Follicle Stimulating Hormone Effects on Immature Human Oocytes: In Vitro Maturation and Hormone Production¹

KAREN L. DURINZI,^{2,6} ANNE COLSTON WENTZ,^{2,3} ERWIN M. SANIGA,^{2,4} DEBORAH E. JOHNSON,² and SUSAN E. LANZENDORF^{2,5}

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Purpose: Our purpose was (1) to determine if in vitro maturation of unstimulated oocytes could be improved with the addition of urofollitropin; (2) to evaluate the output of estradiol, testosterone, progesterone, and androstenedione by the cultured oocyte-cumulus complex; and (3) to ascertain if steroid hormone production of the oocyte-cumulus complex correlates with final oocyte maturation stage.

Methods: Fifty-eight immature oocytes were obtained from 11 regularly cycling women undergoing oophorectomy. The oocyte-cumulus complexes were randomly assigned to control medium (Ham's F-10 supplemented with 7.5% fetal bovine serum) or test medium (control medium supplemented with 75 mIU/ml of urofollitropin).

Results: (1) The addition of urofollitropin to oocyte culture medium does not significantly increase the ability of the oocyte to achieve the metaphase II stage; (2) the addition of urofollitropin significantly increases the production of progesterone, testosterone, and androstenedione by the oocyte-cumulus complex; and (3) there is no difference in the production of estradiol, progesterone, testosterone, and androstenedione by the oocyte-cumulus complex at the germinal vesicle, metaphase I or metaphase II stage of oocyte maturation.

Conclusions: This information is of importance in the use of oophorectomy specimens for patients who must undergo an oophorectomy but desire to attempt pregnancy using their oocytes, in the use of oophorectomy specimens for donor

oocytes, or for patients undergoing in vitro fertilization using immature oocyte collection.

KEY WORDS: immature oocyte collection; in vitro oocyte maturation; oocytes.

INTRODUCTION

From early embryo development until ovulation, the female germ cell undergoes tremendous growth and differentiation. Influencing this process are the endocrine, paracrine, and autocrine effects of steroid hormones, growth factors, and other ovarian regulators. The ovarian follicle is a complex structure containing follicular fluid, theca and granulosa cells, and the oocyte. At the time of ovulation, the oocyte, surrounded by the cumulus cells, is extruded from the follicle.

While the hormonal milieu of follicular fluid has been characterized previously (1–5), hormone production by the oocyte-cumulus complex alone cannot be determined by these studies due to the presence of granulosa cells, theca cells, and the endocrine and paracrine effects on the follicle. Research has also been conducted on the hormone output of cultured human granulosa cells subject to various culture additives (6–9). However, a correlation cannot be made between the hormone production of these granulosa cells and its effect on oocyte maturation in vitro, as the oocytes were removed from the granulosa cells in these studies.

Follicle stimulating hormone (FSH) has been added to culture media for in vitro oocyte maturation of many species (10–12). The results of its ability to improve oocyte maturation, fertilization rates, and blastocyst formation have been mixed. In the human, stimulated cycles for in vitro fertilization have revealed lower FSH levels in the follicular fluid of oocytes at an immature stage of development compared with those which were mature at aspiration (5).

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² Northwestern University Medical School, Chicago, Illinois, and College of Business and Economics, University of Delaware, Newark, Delaware.

³ Present address: P.O. Box 4300, Avon, Colorado.

⁴ College of Business and Economics, University of Delaware, Newark, Delaware.

⁵ Present address: The Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, Norfolk, Virginia.

⁶ To whom correspondence should be addressed at Department of Obstetrics and Gynecology, Kaiser Permanente, 4900 Sunset Boulevard, Los Angeles, California 90027.

The objectives of the present study were (a) to determine if *in vitro* maturation of unstimulated oocytes obtained from oophorectomy specimens could be improved with the addition of urofollitropin to the culture medium; (b) to evaluate the output of estradiol (E_2), testosterone (T), progesterone (P), and androstenedione by the cultured oocyte-cumulus complex; and (c) to ascertain if hormone production of the oocyte-cumulus complex correlates with the final oocyte maturation stage reached *in vitro*.

