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Phosphodiesterase inhibitor Theophylline in the aged laying hens: investigation of the ovarian and fallopian inflammatory mediators, hormones, and functions

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Abstract Inflammatory-dependent events in the aged commercial laying hens reduce production rate and egg quality. Our study was aimed to evaluate the phosphodiesterase (PDE) inhibitor Theophylline on the ovarian and fallopian-inflammatory traits in the commercial laying hens at the late stage of the production period. Twenty-four White Leghorn hens aged 92 weeks were used for four weeks to be orally supplemented by Theophylline (3 mg/kg/Body weight, BW). Ovulation rate and follicular growth were measured by laying frequency and, visual evaluation after euthanizing, respectively. The mRNA expressions of follicular and fallopian cyclooxygenases 1 and 2 (COX-1,-2), and cytokines were detected by real-time PCR. Plasma concentrations of ovarian hormones (Two hours after lighting) were measured via enzyme-linked immunosorbant assay (ELISA). The results showed that the mRNA expression of the IL-1β, IL-6 and TNF-α in the ovary and IL-1β and TNF-α in the infundibulum were significantly decreased ($P<0.01$) by Theophylline treatment; however, the mRNA abundance of COX-2 and IL-10 in both of ovary and infundibulum were increased ($P<0.01$). Plasma concentrations of estradiol and progesterone were elevated ($P<0.05$) in the hens treated with Theophylline. Ovulation rate and follicular size were not significantly ($P>0.05$) influenced by Theophylline. As a phosphodiesterase inhibitor, Theophylline retained the laying frequency in addition to enhancing its anti-inflammatory capability in the late stage of production period in laying hens.

Keywords: aged commercial flock, inflammation, ovarian hormones, ovulation, theophylline

Introduction

In recent decades, the rate of egg production has increased - in the laying hens, mainly, due to the enhancement of genetic and breeding technologies. This improvement has led to a rise in and exacerbated some reproductive consequences like chronic ovarian inflammation in the laying hens compared to the wild birds and the native laying hens (Fleming et al., 2006; Johnson and Giles, 2013). This could be a contributing f-actor to the deterioration of production rate and egg quality in the laying hens (Qi et al., 2016), in particular in the late stage of the production period (Johnson and Giles, -2013). It has been shown that chronic inflammation of the reproductive tract, either in the oviduct or ovary, as well as inflammation-depended reproductive events in the ag-

ed laying hens could be improved by implementing anti-inflammatory strategies like administering the herbal medicines (Barua et al., 2013), non-steroidal anti-inflammatory drugs (NSAIDs) (Urick et al., 2009), and the sources enriched with omega-3 fatty acids (Pal et al., 2019). Therefore, the presentation and evaluation of various anti-inflammatory strategies may modify the inflammatory process of the reproductive system in the aged laying hens.

As an inhibitor of the oxidative stress and anti-inflammatory agent (Tsukagoshi et al., 2000), Theophylline has been commonly used in treatment of the inflammation-originated respiratory disorders like asthma and chronic obstructive pulmonary disease (COPD) (Jilani et al., 2020). Theophylline (dimethylx anthine), found naturally in tea and cocoa beans in trace amounts, has been defined as a nonselective inhibitor of phosphodiesterases (PDE) that decrease intracellular cyclic nucleotides (Barnes, 2013), and thus results in an elevation of the intracellular cyclic adenosine monophosphate (cAMP); which could promote the anti-inflammatory properties in the cell (Eid et al., 2016). Besides, Theophylline has also been shown to inhibit the inflammatory aspects of cancers via the effect on several carcinogenic mediators (Buriani et al., 2017; Hashemi-Niasari et al., 2018; Peng et al., 2018).

Several studies documented the relationship among pro- and anti-inflammatory mediators like cyclooxygenases (COXs) (Hales et al., 2008; Elhamouly et al., 2017), pro- and anti-inflammatory cytokines (Macciò and Madeddu, 2012; Terlikowska et al., 2018), and ovarian function (ovarian steroids, ovulation rate, and follicular growth) (Fleming et al., 2006; Straub, 2007; Zhou et al., 2019); therefore, these factors could be defined as the criteria of infundibular and ovarian inflammatory status. This study aimed to evaluate the effects of dietary supplementation of Theophylline on the relative mRNA abundance of COXs and cytokines in the ovarian and infundibular tissues, and on ovarian function and sexual hormone concentrations in the laying hens during the late stage of the production period.

