Impact of Estradiol Benzoate and Progesterone on the Activity of Carbonic Anhydrase of Erythrocytes in Ducks (*Anatis domesticae* Caro)

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***Abstract*—The impact of estradiol benzoate and progesterone on the duck erythrocyte carbonic anhydrase (CA) activity of *in vitro* culture was studied. The impact of estradiol benzoate and progesterone on CA activity of *in vitro* culture duck erythrocytes was significant and varied with the incubation durations. When the duck erythrocytes were incubated 2, 14 hours, estradiol benzoate increased the CA activity. When the duck erythrocytes were incubated thirty hours, estradiol benzoate at a higher addition (more than 200 pg ml^{-1}) had no facilitation to the CA activity. Progesterone with addition in the range of 0.5 to 50 ng ml^{-1} promoted the CA activity when the duck erythrocytes were incubated fourteen hours. The CA activity was also decreased by progesterone when the duck erythrocytes were incubated two hours in MEM medium containing progesterone at the addition of 1 to 10 ng ml^{-1}. Progesterone at the addition of 0.5 ng ml^{-1} inhibited the CA activity when the duck erythrocytes were incubated thirty hours. These findings can help to understand the interaction between CA and reproductive hormones, and account for the impact of exogenous hormones administration or endogenous estrogens analogs inhalation on the eggshell formation of right thickness and some other biological functions.**

Keywords- Duck (Anatis domesticae Caro, Varity: Gaoyou); carbonic anhydrase; erythrocyte; estradiol benzoate; progesteron

I. INTRODUCTION

Carbonic anhydrase (CA, EC4.2.1.1) is a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide into bicarbonate: $CO_2 + H_2O \longleftrightarrow HCO_3^- + H^+$. It is present in almost all organs and is implicated in various biological functions, the most important of which is participation in the regulation of ion, water, acid-base balance, carboxylation/decarboxylation reactions, and inorganic carbon diffusion between the cell and its environment as well as within the cell [1-3]. The CA was quite sensitive to a calmodulin inhibitor, calmidazolium, and the CA of the shell gland is important to the formation of the eggshells [4, 5]. Some members of the CA gene family have been suggested to promote cell proliferation and to act as trophic growth factors [6].

Estrogen and progesterone are two of the very important reproductive hormones, which affect the domestic fowls' laying of egg [7-12]. Many studies have been conducted about the effects of estrogen and progesterone on CA [13-17]. The results of these previous studies were inconsistent with, even contradictory to, each other. The effects of estrogen and progesterone on CA were documented in a few fowls such as chicken, hen and quail. The hormone-induced effects on CA in other fowls such as duck have not been fully understood.

It has been known for many years that some well-known environmental pollutants, such as the pesticide o,p′-DDT and some of the polychlorinated biphenyls (PCBs), whose action resembles that of endogenous estrogens, are estrogenic [18-20]. Therefore, the present study aims to determine the impact of estradiol benzoate (a species of estrogen) and progesterone on the activity of CA in vitro culture erythrocytes using ducks (*Anatis domesticae* Caro, Varity: Gaoyou), to attain better understanding of the interaction between CA and reproductive hormones. Meanwhile, the result presented by this study is helpful to the understanding of the effects of the estrogenic analogs.

II. MATERIALS AND METHODS

Twenty three-months-old female ducks (*Anatis domesticae* Caro, Varity: Gaoyou) were used for this experiment. 2.5 ml blood collected from every duck was centrifuged at 1 000 g for 10 minutes and the plasma and buffy coat discarded. The erythrocyte pellet was washed 3 times with Earle's balanced salt solution. Washed erythrocytes were re-suspended in minimal essential medium (MEM; Gibco/BRL, Grand Island, NY), which consists of amino acids, potassium chloride, magnesium sulfate, sodium chloride, sodium dihydrogen phosphate, sodium bicarbonate, glucose, vitamins and L-glutamine. The erythrocyte suspended in medium MEM with different additions of estradiol benzoate and progesterone (the *in vitro* concentration of erythrocytes adjusted to 2×10^7 ml^{-1}) was incubated at 37°C in a humidified atmosphere with 5% CO_2. Estrogen additions in different sets of medium MEM

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were fixed at 0, 2, 20, 200, 2000 and 20000 pg ml^{-1}, progesterone additions fixed at 0, 0.5, 1.0, 5.0,10.0 and 50.0 and 100.0 ng ml^{-1}. After 2, 14, 30 hours incubation, the medium with the erythrocytes were centrifuged at 1 000 g for 5 minutes, and the supernatants were discarded. Then, the erythrocyte pellets were washed with Earle's balanced salt solution again. After the supernatants were discarded, the erythrocytes were ready for determining the cell viability, the CA activity and the hemoglobin content.

