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Intrafollicular Milieus and Outcome of Oocyte Retrieval

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The objective of this study was to assess the intrafollicular milieus across different follicle sizes and the associated outcomes of oocyte retrieval. Serum levels of estradiol and progesterone were measured on the day of oocyte retrieval in patients who underwent in vitro fertilization. Gene expression of Kiss-1, GnRH, PACAP, and their corresponding receptors within the cellular components obtained from follicles of different sizes were determined. Larger follicles contained higher levels of sex steroids compared with those in small follicles. The oocyte retrieval rate from the two different follicle sizes were identical. Intrafollicular sex steroid levels were not associated with the success of oocyte retrieval. Expression levels of Kiss-1, GnRH, and their receptor genes as well as PACAP gene expression in follicular cells of different sizes were identical, but gene expression of PACAP receptor was significantly lower in the cellular component of large compared with that of small follicles.

Keywords: follicular fluid, follicular size, sex steroids, oocyte, hypothalamic peptides

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INTRODUCTION

Human follicles develop with a concomitant increase in the synthesis of estradiol（E2）under the control of pituitary gonadotropins. Steroid genesis is crucial for follicle growth and oocyte maturation［1］, and the expected number of retrieved oocytes can be estimated based on circulating E2 levels during in vitro fertilization（IVF）procedures. Follicular size is reported to be associated with the maturity of oocytes, and the intrafollicular E2 environment is associated with oocyte quality, such that elevated E2 in follicular fluid indicates a more advanced stage of oocyte maturation［1］. Elevated E2 in the follicles has also been reported to be associated with successfully achieving pregnancy［2, 3］; however, these reports were not confirmed by other studies［4, 5］. Follicular fluid is a product of blood plasma constituents that cross the blood–follicle barrier. In addition, the secretory activity of cellular components within the follicle, such as granulosa and theca cells, are responsible for forming follicular fluid［6］. Therefore, biochemical characteristics of the follicular fluid and ovarian cells surrounding oocytes may play an important role in determining oocyte quality and subsequent outcomes of IVF treatment.

Follicular development and E2 synthesis in females are primarily regulated by the hypothalamic pituitary gonadal（HPG）axis, which is centrally governed by kisspeptin and gonadotropin-releasing hormone（GnRH）from the hypothalamus［7］. At present, it is generally believed that kisspeptin（encoded by Kiss-1 gene）expressing neurons（Kiss-1 neurons）located in two different areas of the hypothalamus govern the surge secretion and basal pulsatile secretion of GnRH, which ultimately regulates

luteinizing hormone (LH) surge secretion and basal LH/follicle stimulating hormone (FSH) secretion from the pituitary gland [7, 8]. In addition, it is plausible that kisspeptin and GnRH also play a role in the ovary. Kisspeptin and its receptor (Kiss1R) are expressed in the ovarian follicles [9] and are reported to be involved in oocyte maturation [10]. GnRH receptor (GnRHR) is also expressed in the ovary and influence ovarian steroidogenesis [11].

Oocyte maturation is associated with follicle size, and the final size of the follicles on the oocyte retrieval day is one of the determinants of oocyte maturity [12]. It has been reported that oocytes are much more commonly retrieved from medium (13–23 mm) or large (>24 mm) follicles than from small (8–12 mm) follicles [13]. In addition, it has been revealed that human follicular fluid shows diverse metabolic profiles across the different stages of follicle development [14].

In this study, we re-examined the oocyte retrieval rate from small (<12 mm) and large (>12 mm) follicles at the oocyte pick-up (OPU) procedures in IVF treatment. Intrafollicular E2 or P4 levels were compared between the different follicle sizes, and the rates of the presence or absence of oocytes were compared between the two follicle size classes. Furthermore, expression levels of locally expressed hypothalamic peptides and their receptor genes within the follicular cells were compared between the two different follicle sizes.

MATERIALS AND METHODS

Controlled ovarian stimulation

A total of 5 patients, aged 32–40 years and who were undergoing oocyte retrieval for IVF, participated in this study and provided written informed consent. Ethical approval for this study was obtained from the Ethical Committee of Shimane University Hospital (20210818-1). Oocyte retrieval was performed during ovarian stimulation by one of the following methods. The GnRH antagonist method using injection of human menopausal gonadotropin (hMG) (Fuji Pharmaceutical Company, Ltd., Tokyo, Japan) alone or in combination with clomiphene citrate (Clomid Tablets; Shionogi & Company, Ltd., Osaka, Japan). Administration of 0.25 mg GnRH antagonist (Cetrotide) (Shionogi & Company) was started when the diameter of a dominant follicle reached 15 mm and given daily with hMG until follicles reached 18–20 mm in diameter. Choriogonadotropin alfa (Ovidrel) in a syringe (250 mg) for subcutaneous injection (Merck Biopharma Japan, Tokyo, Japan) was administered to patients 34 h prior to oocyte retrieval. Oocytes were collected by manual aspiration with a syringe and an 18 gauge needle (HAKKO & Company, Ltd., Tikuma, Japan)