Materials and methods

Animal care

Twenty-four 92-week-old laying hens (*Gallus domesticus*) of a commercial strain of White Leghorn were housed at the Poultry Research Farm, Department of Animal Sciences, the University of Tehran, Karaj, Iran. The birds were exposed to a photoperiod of 16 h light: 8 h dark with lights on at 06:00 and lights off at 22:00, with food and water provided *ad libitum*. As one of the criteria for ovarian function, laying frequency (ovulation rate) was monitored and, recorded daily. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Tehran, Karaj, Iran. The ingredients and chemical composition of the diet are shown in Table 1.

Table 1. The Ingredients (%) and chemical composition of the diet (Per dry matter)

Ingredients	Value (%)
Corn	61.00
Soybean meal	23.40
Sodium bicarbonate	0.05
D-calcium phosphate	1.52
Fatty acids	2.80
Salt	0.07
Calcium carbonate	10.44
Vitamins and minerals	0.50
DL-methionine	0.13
Calculated analysis	
Crude protein	15.39
Calcium	4.62
Available phosphorus	0.40
Metabolizable energy	2780[1]

[1] (kcal/kg)

The hens were randomly divided into a control group and a *Theophylline* group (n=12 per group) that was orally supplemented with Theophylline (3 mg/kg/ BW, in a premix prepared by corn gluten) for four weeks (Hatefi et al., 2021). The optimum level of Theophylline (Dr. Obidi Co., Tehran, Iran) had previously been obtained by a pre-trial according to production efficiency.

Blood collection and hormone measurement

Blood samples (5 mL/hen) were randomly collected via the brachial vein from 8 hens per group (in EDTA tubes), 2 hours after the morning light on, at the end of the fourth week. The samples were centrifuged (at 3000 rpm for 15 min, 1308 × g) and the plasma was stored at -20°C for determination of ovarian hormones. Concentrations of estradiol, progesterone, and testosterone were determined by ELISA kits (Monobind® Inc, USA), according to the manufacturers' recommendations. The sensitivity of the detection level, and the intra-, and inter-assay coefficients of variations were 6.5 pg/mL, 6.3%, and 8.5% for estradiol; 0.105 ng/mL, 1.5% and below 13% for progesterone; and 0.038 ng/mL, 4.9%, and 4.6% for testosterone.

Tissue sampling

After four weeks, 8 hens per experimental group were euthanized by CO_2 asphyxiation, and necropsied. The ovaries were removed and the yellow follicles were classified based on their diameter (from F1, as pre-ovulatory follicle, to F5 as 5th small yellow follicle) measured from the follicular stigma. Then, the pre-ovulatory follicles (12–35 mm) and the infundibulum were dissected, washed in saline solution, transferred to micro tube, and stored at -80°C for RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated from frozen tissues using Trizol reagent (RNX-plus, Cinagen co., Tehran, Iran) according to the manufacturer's recommendations. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis, respectively. For RNA purification, the samples were treated with DNase I (YT 9054, Yekta Tajhiz Azma co., Tehran, Iran) before reverse transcription reaction. The cDNA was synthesized by the cDNA Reverse Transcription Kit (YT4500, Yekta Tajhiz Azma co., Tehran, Iran). The cDNA synthesis reaction conditions were 42°C for 30 min and 95°C for 3 min. The obtained cDNA was stored at -80°C for gene expression analysis, using the real-time PCR (Hales et al., 2008).

