Multiple Ovulations and *In vitro* Fertilization in the Domestic Fowl (*Gallus domesticus*)*

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ABSTRACT : The aim of this study was to obtain mature ova or embryos at a single cell stage, which can be used in avian transgenesis and nuclear transfer through multiple ovulations, *in vitro* fertilization and culture. Chicken anterior pituitary extract (CAPE) or acetone-dried chicken anterior pituitary extract (ACAPE) was used to induce multiple ovulations in hens pretreated with pregnant mare's serum gonadotrophin (PMSG). *In vitro* fertilization of the multiple ovulated ova was performed by inseminating sperm onto the germinal disks in m-Ringer's solution and incubating the ova at 41°C, 5% CO_2 for 10 h in DME-F12 medium containing 20% liquid albumen. The *in vitro* fertilization process was observed using an environmental scanning electron microscope. When normal laying hens (white Leghorn) were administered daily with PMSG (100 IU), egg laying ceased in most hens within 3 to 8 days. Ovulation began to occur about 7.5 h after injection of CAPE and ACAPE. The number of ovulated ova was 1.00±0.00, 2.33±0.52 and 2.20±0.45, respectively, after receiving 100, 200 and 300 mg CAPE. The number of ovulated ova was 2.00±0.00, 2.86±0.69 and 3.00±1.22, respectively, after receiving 10, 15 and 20 mg ACAPE. The fertilized and cultured ova were able to develop into embryos up to the 32 cell stage. The present experiments demonstrated that multiple ovulations can be induced by CAPE and ACAPE successfully, and the ova resulted from the treatment retained the capability for further fertilization and embryonic development. These data provide new information to support the establishment of an *in vitro* culture system for future avian transgenesis studies. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 12 : 1652-1656)***

Key Words : Fowl, PMSG, CAPE, ACAPE, Multiple Ovulations, *In vitro* Fertilization

INTRODUCTION

Transgenesis has had a great effect on basic as well as applied bioscience (Lee and Piedrahita, 2003). Avian transgenesis and nuclear transfer require a large number of mature ova or embryos at the single-cell stage. However, it is difficult to collect such a number of mature ova each time because the conditions for *in vitro* maturation and ovulation have not yet been optimized. To obtain mature ova or embryos at the single-cell stage, hens have to be sacrificed at specific times, which is time-consuming and labourious. Multiple ovulations in hens were achieved by suppression of endogenous ovulation-inducing hormones through daily injection of pregnant mare's serum (PMS) or pregnant mare's serum gonadotrophin (PMSG), followed by treatment with mammalian or chicken gonadotrophins (Fraps et al., 1942; Imai, 1973). However there is only one report about the *in vitro* fertilizing competency of the multiple ovulated ova (Nakanishi et al., 1991). Many mature ova and embryos at the single-cell stage may be obtained easily through multiple ovulations, *in vitro* fertilization and subsequent culture. In this study, a systematic analysis was pursued (Figure 1); in which chicken anterior pituitary extract (CAPE) and acetone-dried chicken anterior pituitary extract (ACAPE) were used to induce multiple ovulations in PMSG-pretreated hens (*Gallus domesticus*) and the fertilizing competency and early development of these ova were examined. The presented data expanded the knowledge about *in vitro* avian experimental systems and may also be helpful for further understanding of gamete biology and early embryonic development in birds.

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MATERIALS AND METHODS

Preparation of chicken anterior pituitary extraction (CAPE) and acetone-dried chicken anterior pituitary extraction (ACAPE)

White leghorn chicken anterior pituitary collected from broilers of both sexes was stored at -20°C. Immediately before use, the tissues were homogenized in ice-cold physiological saline. The homogenate was centrifuged at 850 g for 15 min and the supernatant was used as CAPE for induction of multiple ovulations.

Chicken anterior pituitary collected from broilers of both sexes was stored for 2 weeks in cold acetone. Immediately before use, the acetone-dried chicken anterior pituitary were homogenized in ice-cold physiological saline and centrifuged at 850 g for 15 min. The supernatant, which was ACAPE, was used as the injection material.

