Effects of Urinary and Recombinant Gonadotropins on In Vitro Maturation Outcomes of Mouse Preantral Follicles

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Abstract

Gonadotropins including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play a crucial role in human-assisted reproduction techniques. Despite wide use of recombinant gonadotropins in clinical practice, the efficacy of urinary gonadotropins and the dosage of LH component have not yet been elucidated. This study was designed to investigate the difference of follicle culture outcomes according to various compositions of gonadotropins during in vitro culture of mouse preantral follicles. Ovaries were obtained from the 14-day-old C57BL/6 mice, and preantral follicles were isolated and cultured in culture media supplemented with human menopausal gonadotropin (hMG) 200 mIU/mL (group 1), recombinant FSH and LH (rFSH + rLH) 200 mIU/mL each (group 2), rFSH 200 mIU/mL + rLH 100 mIU/mL (group 3), or rFSH 200 mIU/mL + rLH 20 mIU/mL (group 4). Follicle survival rate was significantly lower in group 4. Antral follicles in lower doses of LH (groups 3, 4) showed a statistically significant larger diameter and tended to have a higher antral formation rate. However, follicles in group 1 tended to have a higher oocyte maturation rate. Estradiol concentration from conditioned media from 2:1 FSH/LH (group 3) was significantly higher than those from 1:1 FSH/LH (group 2) or 10:1 FSH/LH (group 4). Half dose of rLH to rFSH facilitated upregulation of growth differentiation factor 9 (*Gdf9*) expression in granulosa cells when compared to 1:1 FSH/LH or 10:1 FSH/LH. Conclusively, recombinant gonadotropins provided a comparable condition to hMG, and half dose of rLH to rFSH seems to be more suitable for follicular development during in vitro culture.

Keywords

in vitro maturation, preantral follicles, recombinant gonadotropins, urinary gonadotropin, ovarian stimulation

Introduction

During human-assisted reproduction techniques, gonadotropins including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play a crucial role in follicular growth. Since the introduction of recombinant gonadotropins into clinical practice, “pure” FSH and LH preparations provided powerful new tools in experimental endocrinology.^{1,2} Introduction of recombinant gonadotropin without LH activity in the 1990s resurrected interest in the role of LH and human menopausal gonadotropin (hMG).

Traditionally, the role of LH is restricted to stimulating theca cells’ androgen production in the preantral stage and granulosa cells become receptive to LH in late stage of follicle development. Experimental and clinical experience indicated that exogenous LH administration plays an important role in completing maturation of the follicle.³ Experimentally, androgen which is produced in response to LH have been

shown to stimulate early follicular development and reduce the incidence of apoptosis in rat model⁴ and androgen receptor stimulation is necessary to promote the action of growth differentiation factor (*Gdf9*), to stimulate granulosa cell division and

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progesterone synthesis in porcine model.⁵ Clinical studies using recombinant FSH (rFSH) in hypogonadotropic women have demonstrated that only FSH can induce follicular growth at the preovulatory stage but estradiol (E_2) concentrations were extremely low.⁶ Recently, the amount of LH activity actually necessary for normal follicle and oocyte development has been studied by many investigators. Most of them maintained that optimal dose of LH is not known but is likely to be very low since less than 1% of follicular LH receptors needs to be occupied in order to elicit maximal steroidogenesis.⁷ Clinical and laboratory evidence supports that excessive LH supplementation has a detrimental effect on follicular development^{8,9} and may induce a decline in metabolic activity of granulosa cells in follicles undergoing atresia.¹⁰ Depending on the stage of development, follicles exposed to high concentrations of LH enter atresia in nondominant follicles or become luteinized prematurely in preovulatory follicles and oocyte development could be compromised. However, optimal LH dose or ratio to FSH for proper follicular development and oocyte maturation is still controversial.

The efficacy of hMG as LH activity is still debated because of the difficulty to quantify the exact amount of exposure. This gonadotropin derived from urine of postmenopausal women have been used for over 40 years and its safety is well established, but after the introduction of recombinant gonadotropins, its use was declined because hMG includes gonadotropin-unrelated urinary proteins and has batch-to-batch variation. Nevertheless, the clinical studies with hMG have shown comparable outcomes to rFSH or rFSH plus recombinant LH (rLH)^{11–13} or even better outcomes during some specific conditions such as in patients with high basal FSH/LH.¹⁴

Our study was designed to compare the effects of hMG and rFSH + rLH. As hMG preparation contains a 1:1 ratio of FSH and LH bioactivity, we compared it with 1:1 ratio of rFSH/rLH and furthermore, to 2:1 and 10:1 ratio with fixed dose of FSH in order to observe the differences according to the LH concentrations. We used an in vitro culture and maturation system of young mouse preantral follicles. Follicle development and oocyte maturation were evaluated through assessing follicle growth, E_2 concentration in culture media, and expression of genes related to oocyte maturation and granulosa cell growth.