Table 2. Chicken primers used for real-time PCR

Gene	Accession No.	primers sequences (5'→3')	Orientation
COX-1 [1]	XM_425326	TCAGGTGGTTCTGGGACATCA	Forward
		TGTAGCCGTACTGGGAGTTGAA	Reverse
COX-2 [2]	XM_422297	CTGCTCCCTCCCATGTCAGA	Forward
		CACGTGAAGAATTCCGGTGTT	Reverse
IL-1β [3]	AB559570	CTTCCTCCAGCCAGAAAGT	Forward
		CAGCTTGTAGCCCTTGAT	Reverse
IL-6 [4]	AB559572	CAACCTCAACCTGCCCAA	Forward
		GGAGAGCTTCCTCAGGCATT	Reverse
IL-10 [5]	AB559574	CACAACTTCTTCACCTGCGAG	Forward
		CATGGCTTTGTAGATCCCGTTC	Reverse
TNF-α [6]	AY765397	TGTGTATGTGCAGCAACCCGTAGT	Forward
		GGCATTGCAATTTGGACAGAAGT	Reverse
β-Actin [7]	L08165	CATCACCATTGGCAATGAGAGG	Forward
		GCAAGCAGGAGTACGATGAATC	Reverse

Abbreviations: [1] Cyclooxygenases-1, [2] Cyclooxygenases-2, [3] Interleukin-1β, [4] Interleukin -6, [5] Interleukin -10, and [6] Tumor necrosis factor-α.

[7] COX-1, COX-2, IL-1β, IL-6, IL-10, and TNF-α mRNA data were normalized by β-actin.

Real-time PCR

Target gene mRNA levels were measured using SYBR Green qPCR master mix (YT 2550, Yekta Tajhiz Azma co., and Tehran, Iran). Hen specific primers are shown in Table 2. The β-actin was used as a housekeeping gene to normalize the target gene expression. Quantification of all transcripts was performed in a 20 µL reaction volume containing 1 µL single-strand cDNA, 10 µL of master mix, 0.5 µL of each forward and reverse primers and 8 µL of distilled H_2O in 20 µL by Rotor-Gene 6000 Real-Time PCR software (Corbett Research, Sydney, Australia). The program used for the amplification of genes consisted of a 95°C for 300 s followed by 50 cycles of 95°C for 10s, and 60°C for 30 s. The melting curve analysis was performed at the rate of 0.1°C/s for all genes to check the specificity of the products. Control reactions, lacking the template, were run for each target gene. The relative levels of mRNA were analyzed by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Data were analyzed using the SPSS software (IBM SPSS Statistics, version 26.0, 2019) by the General Linear Model (GLM) procedure using two treatments which were considered significant according to the probability ($P \leq 0.05$) of the F-ratios. Data were presented as the mean ±SD in figures and as the mean ±SEM in Table 3.

Results

Ovarian functions

Changes in the ovulation rate (laying frequency) and size of the ovarian follicles (F1 to F5) are shown in Table 3. Theophylline treatment resulted in larger F4 diameter ($P<0.05$) compared to the control group but it did not significantly influenced the size of other follicles as well as the ovulation rate (judged by the laying rate).

Concentration of plasma estradiol, progesterone, and testosterone

Compared to the control group, the hens supplemented by Theophylline had significantly higher concentrations of estradiol and progesterone ($P<0.01$) but plasma concentration of testosterone was not different ($P>0.05$)

between the Theophylline-treated and control hens (Figures 1a to 1c).

The mRNA expression of pro- and anti-inflammatory mediators

The relative mRNA abundances of cyclooxygenases (COX) 1 and 2, interleukins (IL-1β, IL-6, IL-10), and Tumor Necrosis Factor-α (TNF-α) are reported in Figure 2 (a-f) for pre-ovulatory (F1) follicle and in Figure 3 (a-f) for infundibulum in Fig. 3 (a-f).

In F1 follicles, Theophylline significantly decreased the mRNA expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (P<0.01), increased the COX-2, and anti-inflammatory cytokine IL-10 (P<0.01), but did not impact on COX-1 expression (P>0.05). Figure 3 indicated that Theophylline -treated hens recorded a lower relative abundance of infundibular mRNA expression of IL-1β and TNF-α (P<0.01), but expressed a higher level of the infundibular mRNA for IL-10, IL-6, COX-1, and 2 (P<0.01).