Sample collection and hormone assay

In total, 29 follicular fluid samples were collected from the 5 patients who underwent IVF treatment. During oocyte retrieval, each follicle was measured and then manually punctured, and its follicular fluid was aspirated. Each fluid sample was collected individually. Large follicles were defined as having a diameter greater than 12 mm, while follicles with a diameter less than 12 mm were defined as small follicles. After identifying the presence or absence of oocytes, each fluid sample was centrifuged at 200 × g and frozen at −80°C. From each patient, 3 to 9 follicular fluid samples were collected. The serum fraction was separated rapidly by centrifugation and frozen at −80°C. The blood samples from the patients were also collected on the day of OPU to investigate the relationship between the plasma and follicular fluid sex steroid levels. Concentrations of estradiol and progesterone were measured by chemiluminescent electro-immunoassay with an IMMULYZE 1000 system (LSI medicine, Tokyo, Japan).

RNA preparation from follicular cells, reverse transcription, and quantitative real-time polymerase chain reaction

Total RNA was extracted from harvested follicular fluid cell pellets using TRIzol-LS (Invitrogen, Carlsbad, CA). To obtain cDNA, 1.0 μg of total RNA was reverse-transcribed using oligo-dT primers (Promega, Madison, WI) and prepared using a First-Strand cDNA Synthesis Kit (Invitrogen) in reverse transcription buffer. The preparation was supplemented with 10 mM dithiothreitol, 1 mM each dNTP, and 200 U of RNase inhibitor/human placenta ribonuclease inhibitor (Cat. No. 2310; Takara, Tokyo, Japan) in a final reaction volume of

10 μL. The reaction was incubated at 37°C for 60 min. Using specific primers for Kiss-1 (forward, 5′-AGCTGCTGCTTCTCCTCTGT-3′; reverse, 5′-GCATACCGCGATTCCTTTT-3′), GnRH (forward, 5′-ACTGTGTGTTTGGAAGGCTGC-3′; reverse. 5′-TTCCAGAGCTCCTCGCAGATC-3′), Kiss-1R (forward, 5′-CTGCCACAGACGTCACTTTC-3′; reverse, 5′-ACATACCAGCGGTCCACACT-3′), GnRHR (forward, 5′-CTAACAATGCGTCTCTTGA-3′; reverse: 5′-TCCAGATAAGGTTAGAGTCG-3′), PACAP (forward, 5′-GATGTCGCCCACGAAATCCT-3′; reverse, 5′-GTATGCTATTCGGCGTCCTT-3′), and PAC1R (forward, 5′-CTTGTACAGAAGCTGCAGTC-3′; reverse, 5′- CCGGTGCTTGAAGTCCATAG-3′), mRNA levels of these genes were determined by quantitative RT-PCR (ABI Prism 7000; Perkin-Elmer Applied Biosystems, Foster City, CA) following the manufacturer's protocol (User Bulletin No. 2) and utilizing Universal ProbeLibrary probes and Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). The simultaneous measurement of mRNA and GAPDH permitted normalization of the amount of cDNA added per sample. For each set of primers, a no-template control was also included. Thermal cycling conditions were as follows: 10 min of denaturation at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were followed by melting curve analysis (55°C–95°C).

Statistical analysis

Comparisons between the mean plasma sex steroids levels and those in follicular fluid were performed with Welch's *t*-test. Comparisons of oocyte retrieval between the two follicle size groups were made using the chi-squared test. Comparisons of the means between two groups were performed by using Student's *t*-test. For all statistical analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Correlation between plasma and intrafollicular levels of E2 and P4 on the day of OPU