MATERIALS AND METHODS

Ovarian tissue, collected from patients undergoing oophorectomy for nonovarian causes, was obtained from the Pathology Department at Northwestern University Medical School, following approval by the Institutional Review Board. The patients ($n = 11$) ranged in age from 32 to 46 years (mean age, 42) and had normal cyclic menses prior to surgery. The stage of the menstrual cycle was not available for all patients. Samples were received in sterile saline within 4 hr of the onset of the surgical procedure and varied greatly in the amount of tissue obtained. Oocyte-cumulus complexes were isolated by cutting the tissue into 5-mm square sections. These sections were then teased apart with blunt needles, so that the oocyte-cumulus complex would be liberated from the antral follicles. Only healthy oocyte-cumulus complexes were used in this experiment. Healthy oocyte-cumulus complexes had compact granulosa cells with regular borders surrounding the entire mass. Masses containing degenerate oocytes were easily identified due to the extreme darkness of the oocyte. The germinal vesicle-stage oocytes in large cumulus masses were removed from the saline solution and rinsed twice. These oocyte-cumulus complexes were then randomly assigned to control medium [Ham's F-10 medium supplemented with 7.5% fetal bovine serum (No. 230-6140; GIBCO Laboratories, Grand Island, NY)] or test medium [control medium supplemented with 75 mIU/ml urofollitropin, i.e., Metrodin containing 75 IU follicle stimulating hormone and less than 1 IU luteinizing hormone per ampoule (Serono Laboratories, Inc.)]. The diameter of the oocyte was measured (not including the zona) at collection with a Nikon inverted microscope (Nikon Instrument Group, Garden City, NY) equipped with Hoffman modulation contrast optics (Modulation Optics, Inc., Greenvale, NY). Oocyte-cumulus complexes were cultured singularly in 3 ml medium in organ culture dishes in 5% CO_2 at

37°C. The oocytes within the cumulus complexes were examined twice daily over 72 hr for the presence or absence of a germinal vesicle and first polar body, and they were classified as either prophase I (having a germinal vesicle), metaphase I (showing germinal vesicle breakdown), or metaphase II (exhibiting extrusion of the first polar body). An oocyte was removed from the culture medium if the metaphase II stage was achieved; otherwise, the oocyte remained in culture. At the end of the 72-hr culture, the oocyte-cumulus complex was removed from culture and any adhering cumulus was removed. At this time, the investigators were able to verify that the true stage of arrest (prophase I or metaphase I) had been correctly determined. All medium was then stored at $-70^\circ C$.

The fetal bovine serum used in these experiments was assayed for LH and FSH (<2 and <5 mIU/ml, respectively) using double-antibody kits provided by Diagnostic Products Co. (Los Angeles, CA). The intraassay and interassay coefficient of variation (CV) was $<10\%$ for both LH and FSH. E_2 was measured as described previously (13), with an intraassay CV of 14.7% and an interassay CV of 7%. Androstenedione and T were measured as described (14), with an intraassay CV of 15 and 10.3%, respectively. Progesterone was measured in the culture media as described (15), and its intraassay CV was 16.5%.

Statistical analysis of the oocyte diameter in the two medium was performed using an unpaired *t* test. Evaluation of the stage of maturation of the oocytes cultured in control versus test medium (Table I) was by chi-square analysis. Comparison of the hormone levels generated by the control medium without oocytes and oocyte cumulus-complex in the control and test media (Table II) was performed using ANOVA with Bonferoni *t* posttests. ANOVA was also used to evaluate the hormone levels of the germinal vesicle, metaphase I, and metaphase II oocyte cumulus-complexes within each of the medium treatments (Table III). Statistical analysis was performed using Instat (GraphPAD Software, Inc.) on a Hewlett Packard Vectra LS/12. Significance was defined as $P < 0.05$.

Table I. Final Stage of Oocyte Maturation^a

	Number (%)		
	GV	MI	MII
Control medium	6(21)	12(41)	11(38)
Test medium	3(10)	9(31)	17(59)

^a GV, germinal vesicle; MI and MII, metaphase I and II.

Table II. Hormone Levels (Mean $\pm$ SE)*

	E (pg/ml)	P (ng/ml)	Androstenedione (ng/ml)	T (ng/dl)
Control				
No oocytes	9.03 $\pm$ 4.10	0.11 $\pm$ 0.06	0.08 $\pm$ 0.03 ^b	0.81 $\pm$ 0.16 ^d
With oocytes	37.37 $\pm$ 9.26	2.97 $\pm$ 1.67 ^a	0.14 $\pm$ 0.03 ^c	1.02 $\pm$ 0.18 ^c
Test (FSH), with oocytes	27.60 $\pm$ 8.46	15.70 $\pm$ 6.06 ^a	0.32 $\pm$ 0.06 ^{bc}	1.81 $\pm$ 0.22 ^{dc}

* Superscripts a-e, $P < 0.05$.