Multiple ovulations and ova collection

Ten-month-old hens were kept in individual cages under

Figure 1. General scheme of the experiment. CAPE or ACAPE was used to induce multiple ovulations in hens pretreated with PMSG. *In vitro* fertilization was performed by inseminating diluted sperm onto the germinal disks in m-Ringer's solution and incubating the ova at 41°C, 5% CO_2 for 10 h in DME-F12 medium containing 20% liquid albumen.

14 h light and 10 h dark each day with constant food and water supply. Hens, selected on the basis of regular laying patterns for 2 weeks before the experiment (egg sequences ranging from three to seven eggs), were treated with subcutaneous injection of PMSG (Tianjing Huafu Co. Ltd., China) for 4 to 8 days at daily doses of 75 IU or 100 IU. CAPE or ACAPE was given intraperitoneally to the PMSG-pretreated hens 24 h after the final injection of PMSG. These hens were anesthetized with intravenous injection of 0.9 ml sodium pentobarbital (Abbott Laboratories North Chicago, USA) at 7.2 and 7.5 h after the treatment of CAPE or ACAPE and the abdominal region was disinfected with 70% alcohol. The ovulated ova were carefully collected from infundibulum or body cavity. Naturally ovulated ova were recovered from normally laying hens. These hens were killed 15 to 20 min after oviposition.

Table 1. Effect of pregnant mare's serum gonadotrophin (PMSG) injection on suppression of normal ovulation in ten-month old hens

PMSG dose (IU)	No. of hens	No. of hens stopped ovulation in 3 days	No. of hens stopped ovulation in 8 days
0	10	0	0
75	10	0	3
100	50	0	48

Spermatozoa preparation

Sperm was collected from White Leghorns by the massage method just before the experiment. The spermatozoa concentration in the pooled semen was 1.5×10^8 ml^{-1}, which was diluted at the ratio of 1:200 with the m-Ringer's solution (Tanaka et al., 1994).

***In vitro* fertilization and culture**

In vitro fertilization was carried out as described with a few modifications (Nakanishi et al., 1991). Ova were collected and placed into warm m-Ringer's solution at 41°C in a 100 ml beaker. One hundred µl of diluted semen was inseminated onto the germinal disk of each ovum, followed by a 15 min incubation at 41°C in a 5% CO_2 incubator. The treated ova were then washed twice in m-Ringer's solution and cultured at 41°C for 10 h in DME-F12 medium (Dulbecco's Modified Eagle's medium/Nutrient Mixture F-12 HAM'S, HYCLONE) containing 20% liquid albumen from fresh eggs. These ova were fixed in Bouin's solution for 24 h and then transferred to 60% alcohol. The blastodisc was observed under the microscope directly or after staining with gentian violet (Olsen, 1942).

Environmental scanning electron microscope (ESEM) study on *in vitro* fertilization

The *in vitro* fertilized ova were fixed first with Karnovsky's fixative (4% (w/v) paraformaldehyde and 5% (w/v) glutaraldehyde in Millonig's buffer (0.12 mol NaH_2PO_4 l^{-1} and 1 mol NaOH l^{-1}, pH 7.2) for 1 h at 4°C. After removal of the germinal disc, the ova were refixed overnight in the same fixative at 4°C, followed by examination with Phillips XE 30 ESEM at an acceleration voltage of 20 kV.

Statistical analyses

The statistical significance of differences was determined by the t-test or Fisher's exact probability test. A p-value of less than 0.05 was taken as an indicator of significance.

RESULTS

Dose-dependent inhibition of egg laying by PMSG

When 75 IU PMSG was administered daily for 8 days,

Table 2. Effect of chicken anterior pituitary extract (CAPE) on induction of ovulation in hens pretreated with PMSG

CAPE dose (mg)	No. of hens	Ovulation	
		No. of hens	No. of ovulating follicles/hen[1]
0	3	0	0.00
100	3	3	1.00±0.00[a]
200	6	6	2.33±0.52[b]
300	5	5	2.20±0.45[b]

[1] Mean±SE. [a, b] Values with different superscripts within each column significant different ($p<0.05$).

Table 3. Effect of acetone-dried chicken anterior pituitary extract (ACAPE) on induction of ovulation in hens pretreated with PMSG

ACAPE dose (mg)	No. of hens	Ovulation	
		No. of hens	No. of ovulating follicles/hen[1]
0	3	0	0.00
10	3	3	2.00±0.00[a]
15	7	7	2.86±0.69[b]
20	5	3	3.00±1.22[a]

[1] Mean±SE. [a, b] Values with different superscripts within each column significant different ($p<0.05$).

cessation of egg laying was observed in only 30% of the treated hens. However, the percentage of hens showing the same effects changed significantly (96%) when the amount of PMSG was increased to 100 IU (Table 1).