Materials and Methods

In Vitro Culture of Preantral Follicles

Fourteen-day-old female C57BL6 mice were sacrificed by cervical dislocation. Ovaries were collected in Hanks balanced salt solution with 10% fetal calf serum. Basically, we followed a previous report by Liu et al for the methods of isolation and culture of follicles.^{9,15} The use of mice for this study has been approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

Preantral follicles were mechanically dissected using 28 gauge syringe needles. These follicles sized 100 to 120 μ m in diameter, containing layers of granulosa cells with a healthy,

visible oocyte and superficial thecal cells, were used. The selected follicles were cultured in 20- μ L microdroplets of designed media in a culture dish (BD Biosciences, Irvine, California; 60 $\times$ 15 mm; 1 follicle per droplet, 25 droplets per dish), covered with washed mineral oil, and incubated at 37°C in 5% CO_2 for 12 to 14 days. We used the follicles from same ovary evenly distributed to each group of this study. Media was changed every other day by refreshing half (10 μ L) of the medium. The collected media on the day of human chorionic gonadotropin (hCG) treatment was used to detect E_2 secretion by radioimmunoassay. Morphological changes of the cultured follicles were evaluated under an inverted microscope (TE2000; Nikon, Tokyo, Japan) and the diameter of each follicle was measured and calculated using i-solution program (InnerView, KyungGi-do, Korea).

Media Composition for Follicle Culture

Totally 720 isolated preantral follicles were selected and divided into the 4 groups and cultured in 1 of the following for 12 days. The medium was prepared in accordance with the previous studies made in our laboratory with some modification.^{15,16} The basic components were α MEM medium (Invitrogen, Carlsbad, California) that contained 5% fetal bovine serum, insulin 1 mg/mL, transferrin 0.55 mg/mL, selenium 0.5 μ g/mL, and penicillin–streptomycin solution. In addition to basic medium, the 4 groups differed in gonadotropin components. Gonadotropin was added as follows: hMG (IVF-M, LG Life Sciences, Seoul, Korea) 200 mIU/mL (group 1; n = 180); rFSH (Gonal-F, Merck Serono S.A., Geneva, Switzerland) and rLH (Luveris, Merck Serono S.A.) 200 mIU/mL each (group 2; n = 180); rFSH 200 mIU/mL + rLH 100 mIU/mL (group 3; n = 180); or rFSH 200 mIU/mL + rLH 20 mIU/mL (group 4; n = 180). The medium was changed with each gonadotropin containing fresh medium every other day in 4 different groups.

The hCG Administration for Release of Cumulus–Oocyte Complexes

Cumulus–oocyte complexes (COCs) were released after the administration of 1.5 IU/mL recombinant hCG (Ovidrel, Merck Serono S.A.) plus 5 ng/mL human epidermal growth factor (EGF) on day 12 of in vitro preantral follicle culture.

Measurement of E_2 Production In Vitro

On the day of hCG administration, media was collected from all droplets in which follicle is cultured. The E_2 concentration was measured by radioimmunoassay using a commercial E_2 assay kit (Coat-a-count, Siemens, Illinois). The sensitivity of the assay was 3 pg/mL, and the intra- and intercoefficient variations were 4.0% and 4.2%, respectively. The samples were measured after an appropriate dilution with the kit's zero standard.

Table 1. List of Primers Used for Quantitative RT-PCR.

Gene	Forward (5'→3')	Reverse (3'→5')
<i>GAPDH</i>	GAAGGTCGGTGTGAACGAAT	TTTGATGTTAGCGGGGTCTC
<i>Oct4</i>	GCCCTACAGCAGATCACTCACA	GAACCATACTCGAACCACATCCT
<i>Gdf9</i>	AACCCAGCAGAAGTCACCTC	AGGGGCTGAAGGAGGGAGG
<i>Bmp15</i>	CAGTAAGGCCTCCCAGACGT	AAGTTGATGGCGGTAAACCA

Abbreviation: RT-PCR, reverse transcription-polymerase chain reaction.

Assessment of Oocytes Maturation

Two days after hCG administration, oocytes were evaluated under an inverted microscope. After mechanical denudation of oocyte from the surrounding granulosa cells, the released oocytes were classified as germinal vesicle, metaphase I (MI), and metaphase II (MII).