In total, 29 follicular samples were collected from 5 patients who underwent OPU during IVF procedures. Follicular samples contained fluid that was aspirated from large follicles (>12 mm in diameter) and small follicles (<12 mm in diameter). The characteristics of patients are described in Table 1. Serum Luteinizing Hormone (LH) and Follicle stimulating hormone (FSH) levels were measured between days 1 and 5 of menstruation cycle. Although the number of fluid samples obtained from different follicle sizes differed among patients, average levels of follicular E2 and P4 in each patient were calculated. Average follicular E2 concentration varied among patients, ranging from 563,375 to 742,950 pg/mL and averaging 515,841 ± 226,785 pg/mL. On the other hand, plasma concentrations of E2 in patients ranged from 249 pg/mL to 1,516 pg/mL and averaged 905 ± 466 pg/mL in 5 patients on the day of OPU. On average, the follicular E2 concentration was 570 times higher in patients who underwent OPU. The average follicular E2 concentration in each patient was positively correlated with the serum level of E2 ($r = 0.92$, $P < 0.05$) (Fig. 1A). P4 concentration within the follicle was also much higher than the serum level of P4. The average follicular P4 concentration was 17,056 ± 12,816 ng/mL and ranged from 2,432 ng/mL to 17,546 ng/mL, while the average serum P4 concentration was 3.59 ± 3.74 ng/mL in these patients, ranging from 1.54 ng/mL to 4.28 ng/mL. On average, the follicu-

Table 1. Characteristics of the patients

patient	age	BMI	LH (mLU/mL)	FSH (mIU/mL)	follicle number	large follicle number	small follicle number	oocyte number
1	37	21.2	4.24	7.5	7	3	4	4
2	38	20.6	4.1	6.3	3	1	2	3
3	37	20.8	4.1	8.1	9	6	3	5
4	28	18.9	2.7	11.1	6	6	0	2
5	38	22.4	3.9	6.6	4	4	0	2

BMI: body mass index, LH: lueinzing hormone, FSH: follicle stimulating hormone

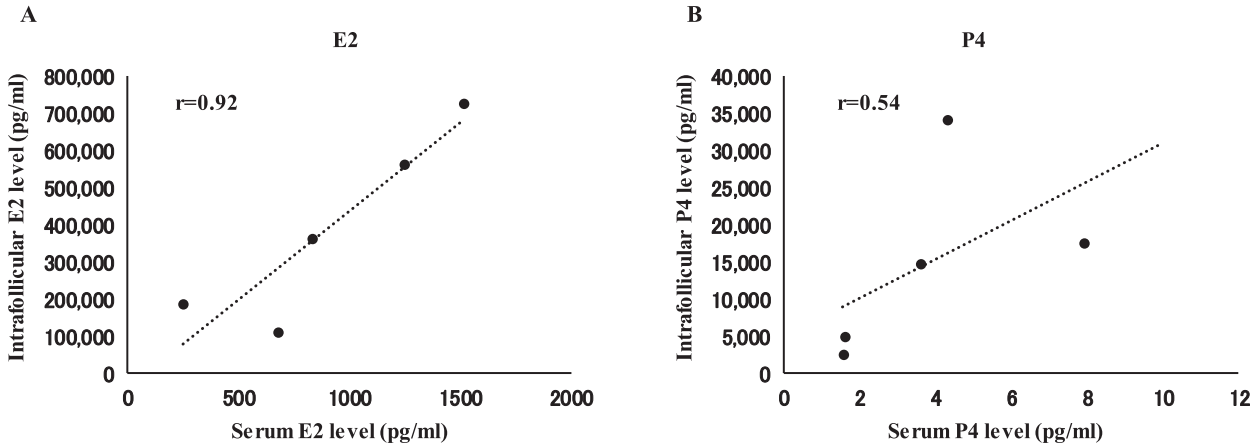

Fig. 1. Correlation between sex steroids levels in follicle and serum samples. (A) Scatter plots showing the relationship between average intrafollicular estradiol (E2) concentration and serum level of E2. (B) Scatter plots showing the relationship between average intrafollicular progesterone (P4) concentration and serum level of P4. Average concentrations of follicular E2 and P4 are positively correlated with their serum levels.

