierarchical follicles remained after artificial forced molting procedures. In the TFE1 group, the egg production rates, relative weights of the fallopian tube, and the follicles of the laying hens were observed to recover quickly after TFE treatments when compared with TFE0 and had returned to nearly normal levels. Dietary genistein, which is a type of soy isoflavones, is similar to estrogen both structurally and functionally and could potentially improve egg production and quality during late egg-laying periods (Lv et al., 2019). The results of this study confirmed that TFE has the potential to increase egg production and also assist in the recovery of the injured follicles and fallopian tube. The development of follicles is known to be closely related to the secretion of follicle stimulating hormone (**FSH**) and luteinizing hormone (**LH**). FSH mainly affects the growth and development of immature follicles, whereas LH participates in choosing dominant follicles and has certain influences on follicular development, corpus luteum formation, and regression (Bhattacharya et al., 2019). This study's results showed that TFE promoted prehierarchical follicles developing into preovulatory follicles. It was suggested

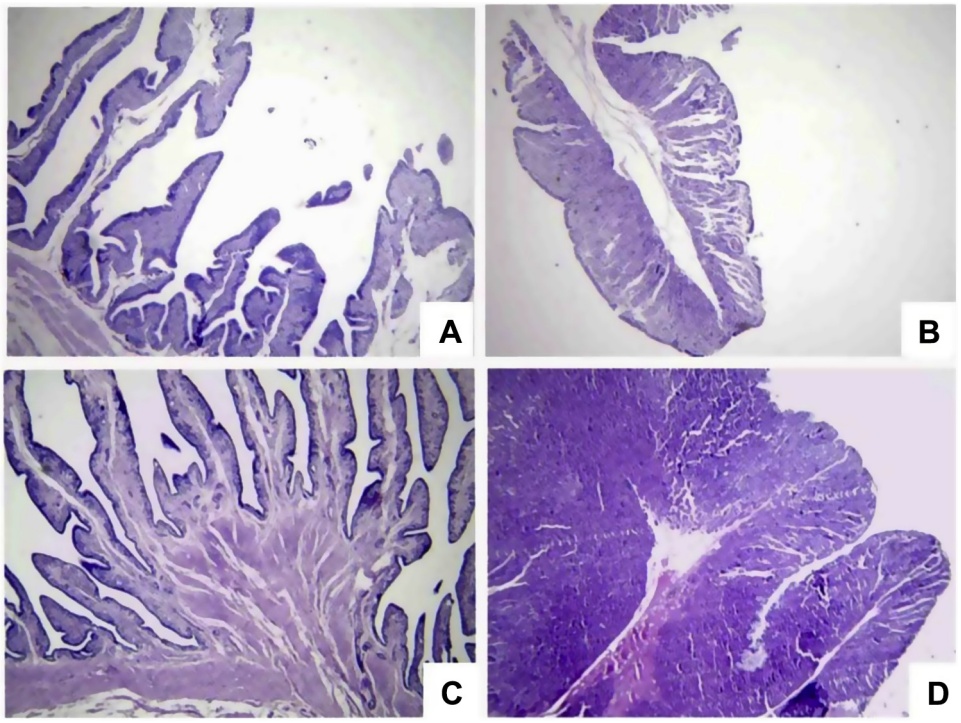

Figure 5. Histomorphology of the uterus and albumen-secreting part of the fallopian tube on the 30th D of the experiment. (A, B) The uterus and albumen-secreting part of TFE0 (hematoxylin-eosin, 10×). (C, D) The uterus and albumen-secreting part of TFE1 (hematoxylin-eosin, 10×). Abbreviations: TFE, total flavonoids of *Epimedium*; TFE1, 1‰ TFE treatment after forced molting; TFE0, no TFE treatment after forced molting.

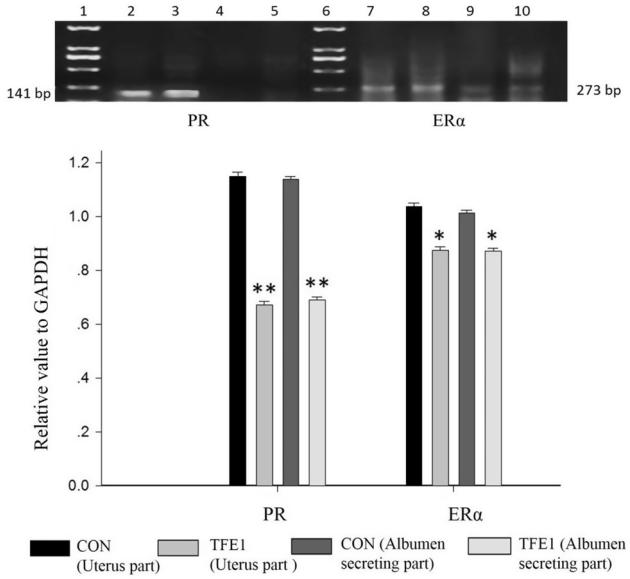

Figure 6. Expressions of PR mRNA and ERα mRNA in the fallopian tube of the laying hens on the 15th D of the experiment. $^{*}P < 0.05$; $^{**}P < 0.01$. (1) DL2000 marker; (2, 3) PCR products of PR in the uterus and albumen-secreting part of CON; (4, 5) PCR products of PR in the uterus and albumen-secreting part of TFE1; (6) DL2000 marker; (7, 8) PCR products of ERα in the uterus and albumen-secreting part of CON; (9, 10) PCR products of ERα in the uterus and albumen-secreting part of TFE1. Abbreviations: CON, control; ERα, estrogen receptor α; PCR, polymerase chain reaction; PR, progesterone receptor; TFE, total flavonoids of *Epimedium*; TFE1, 1‰ TFE treatment after forced molting; TFE0, no TFE treatment after forced molting.