Quantitative Reverse Transcription-Polymerase Chain Reaction

During evaluating the oocyte maturation, ovulated oocytes and granulosa cells from each culture group were collected. Total RNA was extracted from the ovulated oocytes and granulosa cells using TRIzol (Invitrogen) according to the manufacturer's instruction.

Complementary DNA was synthesized using 1 µg of total RNA using Accute RT-premix (Bioneer, Daejeon, Korea). The 3 genes, octamer-binding transcription factor 4 (*Oct4*), bone marrow protein 15 (*Bmp15*), and growth differentiation factor 9 (*Gdf9*) were selected in this study, as oogenesis-specific genes, which had been studied for high expression during oocyte maturation.^{17–22} Quantitative RT-PCR of these 3 genes was carried out using SYBR green PCR mix (Qiagen, Valencia, California). The amplification program included 45 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 30 seconds. All reactions were run in triplicate. Relative gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase expression. Primers used for quantitative reverse transcription-polymerase chain reaction are listed in Table 1.

Statistical Analysis

We repeated each experiment 4 times with 4 different groups. Data were analyzed with SPSS 19.0 for Windows (IBM, Armonk, New York). Student *t* test was used to compare means and chi-square test was used to compare proportions. Differences were considered statistically significant when *P* < .05.

Results

Follicle Survival Rates

Cultured in 4 different conditions of gonadotropins as described, preantral follicles survived or underwent atresia. On day 12 of in vitro culture, follicle survival rate was assessed, which resulted in

80.0% (144 of 180, group 1), 79.4% (143 of 180, group 2), 84.4% (152 of 180, group 3), and 71.7% (129 of 180, group 4). The survival rate in the group 4 was significantly lower than the other 3 groups (*P* < .05). There was no significant statistical difference in follicular survival rate among the groups 1 to 3.

Comparison of Follicle Growth and Antral Formation

Morphological changes of the cultured follicles were evaluated under an inverted microscope every other day from the beginning of culture. Until day 6, the follicles cultured with each medium grew in a similar fashion and, from day 6, the granulosa cells adjacent to the oocyte became prominent as corona (Figure 1B, E, H, K). During growth, the follicle diameters in groups 3 and 4 become larger than those in groups 1 and 2 (Figure 1C, E, I, L).

Antral follicles in group 3 and 4 had flat structures with significant antrum that was easily distinguishable. On the contrary, the follicles in group 1 had compact structures that formed sphere-shaped granulosa cells around COC which makes it difficult to distinguish antral formation. For the precise comparison, follicle diameter in each group was measured on day 12 during in vitro maturation and mean diameter ± standard error of antral follicles was 718.3 ± 31.8 µm in group 1, 745.2 ± 28.5 µm in group 2, 901.9 ± 23.7 µm in group 3, and 875.7 ± 21.5 µm in group 4. Follicles in each group 3 or 4 showed statistically significant larger diameters compared to group 1 and 2 (*P* < .001, Figure 2B). Typical antral follicles are shown in Figure 2A which was taken on day 12.

Comparisons of Antral Formation and Oocyte Maturation Rates

On the day of hCG administration (12th day of in vitro culture), antral formation rates of the survived follicles were 70.8% in group 1, 66.4% in group 2, 77.0% in group 3, and 72.1% in group 4. We had 4 separated experiments and there was no statistically significant difference among the 4 groups, only noticed was a higher tendency of antral formation in group 3.

Two days after hCG administration, oocyte maturity was evaluated under an inverted microscope. Mature oocyte (MII and MI) percentages out of survived follicles were 75.0% in group 1, 69.2% in group 2, 67.8% in group 3, and 70.5% in group 4. There was no statistically significant difference, either.

Figure 1. Development of preantral follicles during in vitro culture. Follicles had been cultured with hMG 200 mIU/mL (A-C), recombinant FSH 200 mIU/mL plus recombinant LH 200 mIU/mL (D-F), recombinant FSH 200 mIU/mL plus recombinant LH 100 mIU/mL (G-I), and recombinant FSH 200 mIU/mL plus recombinant LH 20 mIU/mL (J-L). Typical follicles of each group at days 1, 6, and 10 during in vitro maturation are shown. Original magnification was 60 $\times$ and scale bars represent 100 μ m. In the hMG group, granulosa cells were denser than other groups on day 10 (C). FSH indicates follicle stimulating hormone; hMG, human menopausal gonadotropin; LH, luteinizing hormone.