RESULTS

From the ovarian tissue, 58 healthy germinal vesicle oocytes in cumulus were recovered, ranging in size from 90 to 120 μ m. Between 2 and 10 healthy oocytes were recovered per patient. There was no difference in diameters of the oocytes measured at collection which were cultured in the control or the test medium [109.6 ± 1.68 (SE) vs 110 ± 1.79 μ m, respectively]. Of the 29 oocytes in the control medium, 6 (21%) remained at the germinal vesicle stage, 12 (41%) arrested development at the metaphase I stage, and 11 (38%) progressed to metaphase II. Of the 29 oocytes which were cultured in test medium 3 (10%) remained at the germinal vesicle stage, 9 (31%) advanced to metaphase I, and 17 (59%) developed to the metaphase II stage. There was no significant difference in the maturation rate in the control medium or test medium ($P = 0.09$) (Table I).

The addition of urofollitropin to the control medium was shown to increase significantly the ability of the oocyte-cumulus complex to produce P, T, and androstenedione, compared with the oocyte-cumulus complex cultured in control medium alone. No difference was seen in the ability of the oocyte-cumulus complex to produce estradiol in either medium or compared to the control medium without oocytes (Table II).

No significant difference in the hormone output of the oocyte-cumulus complexes at the germinal vesicle,

metaphase I, or metaphase II stage within the control medium was observed, and similarly, there was no difference in hormone output in any maturation stage of the test medium (Table III). However, a trend in both media was noted, with the oocyte-cumulus complex of the metaphase I oocytes producing the highest level of all hormones studied.

DISCUSSION

The addition of FSH to the culture medium for in vitro oocyte maturation has been described in many species. In rat (10) and monkey (12) oocytes, the maturation frequency did not increase with the addition of FSH. However, in the mouse, its addition to the culture medium significantly increased the rate of germinal vesicle breakdown (16). In the monkey (12) and mouse (17), culture medium with FSH significantly increased the percentage of fertilization and/or embryo development.

In women undergoing oocyte retrieval for stimulated IVF cycles, Laufer and co-workers (5) found the FSH levels in the follicular fluid of metaphase I and metaphase II oocytes to be significantly higher than in follicles containing germinal vesicle oocytes. However, in studies evaluating follicular fluid FSH levels in fertilized and unfertilized oocytes, the results have varied. While Laufer *et al.* (5) found higher levels of

Table III. Hormone Levels by Maturation Stage (Mean $\pm$ SE)^a

	E (pg/ml)	P (ng/ml)	A (ng/ml)	T (ng/dl)
Control				
GV	23.72 $\pm$ 13.09	0.10 $\pm$ 0.04	0.11 $\pm$ 0.021	0.72 $\pm$ 0.14
MI	54.77 $\pm$ 27.58	6.46 $\pm$ 5.27	0.21 $\pm$ 0.10	1.75 $\pm$ 0.47
MII	32.97 $\pm$ 7.43	2.16 $\pm$ 1.23	0.10 $\pm$ 0.02	0.85 $\pm$ 0.16
Test (FSH)				
GV	12.50 $\pm$ 2.47	6.25 $\pm$ 6.24	0.18 $\pm$ 0.03	2.04 $\pm$ 0.68
MI	46.82 $\pm$ 25.14	26.32 $\pm$ 18.82	0.47 $\pm$ 0.17	2.59 $\pm$ 0.56
MII	21.24 $\pm$ 6.63	12.47 $\pm$ 4.28	0.27 $\pm$ 0.06	1.39 $\pm$ 0.18

^a A, androstenedione; GV, germinal vesicle; MI and MII, metaphase I and II.

follicular fluid FSH in oocytes that were successfully fertilized compared to those that did not fertilize, others (4,18) found no significant difference between the two groups.

Salustri *et al.* proposed that FSH induced an increase in cAMP in the cumulus-enclosed mouse oocyte, and that may also promote an increase in cAMP turnover in the cumulus complex, leading to an increase in germinal vesicle breakdown (19). Because FSH stimulates the expansion of the cumulus in culture, sperm access to the oocyte may be facilitated, explaining the increased fertilization rate (20). In addition, the increase in embryo development seen in oocytes matured in culture medium with added FSH may be due to its effect on the process occurring in oocyte maturation that relates to embryo development (21).

