Ultrastructure of "Twin Oocytes" in the Ovaries of the Giant Pandas (Ailuropoda melanoleuca)

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Abstract: *Two pairs of the ovaries were obtained from two giant pandas which accidentally died due to hepatic disease and senility. Of these ovaries, one pair of "twin oocytes" was collected from each pair of ovaries and processed for histological observation with an electron microscope. The results from this study suggest the possibility of the existance of "twin oocytes" in the follicle and of a higher rate of twinning in the giant panda.*

Key words: *Giant Panda, "Twin oocytes", Isolation, Electron Micrograph, Twins*

In general, the majority of ovarian follicles contain only a single oocyte surrounded by several layers of granulosa cells [1]. Nevertheless, some researchers who are working with mammalian reproduction probably pay special attention to the cases in which two or more oocytes may been contained. Hitherto, binovular and multiovular follicles (henceforward called polyovular follicles) have been reported sporadically in most mammalian species including humans [2–5]. Hartman [2] was the first to observe twin oocytes in mammals in which each oocyte in the polyovular follicle had its own granulosa cells. Al-Mufti *et al.* [6] also observed some healthy follicles with 2–24 oocytes in adult rabbit ovaries during all phases of folliculogenesis from primary to preovulatory follicles. In this study, oocytes were classified according to the topographical situation in the follicle and the central oocyte was larger than the peripheral ones which were larger than the surface oocytes. These oocytes showed almost normal growth rate, leading to the developmental stages up to metaphase II and cumulus expansion [6]. Telfer *et al.* [7] studied 15 species of animals, and recorded polyovular follicles in the following species, in descending order of abundance: rabbits, rhesus monkeys, humans, cats and dogs. Polyovular follicles were frequently formed in ovaries exposed perinatally to sex steroids, diethylstilbestrol and tamoxifen, and these oocytes showed a significantly ($P<0.05$) decreased fertilization capacity in vitro [8, 9].

Interest in polyovular follicles has stemmed from two points of view, namely, their ontogenesis and their contribution to fecundity. These follicles have frequently been regarded as pathological entities and this has also led many investigators to pay attention to the incidence of oocyte death and the role of sex steroids and plasma concentrations of gonadotrophins during reproductive cycles [10]. The changing hormonal "balance" after puberty provided an explanation for the apparent fall in the incidence of polyovular follicles as animals matured [11]. The early inquiries were, however, influenced by a limited knowledge of reproductive endocrinology and the widespread and erroneous belief that oocytes are formed continually throughout life. Polyovular follicles are also of interest because they present a natural experiment testing the general rule of one oocyte: one follicle has been altered with possible developmental significance for the follicle and its various components.

For the giant panda, many studies have so far been carried out on reproductive biology in order to rescue this species. Feng *et al.* [12] and Chen *et al.* [13] discovered two oocytes in one follicle when they conducted histological examinations on giant panda's ovaries. They described this phenomenon as "double oocytes in one follicle" [12, 13].

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In the present study, two oocytes surrounded by the same layer of granulosa cells were observed, when oocytes were recovered from one pair of ovaries [14]. Another pair of giant panda's ovaries was also investigated so as to know if this phenomenon is found in other giant pandas, paying special attention to the inferences about ontogenesis. At the same time, this experiment also aimed at making predictions about the contribution of these oocytes to the production of twins in the giant panda.

Materials and Methods

Collection of Giant Pandas' ovaries

Two pairs of ovaries were collected from one giant panda which had died of hepatic cirrhosis and another which had died of senility. The ovaries were kept in physiological saline at 37°C (containing 200 IU/ml Penicillin and Streptomycin) and transported to the laboratory as soon as possible after death.

Isolation of "twin oocytes"

The first pair of ovaries were washed three times with sterile physiological saline and adnexa were trimmed off. The ovaries were then washed repeatedly in PBS and put into dishes (ϕ 6 cm). The cortex of the ovaries was dissected with a blade to a depth of 500 μm. The ovarian fragments suspended in PBS were taken into a watch glass, allowed to stand for 5 minutes and observed under a dissecting microscope.

For the second pair of ovaries, after adnexa were trimmed off, the cortex of the ovaries was cut into small pieces (<3 mm) with scissors, and digested in 15 ml of 0.1% collagenase solution for 1 hr at 37°C. After the cell suspension had been centrifuged 3 times at 200 g for 5 minutes, the collagenase was washed away and the ovarian fragments were transferred onto a mesh filter (ϕ 600 μm). The oocytes were filtered through the same mesh filter and centrifuged again in same manner as before. The supernatant was removed, and the precipitates were collected and put into 10 ml M199 and observed under a light microscope.

Sample preparation for transmission electron microscope

The "twin oocytes" obtained by the collagenase digestion were fixed with 2.5% glutaraldehyde and 1.0% osmium tetraoxide, dehydrated by a graded series of acetone, and embedded in beem capsules with Epon 812. Thin sections were made with glass knives and stained with 1.0% toluidine blue. Ultrathin sections were made with a diamond knife, double-stained with uranyl acetate and lead acetate, and examined with a TEM-2000EX electron microscope.

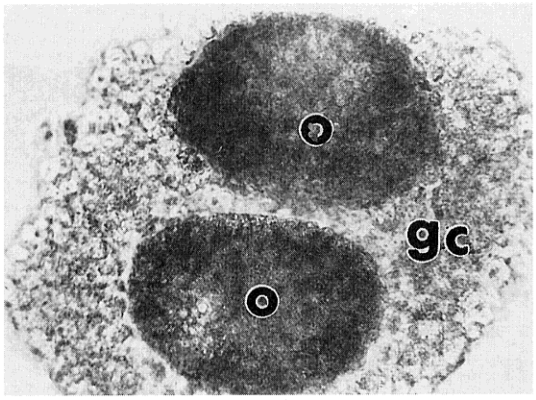

Fig. 1. Twin oocytes (O) surrounded with a mono layer of granulosa cells (gc). A considerably thicker layer of granulosa cells enclosing two oocytes. × 400.