Table 3. Comparison of the ovulation rate (egg laying frequency, %) and follicular size (F1 to F5) in control and Theophylline-treated laying hens

Treatments	*Ovulation rate (%)*	*Follicular size (diameter, mm)*				
		F1	F2	F3	F4	F5
Control	62.69	27.85	25.72	20.77	15.62	11.83
Theophylline	68.64	33.00	32.17	24.83	22.21	16.00
SEM[1]	1.94	2.07	1.84	2.69	1.70	0.67
P- value	0.075	0.084	0.061	0.22	<0.05	0.22

[1]Standard error of the mean.

Discussion

This study indicated that the oral supplementing phosphodiesterase (PDE) inhibitor Theophylline caused down-regulate mRNA abundance of the most pro-inflammatory mediators and up-regulated the anti-inflammatory cytokine IL-10 in the infundibulum and ovary, with increases in the plasma concentrations of estradiol and progesterone, and relative improvement of follicular growth and ovulation. Genetic manipulation of commercial laying hens have led to several physiological disorders, like chronic-ovarian inflammation in aged laying hens (Fleming et al., 2006; Johnson and Giles, 2013); which is due to factors, such as the laying intensify and microbial infection during breeding and production phases (Sevoian and Levine, 1957; Zhang et al., 2020). In addition to the microbial origin of ovarian and fallopian inflammation, some reports indicated that chronic-ovarian inflammation, derived from incessant ovulation, could promote inflammatory conditions in the fallopian tube epithelial cells via exposing these cells to the follicular fluid after ovulation (Karst and Drapkin, 2010; Emori and Drapkin, 2014). Exacerbation in inflammation of the fallopian epithelial cells, on the other hand, could bring about an increase in the ovarian chronic inflammatory status via conduction of inflammatory factors, produced by the fallopian epithelium, to the epithelial surface of the ovary (Emori and Drapkin, 2014; Kollmann et al., 2017). As a result, depression in laying rate and egg quality could be predicted in older laying hens via increased microbial infection and inflammatory conditions in the ovary and infundibulum. Therefore, implementation of anti-inflammatory strategies may restore the laying frequency and egg quality in the aged laying hens. As a natural compound extracted from coc-oa and tea, Theophylline (*methylxanthine*) is a bronchodilator widely used to treat respiratory disorders like COPD and asthma through its anti-inflammatory and antioxidant properties (Jilani et al., 2020).

In the current research, Theophylline (Figures 2 and 3) caused a considerable decrease in mRNA relative abundances of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, while increasing the anti-inflammatory cytokine IL-10 expression in the pre-ovulatory follicle, without a significant effect on the infundibular IL-1β, TNF-α and IL-10 expressions. On the other hand, Theophylline up-regulated the expression of COX-2 mRNA in both the ovarian and infundibulum, and that of COX-1 in infundibulum. These results were in agreement with the majority of studies reporting an inhibitory effect of this drug on these cytokines in the tissues like the lungs and adipocytes (Juergens et al., 1999; Iiboshi et al., 2007, He et al., 2014; Ghasemi-Pirbaluti et al., 2017; Mitani et al., 2018). Theophylline exerts its anti-inflammatory properties via several cellular pathways; such as: PDE inhibition, adenosine receptor antagonism, and histone acetyltransferase (HDAC) pathway activation and restoration (Barnes, 2006; Barnes, 2013). All of these mechanisms contribute to the anti-inflammatory and antioxidant properties of Theophylline which are closely accompanied with increased intracellular cAMP concentration (Tsukagoshi et al., 2000; Barnes, 2006). Theophylline increased the ovarian and infundibular mRNA expressions of COX-2 in the present study, and there are reports that Theophylline induces COX-2 expression via cAMP-depended downstream pathway PKA-CREB-AP-1 (Juergens et al., 1999; Klein et al., 2007). In fact, Theophylline could also enhance the COX-2 as a pro-inflammatory mediator.