Cell viability was determined with a trypan blue dye exclusion test [21]. The cells were removed from the plate with 0.1% trypsin. 500 µl of diluted cell preparation was combined with 500 µl of the 4% trypan blue dye solution. Living cells do not take up the dye, whereas dead ones do. The cells were counted using a hemocytometer and phase contrast microscope (Olympus 1M, Japan). CA activity was assayed according to the method described by Wilbur and Anderson [22]. CO_2– Hydratase activity was recorded in units of Wilbur-Anderson (WA) enzymatic activity, defined as $[(T_0/T)-1]$, where T_0 and T are the time for pH change occurring in the non-enzymatic and enzymatic reaction, respectively. The hemoglobin (Hb) content was measured using the cyanmethemoglobin method [23]. Each treatment consisted of five replicates. The mean and standard errors are calculated for each treatment. One-way ANOVA and pairwise comparison test (Tukey) was conducted for each incubation duration. The significance of differences between incubation durations was evaluated by two-way ANOVA for changes from baseline (control) to different levels of hormone addition. Post hoc test (Tukey) was applied in case of significant incubation duration's difference. $P<0.05$ was considered statistically significant.

III. Results

A. The effect of estradiol benzoate on the CA activity and hemoglobin content

The results of cell viability show that the erythrocyte mortality in the MEM medium is below 1% no matter how much the addition of the estradiol benzoate in MEM medium is. And the hemoglobin content of erythrocytes was little influenced by estradiol benzoate no matter how long the duration of incubation was (Table I).

There was a significant interaction between the duck erythrocyte CA activity and the addition of estradiol benzoate ($P<0.0001$) (Fig. 1). Post hoc testing revealed a significant increase in CA activity for the duration of 2, and 14 hours incubation, whereas a significant increase in CA activity was only observed for the duration of 30 hours incubation at the estradiol benzoate of 2 and 20 pg ml^{-1} addition levels in MEM medium. No change was observed for the duration of 30 hours incubation at a higher addition (200 to 20000 pg ml^{-1}). The maximum value of the duck erythrocytes CA activity for 14 hours incubation appeared when the addition of estradiol benzoate in the MEM medium was 2 pg ml^{-1}, while that for 30 hours incubation appeared when the addition of estradiol benzoate in the MEM medium was 200 pg ml^{-1}.

B. The effect of progesterone on the CA activity and hemoglobin content

The results of cell viability showed that the erythrocyte mortality in MEM medium with different progesterone additions was also below 1%. The hemoglobin content of erythrocytes was little influenced by estrogen (Table. II).

By two-way ANOVA, there was a significant effect of incubation duration on the duck erythrocyte CA activity for different addition of progesterone ($P<0.0001$) (Fig. 2). When the erythrocyte was incubated two hours, and the addition of progesterone was in the range of 1 to 5 ng ml^{-1} in MEM medium, the CA activity was decreased. When the erythrocyte was incubated fourteen hours, the CA activity was increased with progesterone additions in the range of 0.5 to 50 ng ml^{-1}, and the increment of CA activity was positively correlated to progesterone addition in MEM medium. When the erythrocyte was incubated thirty hours, the erythrocyte CA activity with the progesterone addition at 0.5 ng ml^{-1} was the lowest, and that with the progesterone addition at 50 ng ml^{-1} in MEM medium remained the highest.