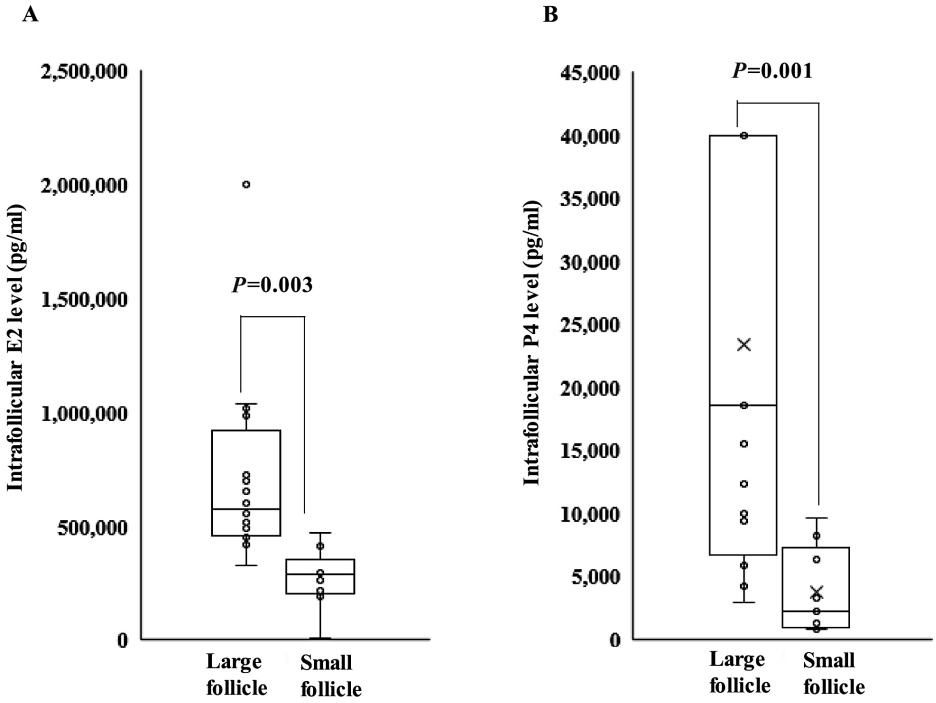

Fig. 2. Intrafollicular sex steroid levels in large and small follicles. Estradiol (E2) (A) and progesterone (P4) (B) concentrations were measured in large follicles (diameter >12 mm) and small follicles (diameter <12 mm) and compared. Both intrafollicular E2 and P4 levels were significantly higher in large follicles.

lar P4 concentration was 4,751 times higher in each patient who underwent OPU. As observed for E2, the average follicular P4 concentration was positively correlated with the serum level of P4 in each patient (r = 0.54, P < 0.05) (Fig. 1B).

Intrafollicular steroid levels among large and small follicles

Intrafollicular E2 levels were compared between large and small follicles. The average intrafollicular E2 level in large follicles was 697,679 ± 371,744 pg/mL and that in small follicles was 270,502 ± 127,683 pg/mL, respectively; moreover, there was a significant difference in intrafollicular E2 concentration between large and small follicles (P = 0.003) (Fig. 2A). The average level of intrafollicular P4 in large and small follicles was 23,312 ± 15,852 ng/mL and 3,688 ± 3,355ng/mL, respectively. As ob-

served for E2, the intrafollicular P4 level was significantly higher in large follicles (P = 0.001) (Fig. 2B).

Follicle size and oocyte retrieval

In a total of 29 follicular samples from 5 women, we obtained 20 follicular fluid samples from large follicles and 9 from small follicles. Among the 20 samples from large follicles, oocytes were retrieved from 12 follicular samples. Oocytes were not found in the remaining 8 large follicles. From 9 follicular fluid samples aspirated from small follicles, we found 4 oocytes. The oocyte retrieval rate from large and small follicles was not statistically different (P = 0.68) (Fig. 3).

Oocyte	+	−
Large follicle	12	8
Small follicle	4	5

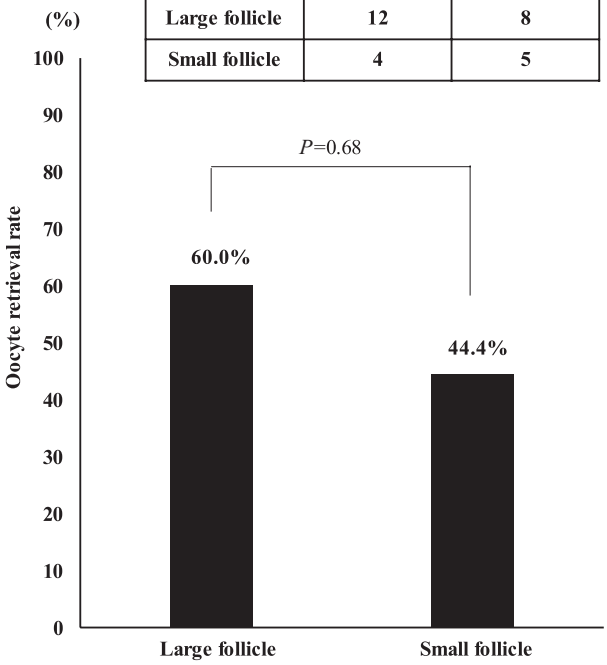

Fig. 3. Oocyte retrieval rate from follicles of different sizes. The oocyte retrieval rate from large follicles (diameter >12 mm) and small follicles (diameter <12 mm) were compared. The retrieval rate was not statistically significant (P = 0.68).