Comparison of E_2 Production

On day 12, the day of hCG administration, media was collected from all droplets in which the follicles are cultured. The E_2 level of the collected culture media was too high to measure using commercial kits; therefore, the samples were measured after an appropriate dilution. The E_2 concentration was measured as 14.3 ± 0.1 ng/mL in group 1, 11.2 ± 0.1 ng/mL in group 2, 16.7 ± 0.4 ng/mL in group 3, and 8.7 ± 0.2 ng/mL in group 4 (Figure 3). The E_2 concentration in either group 2 or group 4 was significantly lower than that of group 1 or 3

($P < .05$); however, there were no significant differences between groups 1 and 3 or groups 2 and 4.

Expression of Oocyte Development-Related Genes in Oocytes and Granulosa Cells

The 3 oogenesis-specific genes, *Oct4*, *Bmp15*, and *Gdf9*, were selected in this study, which are known for high expression during oocyte maturation.^{17–21} No differences were observed in the relative expression of 3 genes among the 4 groups, in

Figure 2. A, Typical antral follicles are shown. Original magnification was 60× and scale bars represent 100 μm, applied to all images. B, Mean diameters of antral follicles cultured with 4 different gonadotropin components on day 12 during in vitro maturation. Data represent diameters (mean ± standard error [SE]). (A-1) hMG 200 mIU/mL, (A-2) rFSH: rLH 200 mIU/mL: 200 mIU/mL, (A-3) rFSH: rLH 200 mIU/mL: 100 mIU/mL, and (A-4) rFSH: rLH 200 mIU/mL: 20 mIU/mL. *Follicles in group 3 or 4 showed a statistically significant larger diameter compared to group 1 or 2 ($P < .001$). No significant difference was observed between groups 3 and 4. rFSH indicates recombinant follicle stimulating hormone; hMG, human menopausal gonadotropin; rLH, recombinant luteinizing hormone.

ovulated oocytes (Figure 4A). However, in the analysis with granulosa cells, relative expression of *Oct4* in group 4 was lower than that in group 3 ($P < .05$) and relative expression

of *Bmp15* in group 2 was lower than that in group 1 ($P < .05$). Relative expression of *Gdf9* in group 3 was higher than each of group 2 or 4 ($P < .05$; Figure 4B).

Figure 3. Comparison of estradiol concentrations from conditioned media. Data present estradiol level (ng/mL, mean $\pm$ standard error [SE]) measured from conditioned media on day 12 of in vitro culture. Different letters indicate a significant difference ($P < .05$).

Discussion

Prior to searching the optimal LH component and dose, we reconfirmed that follicle parameters including survival rate, antral formation rate, and oocyte maturation rate of FSH-only group were all significantly lower than those of FSH + LH groups (data not shown), which were compatible with our previous report¹⁵ and other study from Belgium.²³

Although there have been numerous clinical and experimental studies that compared between urinary and recombinant gonadotropins or, between high-dose and low-dose LH, it is difficult for physicians to choose optimal gonadotropins when LH activity is needed in human-assisted reproduction techniques, since most of investigations present comparable or inconclusive outcomes.

In comparison of urinary and recombinant gonadotropins, urinary proteins, batch-to-batch variation, and different FSH/LH ratios have been at the center of focus. Previous studies demonstrated that batch-to-batch consistency was high in contrast to our concerns, proved via oligosaccharide isoform profiling, immunoassay, chromatography analysis, and in vitro bioassay.^{24,25} However, the role of urinary proteins is not known yet, only to be known that those can result in allergic or other hypersensitivity reactions. Some investigators revealed that urinary FSH contains more carbohydrate complexity and acidic compositions compared to rFSH,^{26,27} but did not reveal the effects of those findings.

In regard to clinical outcomes, hMG was superior to rFSH with regard to pregnancy and live birth rates.^{28,29} When compared to rFSH plus rLH, hMG achieves comparable results in terms of pregnancy rate, implantation rate, and embryo quality, but better results in oocytes quality.¹² We cannot still

Figure 4. Relative expression of oocyte development-related genes (*Oct4*, *Bmp15*, and *Gdf9*) in oocytes (A) granulosa cells (B). The expression of each gene was calculated relative to that of group 1. A, All measures in oocytes have no significant difference with regard to relative expression of all 3 genes. B, *Relative expression of *Oct4* in group 4 was lower than that in group 1 ($P < .001$) and 3 ($P < .01$) and relative expression of *Bmp15* in group 2 was lower than that in group 1 ($P < .001$). Relative expression of *Gdf9* in group 3 was higher than that in group 2 ($P < .05$) and 4 ($P = .001$). A statistical difference was also observed between group 1 and group 4 in terms of *Gdf9* expression ($P < .001$).

understand what makes hMG superior to recombinant gonadotropins.