Results

Isolation of "twin oocytes"

Oocytes were recovered by the dissecting method from the ovaries of a giant panda which had died of hepatic cirrhosis. In the present study, "twin oocytes" were considered to number about 1–5 per 100 oocytes in one ovary. Under the microscope, two oocytes were observed to be linked with each other and could not be separated by repeated pipetting and aspirating with a small pipette. Two oocytes were also found to be close to each other under a magnification of ca. X 400, but each oocyte had its own intact zona pellucida, surrounded by a layer of normal granulosa cells. There was 2–3 μm distance between the two oocytes (Fig. 1).

Another pair of the ovaries were processed for histological examination with the same digestion method described above. We also obtained another one ovary in which two oocytes were also linked to each other, showing a much clearer appearance after some of the granulosa cells were removed.

Electron microscopic observation of "twin oocytes"

Two zonae pellucidae of two oocytes showed a slightly thinner layer compared with those of maturated oocytes, and were linked together at the narrowest parts between the zonae pellucidae. Each side had one layer of granulosa cells between the zonae pellucidae. It was

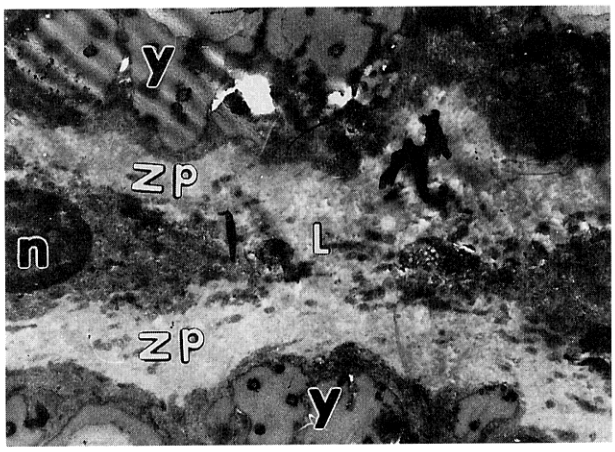

Fig. 2. An electron micrograph of twin oocytes which were separated by two layers of zona pellucida (zp). A very narrow part of the zona pellucida linked each oocyte (L), though a single layer of cells (n) was observed between the zonae pellucidae. The oocytes of the giant panda were characterized by a lot of yolk bodies (Y) which is completely different from the oocytes of other animals such as cows or goats. × 7500.

also of interest to observe a lot of yolk bodies in the cytoplasm of the oocytes (Fig. 2).

Discussion

In general, a few percent of the follicles in the giant panda have been considered to be "twin oocytes". Two oocytes surrounded with the same layer (one layer) of granulosa ceus were observed in the oocytes recovered from the ovaries of the first giant panda. It is inferred that this phenomenon may be observed in other giant panda's ovaries, since another two oocytes surrounded by granulosa cells (one layer) were found in another pair of ovaries digested with collagenase solution.

Electron microscopic observation depicted two zonae pellucidae of two oocytes connecting with each other. This is probably different from polyovular follicles, since each oocyte was surrounded with its own granulosa cells [6]. This is referred to as "twin oocytes" in this study.

Papadaki [15] studied binovular follicles in the adult human ovary, indicating that binovular follicles were formed by the fusion of two adjacent primordial follicles with a mechanism similar to the depolymerization of the ground substances in the apex of the Graafian follicle that leads to ovulation. In the present study, the electron micrograph showed a tight connection between two zonae pellucidae of the "twin oocytes". In this case, the granulosa layer was not separated between the "twin oocytes", suggesting the development of two single oocytes during their early proliferating stage.

The giant panda's reproductive ability is very low both in the wild and in captivity, but its birth rate of twinning is considerably high compared with other monotocous animals such as the cow and some species of goat [16, 17]. According to the report by Zhang *et al.* [18] and recently published statistical information about twin births of giant pandas in captivity, the birth rate of twinning is about 50% (2000). Fang *et al.* [19] also demonstrated the results of DNA fingerprinting using hairs, blood and semen for paternity determination in the filial generation of artificial breeding of the giant panda. Three pairs of twins were examined for DNA analysis, and their hybrid bands were also observed in their parents. The DNA fingerprinting of each individual was highly individual-specific, proving that all these three pairs of twins were fraternal twins. Some other researches have also shown that the twins born to captive paudas were not identical twins [20, 21], and it was also suggested that repeated artificial insemination, multi-artificial insemination, might have led to the chance of fertilization of two oocytes which were ovulated separately on different days during the estrus cycles [22]. However, Zeng *et al.* [23] and Lin *et al.* [24] determined the concentration of urinary estrogen during the estrus period, and demonstrated that only one peak of hormone level was found at the time of ovulation, which was well coincident with the estrus behavior of giant pandas. Multi-artificial insemination has usually been employed in captivity to ensure the success of fertilization during the breeding season [25]. It is suggested by the recent experiments that "twin oocytes" may reside in one follicle during the developmental period and that two oocytes could be ovulated simultaneously just at the time of ovulation. This phenomenon may, thus, contribute to the higher rate of twinning in the giant panda. Therefore, "twin oocytes" may also give us a clue for explaining the higher rate of twinning, though further studies will be needed to provide more useful information about "twin oocytes" in the giant panda.

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