Intrafollicular steroid levels and the presence of oocytes

The relationship between follicular steroid level and the presence of oocytes in follicular samples was examined. Out of 29 follicular samples obtained from both large and small follicles, a total of 16 oocytes were retrieved. The average level of intrafollicular E2 in the follicles from which oocytes were retrieved was 791,577 ± 359,232 pg/mL, and

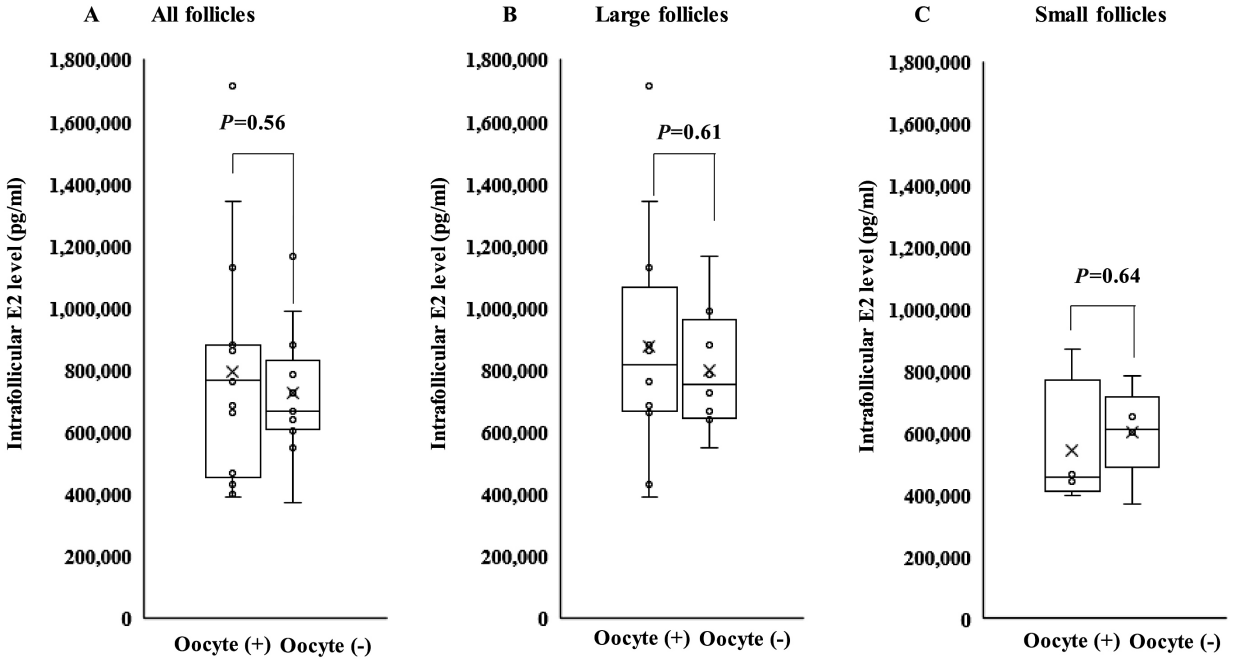

Fig. 4. Comparison of intrafollicular estradiol (E2) levels in follicles with and without oocytes. (A) Intrafollicular E2 levels were compared between all follicles with or without oocytes. (B) Intrafollicular E2 levels were compared between large follicles (>12 mm) with or without oocytes. (C) Intrafollicular E2 levels were compared between small follicles (<12 mm) with or without oocytes. There was no significant difference between them.

that from which oocytes were not retrieved was 724,333 ± 199,174 pg/mL. There was no significant difference in the concentration of E2 between the follicles from oocytes were obtained or not (Fig. 4A). Subsequently, intrafollicular E2 concentrations were compared within the same follicle size class. There was no difference in E2 concentration in the follicular fluid, whether in the presence or absence of oocytes, both in large and small follicles (Fig. 4B). In result of the combined large and small follicles, the mean intrafollicular P4 concentration of follicles from which oocytes could be retrieved was 57,966 ± 121,074 ng/mL, while the mean intrafollicular P4 concentration of follicles from which oocytes could not be retrieved was 76,612 ± 99,186 ng/mL, showing no significant difference in intrafollicular P4 concentration (Fig. 5A). When compared within the same follicle size class, P4 concentrations in the follicular fluid did not differ by the presence or absence of oocytes within each large and small follicle (Fig. 5B and C). Basically, there were no differences in steroid hormone concentrations in the follicular fluid with or without oocytes, regardless of follicle size.

Hypothalamic peptides and their receptor gene expression in the cellular component of follicular fluids

Locally expressed hypothalamic peptides and their receptor gene expression in cellular components from follicles of different sizes were examined. Kisspeptin and GnRH gene expression levels within the follicular cells were not significantly different between the cells from small follicles and large follicles. Gene expression levels of receptors for kisspeptin (Kiss1R) and GnRH (GnRHR) were not significantly different between the cells from small follicles and large follicles. Pituitary adenylate cyclase-activating polypeptide (PACAP) gene expression in follicular cells did not significantly differ between small and large follicles, but gene expression of its PACAP type I receptor (PAC1R) was lower in large follicles than in small follicles (Fig. 6).