CAPE and ACAPE stimulate multiple ovulations

Ovulation was induced in all CAPE-treated (Table 2) and ACAPE-treated hens (Table 3). The number of ovulating follicles increased by more than twice as the amount of CAPE increased from 100 mg to 200 mg ($p<0.05$). However, no further change was observed when 300 mg CAPE was used. The number of ovulating follicles was significantly increased as the ACAPE amount increased from 10 mg to 15 mg ($p<0.05$). All ovulated ova were mature (germinal vesicle disappeared) with the diameter similar to those of naturally ovulated ova (Figure 2).

Timing of multiple ovulations after CAPE injection

When hens were sacrificed at 7.2 h after CAPE or ACAPE injection, no ovulation occurred in most hens except for one, which began multiple ovulations about 6 h after injection of ACAPE. However, multiple ovulated ova were found in the infundibulum or body cavity at 7.5 h post injection. At that time, most hens had one ovum captured in the infundibulum and another one or two located in the body cavity. Two ova rarely resided in the infundibulum simultaneously.

(A) (B)

Figure 2. A dose-dependent induction of ova with CAPE injection. (A) one ovum (arrow) was ovulated in a hen treated with 100 mg CAPE, (B) three ova (arrow) were ovulated in a hen treated with 200 mg CAPE.

(A) (B)

Figure 3. Early development of the multiple ovulated ova after *in vitro* fertilization. (A) 4 cell stage, (B) 16 cell stage.

In vitro fertilization and culture

Nine multiple ovulated ova were used for *in vitro* fertilization experiment (Table 4). After incubation for 10 h, one developed to the 4 cell stage (Figure 3, left), four to the 8 cell stage, one to the 16 cell stage (Figure 3, right) and two to the 32 cell stage. Only one egg did not show any signs of embryo development. Naturally ovulated ova developed to 4 to 32 cell stages after *in vitro* fertilization and culture. The multiple ovulated ova recovered 7.5 h after CAPE and ACAPE stimulation have shown the same capability as the naturally ovulated ova for *in vitro* fertilization and development ($p>0.05$).

ESEM study on *in vitro* fertilization

In vitro fertilization and multiple stages of the sperm penetrating process were observed by ESEM. For example, sperm was found on the perivitelline layer of an ovum 5 min after fertilization (Figure 4A). Ten min later, several

Table 4. Early development of the multiple ovulated ova after *in vitro* fertilization and culture

	No. of eggs	No. (%) of eggs developed	No. of fertilized eggs developed to stages				
			2 cell (%)	4 cell (%)	8 cell (%)	16 cell (%)	32 cell (%)
Multiple ovulated	9	8 (89%)[a]	0	1 (13%)	4 (50%)	1 (13%)	2 (25%)
Naturally ovulated	7	6 (86%)[a]	0	1 (17%)	2 (33%)	2 (33%)	1 (17%)

[a] Values with same superscripts within each column observed no significant differences ($p>0.05$).

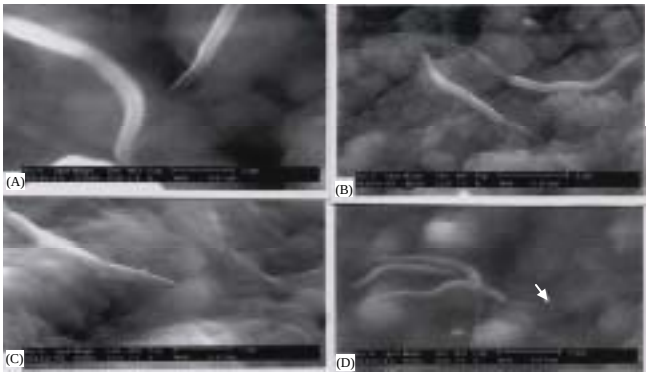

Figure 4. *In vitro* fertilization process observed under ESEM. (A) 5 min after *in vitro* fertilization, (B), (C) and (D) 15 min after *in vitro* fertilization.

sperms were found to be penetrating one ovum simultaneously (Figure 4C, 3D), and one sperm was found to have almost entered into the ovum but its tail left outside (Figure 4D, arrow).