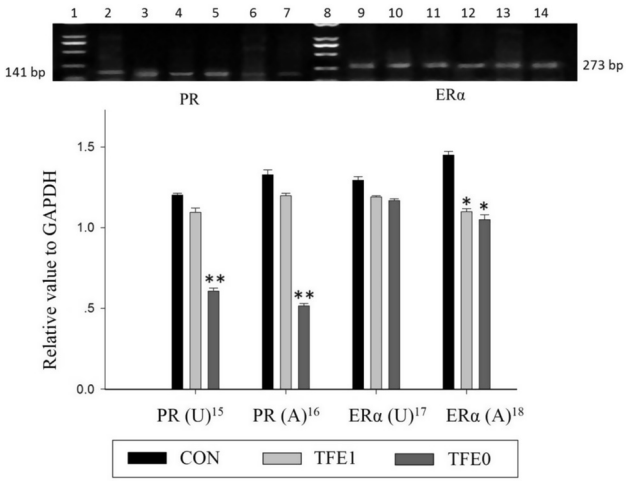

Figure 7. PR mRNA and ERα mRNA expressions in the fallopian tube of the laying hens on the 30th D of the experiment. $^{*}P < 0.05$; $^{**}P < 0.01$. (1) DL2000 marker; (2, 3) PCR products of PR in the uterus and albumen-secreting part of CON; (4, 5) PCR products of PR in the uterus and albumen-secreting part of TFE1; (6, 7) PCR products of PR in the uterus and albumen-secreting part of TFE0; (8) DL2000 marker; (9, 10) PCR products of ERα in the uterus and albumen-secreting part of CON; (11, 12) PCR products of ERα in the uterus and albumen-secreting part of TFE1; (13, 14) PCR products of ERα in the uterus and albumen-secreting part of TFE0; (15, 16) relative value to GAPDH of PR in the uterus (**U**) and albumen (**A**)-secreting part of 3 groups; (17, 18) relative value to GAPDH of ERα in the uterus (**U**) and albumen (**A**)-secreting part of 3 groups. Abbreviations: CON, control; ERα, estrogen receptor α; PCR, polymerase chain reaction; PR, progesterone receptor; TFE, total flavonoids of *Epimedium*; TFE1, 1‰ TFE treatment after forced molting; TFE0, no TFE treatment after forced molting.

that the functions of TFE in the development of follicles may have been related to the components of TFE playing roles similar to FSH and LH.

It was determined in this study that TFE improved the secretion of estrogen. Differing completely from mammals, the progesterone and small amounts of estrogen in poultry are synthesized by granulosa cells, and androgen and estrogen are synthesized in the inner and outer layers of membrane cells (Sechman et al., 2014). Estrogen and progesterone play important roles in the production performances of laying hens, and the effects of estrogen and progesterone are generally mediated by their receptors (Li et al., 2017).

In this study, the expressions of ERα and PR mRNA in the fallopian tube of each group were examined. It was found that the expressions of ERα and PR mRNA were downregulated, and the E_2 levels had decreased to 201.2 ± 10.5 pmol/L after forced molting. The concentration levels of E_2 were observed to have increased to normal levels, and the expressions of ERα and PR mRNA had returned to a relatively normal level similar to those of the CON group after TFE treatments. Meanwhile, the estrogen levels of the TFE0 group were lower than that of the CON group, and the expressions of ERα and PR mRNA were found to have not increased to normal levels. These results indicated that TFE had displayed the ability to promote the expressions of ERα and PR and improve the secretions of endogenous estrogen. In our former study, TFE had promoted ovariectomized mice showing estrus in the low level of endogenous estrogen. Therefore, it was concluded that TFE played a role with uterine which was similar to estrogen. The reasons for the TFE-related improvements in the development of the fallopian tube may be attributed to its estrogen-like effects. However, the functions of TFE were found to be weaker and slower than estrogen. In regard to how TFE promotes the secretion of endogenous estrogen, this study speculated that certain ingredients of TFE play roles similar to FSH and LH. TFE is known to have complex composition in which there are 17 flavones with dehydrated icariin as a mother nucleus, such as Epimedium, Epidemin A, Epidemin B, Epidemin C, and so on (Ma et al., 2019). The functions of each of these components, as well as the molecular targets, will be the focus of further studies.

TFE was a multicomponent natural product, which could be implemented to improve the development of follicles and atrophic fallopian tube of laying hens. The components of TFE play multifunctional roles, such as estrogen-like effects and gonadotropin-like effects. This study provided important references for the development of medicines specifically for the treatment of reproductive diseases.

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