DISCUSSION

In this study, by examining follicular samples from women who underwent OPU, we determined the intrafollicular levels of E2 and P4. The average intrafollicular E2 concentration in each follicle re-

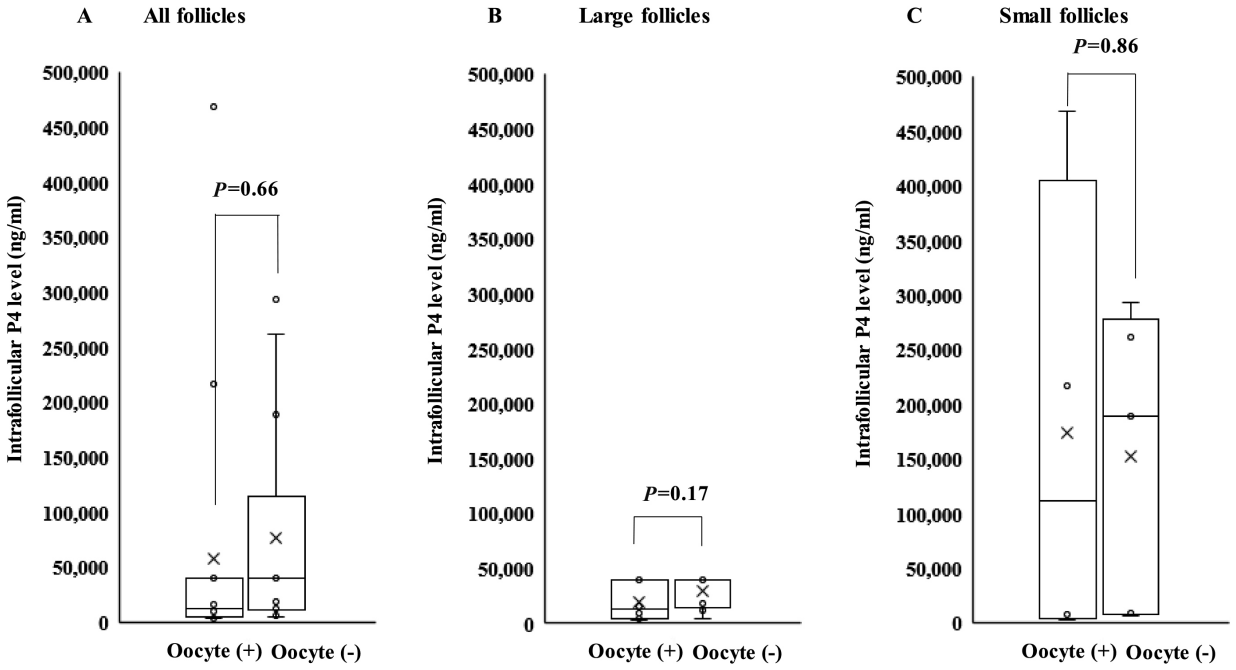

Fig. 5. Comparison of intrafollicular progesterone (P4) levels in follicles with and without oocytes. (A) Intrafollicular P4 levels were compared between all follicles with or without oocytes. (B) Intrafollicular P4 levels were compared between large follicles (>12 mm) with or without oocytes. (C) Intrafollicular P4 levels were compared between small follicles (<12 mm) with or without oocytes, and no significant difference was found.