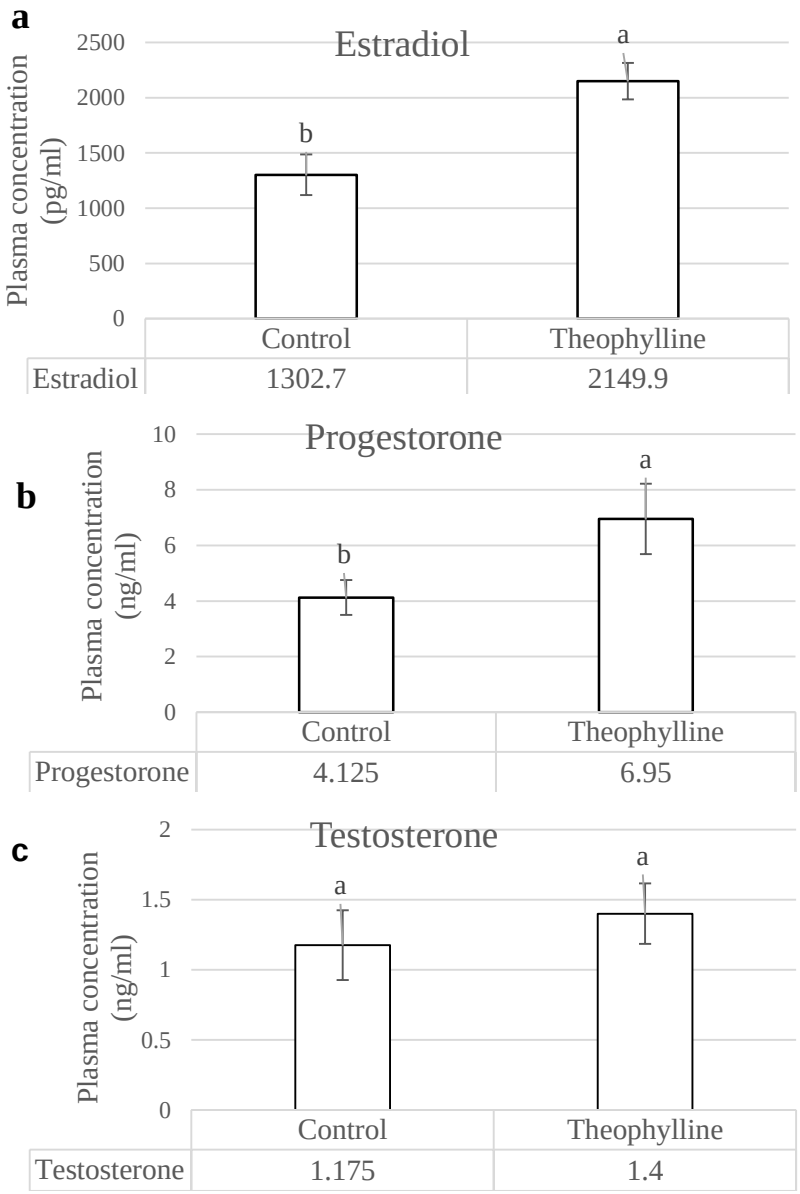

Figure 1. Comparison of plasma Estradiol (a), Progesterone (b) and Testosterone (c) concentrations between control and Theophylline-treated aged layer hens. Different letters (a-b) indicate significance between treatments (P<0.05).

As the main reproductive steroids, estradiol, progesterone, and testosterone (androgen) are critical in regulating the growth, differentiation, and function of a wide range of target tissues in the female reproductive organs (Jeon et al., 2016). Unlike mammals, hens do not form corpus luteum, and their progesterone is made by the granulosa cells in mature follicles and reaches maximum concentration, approximately 4-6 hours before the ovulation, like estradiol that is produced by theca externa layer (Elnagar et al., 2002; Apperson et al., 2017). It is now well-established that estrogen plays a crucial role in follicular growth and ovulation in hens. On the one hand, progesterone stimulates the ovulation via-acting directly on the ovarian follicle (Nakada et al., 1994) and indirectly, on the hypothalamus to induce a positive feedback response for secretion of gonadotropin-releasing hormone (GnRH) and then luteinizing hormone (LH) from the anterior pituitary (Mishra et al., 2019). On the other hand, estradiol plays an important role in follicular growth by stimulating the avian liver to produce the yolk precursor, vitellogenin, and very-low-density lipoproteins (VLDL) (Redshaw and Follett, 1972). However, these hormones have different inflammatory effects. Researchers confirmed that estradiol demonstrates a dual effect depending on its concentration. In chronic inflammatory disorders, estradiol inhibits important pro-inflammatory cytokines -