IV. Discussions

Hormones are known to regulate the expression of the CA isoenzymes [15, 24-27]. Estrogen and progesterone can regulate CA isoenzymes in guinea pig and rabbit uterus [14]. The rat hepatic CAII activity was increased by diethylstilboestrol addition [16]. There was a 2-fold increase in hepatic CA activity when mature female rats were treated with beta-estradiol for 3 days, and then followed by progesterone for 2 days. Either hormone alone produced no change [13]. Testosterone promoted synthesis of CAIII in rat liver and estrogen to inhibit it [28]. *In vivo* treatment with ethynylestradiol resulted in a loss of CA activity in quail tubular glands while the surface epithelium showed strong induction of both membrane bound and cytoplasmic CA activity [15]. CA IX expression is correlated negatively with estrogen receptor, and progesterone receptor [6]. However, the levels of hemolysate CAII and CAIII of normal pregnant women were increased from the middle of the second trimester, and the CAII or CAIII level is correlated with serum progesterone [29]. Erythrocyte CA had a significant correlation with plasma progesterone concentrations in women [17].

Erythrocytes are known to contain two soluble cytoplasmic isoenzymes of carbonic anhydrase (CA) designated CA I and CA II. CA II is a high activity form, and CA I is a low-activity one (with respect to hydration of CO_2 and dehydration of H_2CO_3), where it facilitates CO_2 transport [30]. The present study shows that the influence of estradiol benzoate and progesterone on CA activity of *in vitro* culture duck erythrocytes was significant and varied with the incubation durations, which suggested that these hormones have some impact on CO_2 transport of blood cell. Exogenous estrogen and progesterone administration or endogenous estrogens analog inhalation may promote the plasma hormone concentration in duck. The turbulence of CA in erythrocyte may induce the malfunction of CO_2 transport. Some other functions, such as the regulation of ion, water, and acid-base balance,

carboxylation/decarboxylation reactions, would similarly get influenced profoundly, which even causes cancer [31].

The variation of reproductive hormone concentration is related to ovulation of domestic fowls. Total estrogen concentration in hen plasma varied from less than 25 pg ml^{-1} to over 600 pg ml^{-1} [32]. The beta-estradiol concentration in peripheral plasma of laying hens was increased from 66 to 180 pg ml^{-1} during two to six hours before ovulation [33]. A consistent relationship appeared to exist between peaks of estradiol and estrogen concentrations in the domestic fowls. And the peaks of estrogen and progesterone precede ovulation by 4-7 hours [10]. Progesterone levels were the highest (2-4 ng ml^{-1}) in breeding females of Harris' Hawk, Parabuteo unicinctus just before egg laying [34]. Ovulation and egg laying of domestic fowls are almost occurring simultaneously.

It is known that the role of CA in eggshell calcification relates to CA activity within cells of the shell gland. There was a direct relationship between the variation of the CA activity in shell gland and that in erythrocytes [35]. The present study shows that the effect of reproductive hormone on duck erythrocyte CA can also suggest its influence on the formation of the eggshells. After about two hours of ovulation, the ovum stayed about 20 hours in womb where eggshells were formed. Meanwhile, estrogen and progesterone at the peak of concentration (prepared in the shell gland) induced the higher activity of CA in the shell gland mucosa according to the result from two hours' incubation of the duck erythrocytes. It facilitated the formation of eggshell. According to the result of a fourteen-hour-incubation experiment, the CA activity was sensitively modulated by estrogen and progesterone in the range of physiological concentration. The modulation can bring about right thickness of eggshell owing to the longer duration of the formation of eggshell in the shell gland where the formation lasted from about 2 hours after ovulation to 2 hours before the egg laying. In addition, the result from thirty hours' incubation of the duck erythrocytes suggests that estrogen and progesterone, especially in the range of physiological concentration, cannot regulate the thickness of eggshell well when hormone concentration remains constant for a long time. Evidently, it does not agree with the variation of reproductive hormones in peripheral plasma of laying poultry [7-12, 33]. Therefore, exogenous hormones administration or endogenous estrogens analog inhalation may have a greater impact on the eggshell formation of right thickness.

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TABLE I. THE HEMOGLOBIN CONTENTS OF DUCK ERYTHROCYTES INCUBATED FOR DIFFERENT DURATIONS (2, 14, AND 30 HOURS) AND WITH VARIOUS ADDITIONS OF ESTRADIOL BENZOATE IN MEM MEDIUM. SE IS SHOWN IN BRACKETS (N=5).