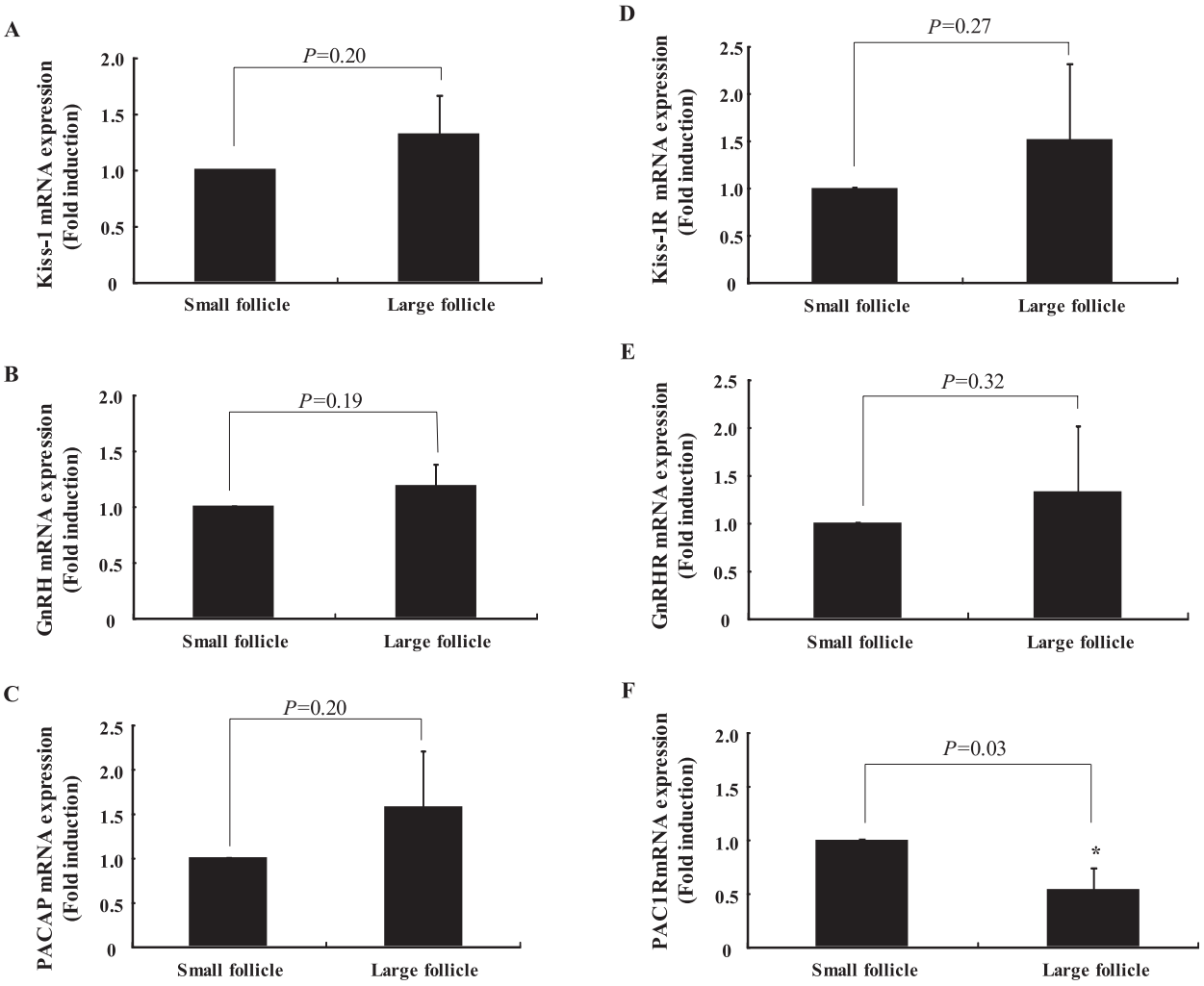

Fig. 6. Gene expression of hypothalamic peptides and their receptors in the cellular component of ovarian follicles. Cellular components were obtained from oocyte pick-up (OPU). After mRNA extraction and reverse transcription, the gene expression levels of Kiss-1, GnRH PACAP, and their receptors were determined by quantitative real-time PCR and compared between small and large follicles. The results are expressed as the fold elevation of follicular cells relative to small follicles and presented as mean ± SEM values (*$P < 0.05$ relative to small follicles).

gardless of its size was almost 400- to 1000-fold higher compared with circulating levels of E2. Similarly, the intrafollicular concentration of P4 was highly concentrated compared with serum level P4, at a concentration almost 1000- to 5000-fold higher. Although ovarian follicles are the principal organ that synthesizes and releases sex steroids, it was unexpected that sex steroid hormones from ovaries were so much less concentrated in general circulation. A previous report indicated that follicular size is positively correlated with absolute steroid levels [15]. We also observed a significant difference in intrafollicular E2 concentrations between large (>12 mm) and small (<12 mm) follicles. Similarly, the intrafollicular P4 concentration was also much higher in large follicles than in small follicles. Because large follicles contain much more fluid volume, the absolute amount of sex steroids may be much greater in large follicles. Notably, the average intrafollicular E2 and P4 levels in individual patients were positively correlated with their E2 and P4 serum levels. Because follicular fluids obtained from each patient included samples from various follicle sizes, a positive correlation of sex steroid levels between follicular fluid and general circulation might reflect the steroid synthesis ability of each patient.