such as TNF, IL-1β, and IL-6 at high levels; whereas, these cytokines are stimulated at a lower levels of estradiol (Straub, 2007). Progesterone has a protective role in inhibiting inflammation during pregnancy by decreasing IL-6 and TNF-α; and by the recovery of anti-oxidant enzyme function in some tissues (Zhou et al., 2019). Testosterone therapy reduces the inflammatory process and decreases the intensity of disease by the mechanisms which inhibit inflammatory cytokines mRNA expression and performance like TNF-α, IL-1β, and IL-6 (Traish et al., 2018).

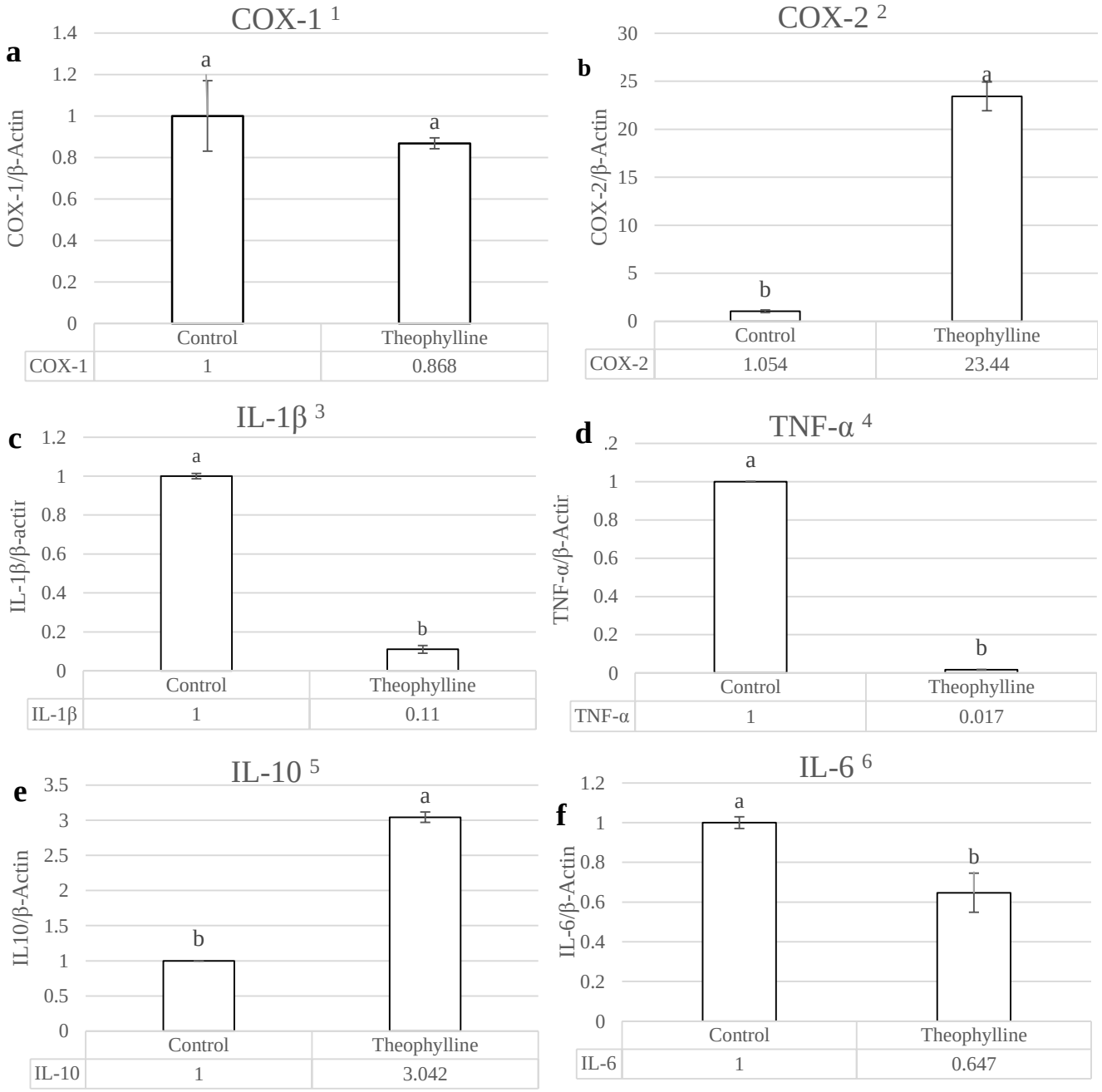

Figure 2. Comparison of COX-1 (a), COX-2 (b), IL-1β (c), TNF-α (d), IL-10 (e), and IL-6 (f), mRNA expressions (normalized by β-actin) in pre-ovulatory follicles (F1) between control and Theophylline-treated aged layer hens. Different letters (a-b) indicate significance between treatments (P<0.05) 1) cyclooxygenases-1, 2) cyclooxygenases-2.