As a prominent outcome of the present study, the follicles cultured in media with recombinant gonadotropins at lower dose of LH component (groups 3 and 4) appeared larger in diameter than those cultured at higher doses of LH component (group 1 or 2). Moreover, hMG as a urinary preparation stimulated the proliferation of denser and sphere-shaped granulosa cells around COC, which may imply that recombinant gonadotropins with 1:1 FSH/LH ratio stimulated flat granulosa cells with small diameters and that recombinant gonadotropins

with 2:1 and 10:1 FSH/LH ratio made flat granulosa cells with large diameters. These results in contrast to a previous study¹⁶ assessed the differences between urinary and rFSH without LH activity in in vitro maturation model of mouse preantral follicles. Calongos et al reported that follicles cultured with urinary FSH appeared with a larger diameter and flat structure compared to rFSH, but did not investigate what that structural difference means.¹⁶ They only revealed the group used rFSH which made dense granulosa cells show statistically significant higher mature oocytes rate, similar to our results.

To explain the morphological difference of granulosa cells between urinary and recombinant preparations, we have observed the cytoskeleton proteins via immunostaining. Zampolla et al reported the interaction between mitochondria and cytoskeletal proteins such as actin and tubulin in granulosa cell of zebrafish³⁰ and Grieshaber et al, presented that FSH induces the massive reorganization of the cytoskeletons and morphological changes in rat ovarian granulosa cells.³¹ Our findings revealed that actin and α -tubulin are distributed over the whole granulosa cell with organized arrangements. However, we could not observe any difference in density or distribution among the 4 groups (data not shown). Other cytoskeleton proteins or extracellular matrix components such as laminin related to granulosa cell structure should be subjected to further investigations.³²

From our comparison between urinary and recombinant gonadotropins, group 1 with urinary preparation showed a significantly higher E₂ concentration from conditioned media and a tendency of higher oocyte maturation rate than group 2. Those results are comparable to a previous study that showed a higher E₂ production in hMG group than rFSH with LH group.⁹

Both *Bmp15* and *Gdf9* are the members of transforming growth factor β superfamily which are expressed during all oocyte maturation phases.^{20,21} The 2 molecules are well known in that they are expressed in an oocyte-specific manner from early stage and play crucial roles in promoting follicle growth beyond the primary stage.²² The *Oct4* is a nuclear transcription factor that belongs to the POU family and its high expression was shown during folliculogenesis, especially during the recruitment of follicles for initiating growth and the selection of oocytes for ovulation.²⁰

Our results have shown a significant lower expression of *Bmp15* in recombinant gonadotropins (group 2) compared to urine-derived gonadotropins (group 1). Both *Oct4* and *Gdf9* showed a similar expression level between urinary and recombinant gonadotropins. A low level of *Bmp15* may be deemed as a clue that explains why oocyte maturity was high in the hMG group.

We need to explore additional oocyte maturation-related genes for future studies. Grondahl et al showed that there was a trend of decreased expression of vascular endothelial growth factor in hMG-treated patients with a borderline statistical significance and speculated that the higher expression of the anti-apoptosis protein in hMG-treated granulosa cells suppresses apoptosis.³³

As for the LH dose, we compared groups 2, 3, and 4. Group 4 showed inferior outcomes compared to group 3 in several aspects such as follicle survival rate, E₂ concentration from conditioned media, and relative expression of oocyte development related genes (*Oct4*, *Gdf9*) in granulosa cells. This study showed the follicles in group 3 with half dose of LH to FSH have a tendency of higher antral formation than those in group 2 and higher E₂ concentration than groups 2 and 4. These results support the fact that high concentrations of LH cause atresia in growing follicles and 2:1 ratio of FSH:LH seems to yield better results than 1:1 ratio.³⁴

Indeterminate outcomes of oocyte maturation rate is 1 of our limitations, which cannot explain why oocytes cultured with hMG have high maturity regardless of lower antral formation rate and dense granulosa cells. Another limitation of our study is that we did not evaluate the fertilization and subsequent development for verifying ultimate oocyte quality.

Conclusively, we tried to compare the efficacy of urinary and recombinant gonadotropins during in vitro culture of murine follicles and found that recombinant gonadotropins provided a comparable condition to hMG, and that half dose of rLH to FSH seems to be more suitable for follicular development during in vitro culture.

Declaration of Conflicting Interests

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