To evaluate the effect of FSH in *in vitro* hormone production, human granulosa cells obtained from hormonally stimulated follicles were cultured with FSH by Tapanainen *et al.* (8) and Sjögren *et al.* (9). Both groups found that FSH stimulated the formation of P by the granulosa cells. In a human granulosa cell tumor culture, the addition of 1.0 and 0.1 $\mu\text{g/ml}$ FSH to the culture media significantly increased the production of P by the granulosa cells, however, the production of E_2 was increased only when 1 $\mu\text{g/ml}$ was added (22). When Dain and co-workers (7) obtained granulosa cells from stimulated IVF patients, their examination revealed that the levels of follicular fluid FSH was positively correlated with both P and E. Studies have conflicted regarding the production of T and androstenedione in granulosa cell cultures. Tsang *et al.* found that the granulosa cells produced little or no androgen, even with FSH added to the culture medium (23). However, McNatty *et al.* found that P, E_2 , T, and androstenedione did accumulate in the medium of granulosa cells cultured *in vitro* and proposed that the granulosa cells of most follicles were capable of *de novo* synthesis of all of these steroids (24).

This study examined the results of the hormone output of the oocyte-cumulus complex with and without added FSH, and the results showed similarly elevated levels of P but not elevated levels of E_2 . The higher production of P by the oocyte-cumulus complex cultured in FSH was probably achieved by an FSH increase in cytochrome P-450 scc (25). That there was no increase in E_2 when the medium without the oocyte-cumulus complex was compared to that with oocyte-cumulus complex may be due to the limited number of cells available to aromatize androgens to estrogens, the amount of FSH added to the control medium, or to the fact that the decreased aromatase activity

observed in the cumulus cells of other species compared to membrana granulosa cells may hold true in the human as well (26). The oocytes cultured in control medium showed no increase in T and androstenedione production, while those cultured in test medium showed an increase in their production. These results, as well as the conflicting results in the above studies on the production of androgens by the granulosa cell, indicate that this is an area for investigation.

Investigators have studied the hormone levels of the follicular fluid of developing oocytes in both natural and stimulated cycles (1-5). In the natural cycle, Seibel *et al.* (27) measured the follicular fluid levels in healthy oocytes and found no difference in E_2 and androstenedione levels at any stage of oocyte maturation. The mean P levels were significantly higher in follicular fluid containing metaphase I and metaphase II oocytes compared to those containing germinal vesicle oocytes, while the highest T levels were seen in the germinal vesicle oocytes.

From oophorectomy specimens, McNatty and co-workers (28) determined the *in vivo* maturation stage of an oocyte after removal from its follicle and the concentration of the steroids in the corresponding antrum. There was no difference in E_2 , T, P, or androstenedione in those healthy oocytes at the germinal vesicle stage or after germinal vesicle breakdown.

In this study, there was no difference in hormone levels at any stage of the oocytes matured *in vitro*. However, a trend was noted in the metaphase I oocytes. They had the highest level of hormone output for all of the hormones assayed. This may be due to their longer incubation time compared to the metaphase II oocytes, as the metaphase II oocytes were removed from their medium when a polar body was seen. This does not explain the increase in hormone production over the germinal vesicle oocytes, as both the metaphase I and the germinal vesicle oocytes were cultured for the same length of time (72 hr). These results indicate that elevated hormone levels may not be sufficient for oocyte maturation *in vitro*. Also, oocytes that are matured *in vitro* and arrest their development at the germinal vesicle stage may have granulosa cells which are not as active in the production of hormones as those arresting development at the metaphase I and II stage.

The stimulatory effects of LH in this study (from the fetal bovine serum in the control medium and from the fetal bovine serum and the urofollitropin in the test medium) on oocyte maturation and oocyte-cumulus complex hormone output are unknown. In mouse studies, oocytes matured in medium supple-

mented with either FSH or LH alone increased oocyte maturation or blastocyst development (17,29). While in human granulosa cell culture supplemented with either FSH or LH alone, the formation of P was increased (9).

Although the stage of the menstrual cycle at which the oocytes were obtained was unavailable for some patients, other investigators have indicated that there is no difference in oocyte maturation rate regardless of the time of the cycle at which the oocyte is retrieved from the oophorectomy specimen (30,31).

CONCLUSIONS

The results of this study show that (a) the addition of urofollitropin to oocyte culture media does not significantly ($P = 0.09$) increase the ability of the oocyte to achieve the MII stage; (b) the addition of urofollitropin significantly increases the production of P, T, and androstenedione by the oocyte-cumulus complex; and (c) there is no difference in the production of E_2 , P, T, and androstenedione by the oocyte-cumulus complex at various stages of oocyte maturation.

Clinically, this information is of importance in the use of oophorectomy specimens for patients who must undergo oophorectomy but desire to attempt a pregnancy using their oocytes, in the use of oophorectomy specimens for donor oocytes, or for patients undergoing in vitro fertilization using immature oocyte collection.

Further investigation regarding FSH are needed. For while FSH may not increase the maturation rate, it may improve the fertilization rate and embryo development in the immature human oocyte matured in vitro.

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