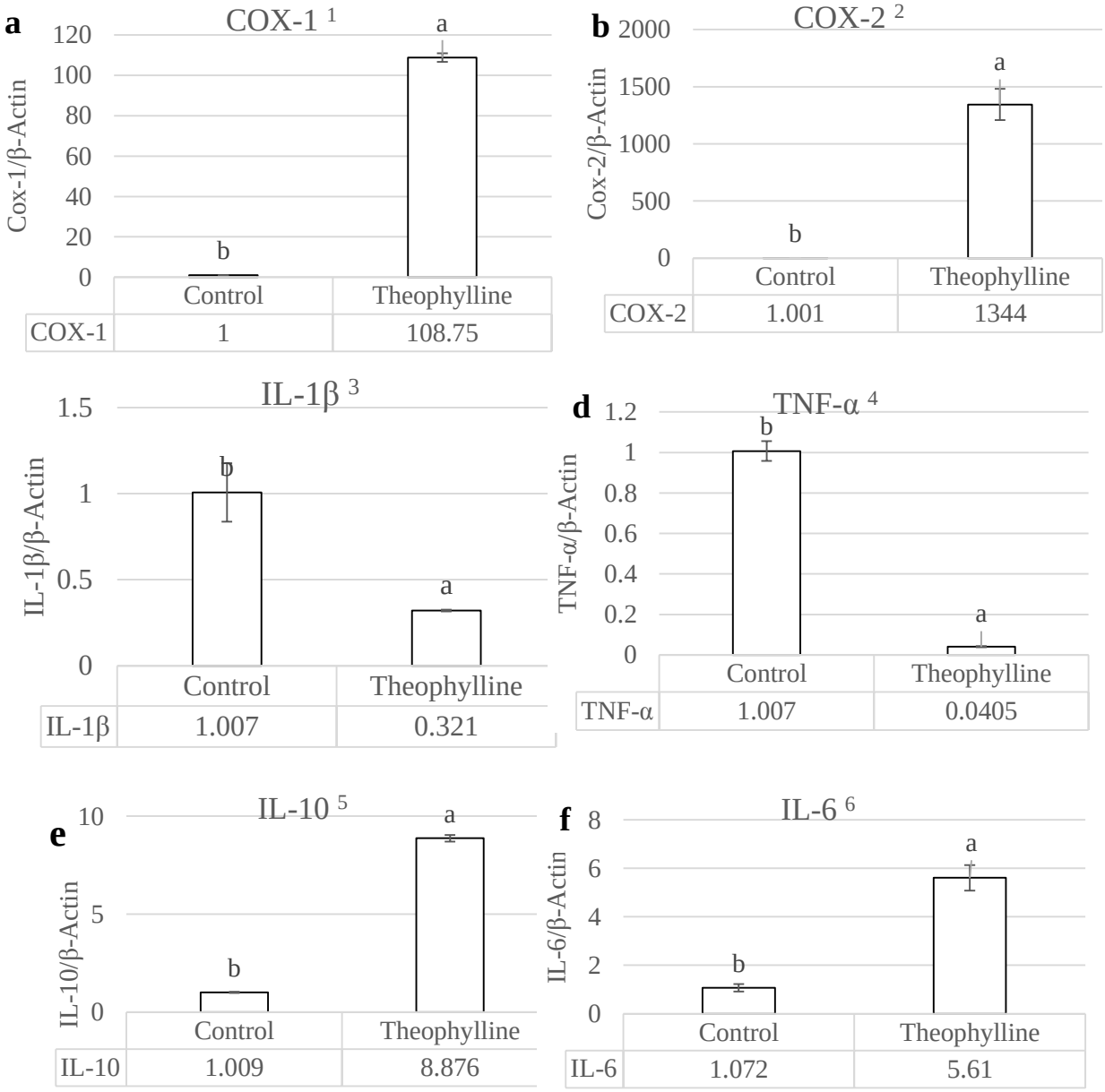

Figure 3. Comparison of COX-1 (a), COX-2 (b), IL-1β (c), TNF-α (d), IL-10 (e), and IL-6 (f), mRNA expressions (normalized by β-actin) in infundibulum between control and Theophylline-treated aged layer hens. Different letters (a-b) indicate significance between treatments (P<0.05). 1) cyclooxygenases-1, 2) cyclooxygenases-2, 3) Interleukin-1β, 4) Tumor necrosis factor-α, 5) Interleukin-10, and 6) Interleukin.

Hens treated with Theophylline recorded higher plasma concentrations of estradiol and progesterone (Figure 1). Although few reports have been published concerning the effect of Theophylline on ovarian steroid secretion, there is similarity of action between the beta-2 adrenergic agonists and PDE inhibitor Theophylline on cAMP-depended downstream pathways; therefore these drugs could lead to pharmaceutically identical results. Regarding to this identical pathway, a number of studies demonstrated there is a positive correlation between beta-2 adrenergic signaling and ovarian hormones (Unsicker et al., 1983; Breuiller et al., 1988; Ebeid et al., 2008). This correlation are usually created via direct stimulation of follicular layer in the ovarian follicles (Ebeid et al., 2008) and indirect effect on the GnRH, LH, and FSH responses which are targeted by current beta-2 adrenergic signaling for cAMP formation (Swartz and Moberg, 1986). Therefore, less mRNA expression of the ovarian pro-inflammatory cytokines in this study could also derive from the anti-inflammatory behavior at higher concentrations of estradiol and progesterone in Theophylline group. Moreover, elevation of the ovarian estradiol and progesterone in the Theophylline- treated -