Oocytes could be retrieved proportionally from both large and small follicles. Oocytes were retrieved from 60% of large follicles (12 oocytes from 20 follicles) and 44.4% of small follicles (4

oocytes from 9 follicles), respectively. The number of follicles we evaluated was limited, and the results might differ for a greater number of samples; however, it was unexpected that a similar oocyte recovery rate would be obtained for the two different follicle size classes. Larger follicles often retrieve mature oocytes, while smaller follicles often retrieve immature oocytes. However, recent reports indicate that there is no relationship between follicle diameter and normal fertilization rate [16, 17]. In the comparison of follicular E2 concentrations between the follicles from which oocytes were retrieved and those without oocytes, there was no significant difference. Furthermore, there was no significant difference in P4 levels between follicles from which oocytes could and could not be retrieved. These observations indicate that the final concentrations of sex steroids are neither crucial to nor a predictor of the success of oocyte retrieval. A similar observation was already reported by Wen *et al.*, who also found no difference in follicular fluid steroid level between those oocytes that were or were not fertilized [15].

With a concomitant increase in the intrafollicular steroid levels, follicular fluid shows diverse metabolic profiles across different developmental stages [14]. In this study, we focused on hypothalamic peptides that are expressed within the ovary. Ovarian function, including follicle development and steroidogenesis, is principally controlled by the HPG axis. Kisspeptin neurons in the hypothalamic arcuate nucleus govern the basal pulsatile release of GnRH, which maintains gonadotropin secretion from the pituitary, and then, gonadotropins, LH, and FSH regulate follicular development within the ovary. Kisspeptin neurons are also located in the anteroventral periventricular nucleus in the brain, and kisspeptin neurons in this area are involved in the elevated E2-evoked GnRH/LH surge that induces oocyte to mature until ready for fertilization and ovulation [7]. Although kisspeptin and GnRH are principal peptides regulating ovarian function through pituitary gonadotropins, these peptides and their receptors are also expressed in ovary tissue [9, 18]. In addition, local production of gonadotropin subunit genes within the ovary was also previously noted [19]. These previous observations suggested that locally produced hypothalamic peptides, regulators of the HPG axis, also have roles at the level of the ovary. In the cellular component of follicular fluid that probably originated from ovarian granulosa cells or cumulus discus oophorus, neither Kiss-1 nor GnRH gene expression differed between small follicles and large follicles. Kisspeptin signaling was found to be involved in the initial and cyclical recruitment of ovarian follicles in rats; specifically, a kisspeptin antagonist increased primordial follicle activation [20]. Kisspeptin expression was also reported to be upregulated during preovulatory periods in the late proestrus of rats [9]. It was also reported that intrafollicular kisspeptin levels increased as follicle development progressed in patients who underwent IVF treatment [21]. In addition, gene expression levels of GnRH and its receptor within the ovary were reported to increase in the morning of diestrus and early afternoon of proestrus. Because expression levels of both Kiss-1 and GnRH as well their receptors did not differ between large and small follicles, the expression levels of these gene might be synchronized after exogenous choriogonadotropin alfa administration.

In this study, we also examined PACAP gene expression in the cellular component of follicular fluid. PACAP was originally isolated from the hypothalamus and is able to increase cAMP levels in pituitary cells [22]. PACAP is a multifunctional peptide distributed within various tissues [23], and PACAP is also involved in the regulation of GnRH neurons and pituitary gonadotrophs [24]. In rat, granulosa cells in large follicles express much more PACAP compared with those in immature follicles [25, 26]. In addition, a study examining the concentration of PACAP in human follicular fluid showed that higher concentrations of PACAP were associated with a lower number of developing oocytes, while a lower concentration of PACAP was reported to be correlated with a higher oocyte retrieval rate [27]. Thus, the expression levels of PACAP and its receptor have some role within the follicles and oocytes. It still unknown why PAC1R gene expression within the cellular component of large follicles was lower than those in small follicles, but reduction of PACAP activity might be necessary to the final step of oocyte maturation.

In this study, we found that large follicles con-

tained much higher concentrations of sex steroids compared with those in small follicles; however, the average levels of intrafollicular sex steroids in each patient were positively correlated with the serum levels of the corresponding sex steroids. The oocyte retrieval rate did not significantly differ between these two follicle size classes. The concentrations of E2 and P4 in the follicles from which oocytes were retrieved were similar to those in the follicles from which oocytes were not retrieved. Expression levels of Kiss-1 and GnRH genes as well as their receptors in the cellular component of large and small follicles were identical, but PACAP receptor gene expression was significantly lower in follicular cells of large follicles. Since the sample size was small in this study, it is necessary to increase the sample size and confirm the veracity of the results of this study in the future. In addition, since PACAP receptor expression varies with follicle size, the relationship between PACAP and follicle development needs to be clarified through molecular biological studies.

Conflict of interests

The authors report no conflicts of interest.

Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Data Availability Statement

The datasets used and/or analyzed during this study are available from corresponding authors on reasonable request.

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