DISCUSSION

Multiple ovulations in hens could be achieved by suppression of endogenous ovulation through daily injection of large amount of PMS or PMSG and subsequent induction of ovulation with mammalian or chicken gonadotrophins (Fraps et al., 1942b; Imai, 1973). An important factor for successful multiple ovulations was to use the PMSG-treated hens that ceased ovulation within a certain umber of days. In this experiment, the effect of multiple ovulations was better if hens ceased ovulation within 3 to 6 days after PMSG injection. If hens stopped ovulation within 3 days, no ovulation occurred after CAPE or ACAPE injection. If hens stopped ovulation 6 days after PMSG injection, CAPE or ACAPE treatment failed to induce ovulation and many little immature yellow ova were observed in the ovary (diameter about 2 cm smaller). Additionally, it was reported that more ovulating ova were induced by extracts from chicken pituitary than by ovine LH (Imai, 1973). Instead of 3 to 5 multiple ovulated ova in the hens treated with chicken pituitaries, only 2.0 to 2.3 ova were found in ovine or bovine-injected hens (Imai, 1973; Johnson and Leone, 1985). In our study, 1 to 5 multiple ovulated ova were found in each hen after CAPE or ACAPE stimulation. One possible explanation for the lower ovulation rate is that the hens had been sacrificed a little too early and did not have enough time to ovulate more ova, as the number of ovulated ova was reported to increase gradually with the incubation time after CAPE injection (Nakanishi et al., 1991).

Multiple ovulations occurred 6.1 h to 7.2 h after injection of luteinizing preparation from equine pituitary (Fraps et al., 1942b) and 7.5 h after CAPE injection (Nakanishi et al., 1991). We observed that most hens began multiple ovulations 7.5 h after CAPE or ACAPE injection. Only one hen began multiple ovulations about 6 h after injection of ACAPE. In the hens treated with CAPE or ACAPE for 7.5 h, one or two ova were just engulfed by the infundibulum and others resided in the body cavity.

Multiple ovulations may cause atretic ova (Imai, 1973; Nakanishi et al., 1991), which can be classified into non-bursting atresia and bursting atresia (Gupta et al., 1988). Both types of atretic ova were observed in our study; however, the percentage was not as gresat as reported by (Nakanishi et al., 1991). Only three ruptured ova were found and one mature ovum located in the body cavity showed non-bursting atresia.

The multiple ovulated ova recovered 7.5 h to 8.5 h after CAPE stimulation have shown the same capability as naturally ovulated ova for *in vitro* fertilization and development (Nakanishi et al., 1991). As demonstrated in this report, multiple ovulated ova could develop to 4 to 32 cell stage embryos in culture, just like naturally ovulated ova. Their competency for further development is being tested in our lab now. The optimal number of spermatozoa for *in vitro* fertilization needs to be studied further because excessive polyspermy following *in vitro* fertilization might affect the development of embryos, as the so-called ‘sperm selection mechanism’ is absent in the *in vitro* fertilization system (Tanaka et al., 1994). More details about the composition of oviduct fluid should be known and the *in vitro* culture conditions need to be optimized. After modifying the culture medium (using thick albumen), multiple ovulated ova after *in vitro* fertilization and culture could develop to the primitive streak stage *in vitro* in our lab (unpublished data). A major structural difference detected between the fertilized ova *in vivo* and the multiple ovulated ova in culture was that the latter did not have an outer perivitelline layer. This outer perivitelline layer was formed to block polyspermy when the ovum transits through the distal infundibulum and proximal magnum (Bakst and Howarth, 1977). Whether it has any influence on embryonic development remains to be examined.

A complete *in vitro* culture system starting from fertilization to hatch has been reported (Perry, 1988), but the fertilized ova used were taken from the oviduct and had a surrounding layer of albumen. This layer of albumen is required for further development of embryos. Since multiple ovulated ova did not have this thin layer of albumen, this reported system could not be used for all purposes. With a modified method, we present data to improve the current culture conditions. These results should be beneficial in establishing a new *in vitro* culture systems for multiple ovulated ova and to support future avian transgenesis studies.

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