birds could be a reason for the increase in ovulation rate and follicular growth (Liu et al., 1983; Amer, 2007; Holesh et al., 2020), which was significantly observed in F4 follicles (Table 3. The hypothalamic-pituitary-ovarian axis plays fundamental roles in ovarian functions. In this regard, GnRH, gonadotropins, and ovarian hormones are pivotal in follicular development and ovulation (Holesh et al., 2020). For these reasons, the tendency for a significant increase in ovulation rate and follicular growth could probably be due to elevated plasma estradiol and progesterone in the birds administrated by PDE inhibitor Theophylline. Therefore, as one of the contributing factors to ovulation, pro-inflammatory mediators whose mRNA expressions were down-regulated in the treated laying hens did not seem to be as effective as the ovarian steroids on ovulation rate and follicular growth in this group.

Conclusion

The results of the present study indicated that the administration of phosphodiesterase (PDE) inhibitor Theophylline, as an anti-inflammatory agent, resulted in down-regulation in mRNA expression of most pro-inflammatory cytokines and up-regulation in anti-inflammatory cytokine IL-10 in the aged White Leghorn infundibulum and ovary, increases in the plasma concentration of ovarian steroids, and relative improvement of the follicular growth and ovulation. Therefore, Theophylline is capable of enhancing its anti-inflammatory traits in addition to keeping laying frequency in the late stage of laying hens' production period.

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