Estradiol benzoate (pg ml^{-1})	**Incubated Duration (hours)**		
	2	***14***	***30***
0	124.6a (5.4)	129.7a (5.6)	134.7a (5.7)
2	126.3a (3.5)	116.8a (4.6)	125.9a (5.27)
20	122.50a (4.9)	118.1a (5.1)	119.3a (4.7)
200	112.8a (4.5)a	115.6a (4.5)	131.6a (5.2)
2000	113. 7a (5.4)	114.5a (4.9)	133.1a (5. 6)
2000	116.8a (4.6)	129.3a (5.6)	117.2a (4.6)

Notes: The same letters following the figures in the same columns do not mean significant difference at P<0.05 by two-way ANOVA and pairwise comparison test (Tukey).

TABLE II. THE HEMOGLOBIN CONTENTS OF DUCK ERYTHROCYTES INCUBATED FOR DIFFERENT DURATIONS (2, 14, AND 30 HOURS) AND WITH VARIOUS ADDITIONS OF PROGESTERONE IN MEM MEDIUM. SE IS SHOWN IN BRACKETS (N=5).

Progesterone (ng ml^{-1})	**Incubated Duration (hours)**		
	2	***14***	***30***
0	117.8a (5.8)	126.6a (5.7)	131.1a (5.1)
0.5	117.3a (5.4)	123.9a (3.5)	130.9a (5.1)
1	119.2a (4.9)	125.0a (3.3)	136.5a (5.9)
5	122.5a (5.7)	119.1a (5.8)	133.3a (5.1)
10	110.3a (4.3)	116.6a (4.3)	122.4a (4.8)
50	109.7a (5.3)	128.6a (4.0)	127.3a (5.1)
100	107.5a (6.0)	128.5a (5.8)	137.5a (5.4)

Notes: The same letters following the figures in the same columns do not mean significant difference at P<0.05 by two-way ANOVA and pairwise comparison test (Tukey).

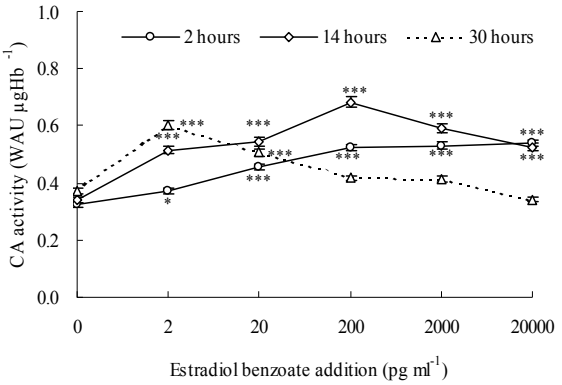

Figure 1. The CA activity of duck erythrocytes incubated for 2(circle), 14(arris), and 30(triangle) hours with various additions of estradiol benzoate in MEM medium (Means±SE, n=5). Comparisons between baseline (control) and each level of estradiol benzoate additions: *P<0.05; ***P<0.001.

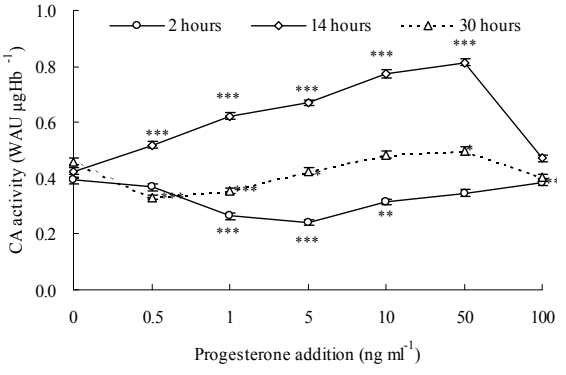

Figure 2. The CA activity of duck erythrocytes incubated for 2(circle), 14(arris), and 30(triangle) hours with various additions of progesterone in MEM medium (Means±SE, n=5). Comparisons between baseline (control) and each level of progesterone additions: *P<0.05; **P<0.01; ***P<0.001.