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ORIGINAL ARTICLE *Gynaecology*

Post-operative oral contraceptive use reduces the risk of ovarian endometrioma recurrence after laparoscopic excision

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BACKGROUND: The aim of this study was to evaluate the impact of post-operative oral contraceptives (OCs) use on the rate of recurrence after laparoscopic excision of ovarian endometrioma.

METHODS: In May 2005, we introduced a 'post-operative OC recommendation' for patients treated with laparoscopic excision of endometrioma. That is, at the time of the operation, we provided each patient with information about OC, known and possible benefits and risks and let her decide whether to take OC. A retrospective cohort study included 87 patients who underwent a laparoscopy after May 2005. The endometrioma recurrence rate at 24 months was compared between those who used OC for the entire follow-up period OC ($n = 34$) and all of the others ($n = 53$). We also performed logistic regression analysis to identify variables associated with recurrence. A before–after study included another 224 patients who underwent a laparoscopy before May 2005 and compared the recurrence rate before and after introduction of the 'post-operative OC recommendation'.

RESULTS: The recurrence rate in those who used OC for the entire period was significantly lower than in the 'others' group (2.9 versus 35.8%, relative risk 0.082, 95% CI 0.012–0.58, $P < 0.001$). Post-operative OC was determined as an independent variable associated with lower recurrence (OR 0.054, 95% CI 0.007–0.429, $P < 0.001$). The overall recurrence rate in patients who underwent laparoscopy after the introduction of the 'post-operative OC recommendation' was significantly lower than that in patients who received laparoscopy before the introduction (18.6 versus 33.1%, relative risk 0.56, 95% CI 0.32–0.97, $P < 0.05$).

CONCLUSIONS: Post-operative OC use reduces the risk of ovarian endometrioma recurrence after laparoscopic excision. This information will help in appropriate planning of pre- and post-operative management.

Key words: endometriosis / laparoscopy / recurrence / oral contraceptives / ovary

Introduction

Ovarian endometrioma accounts for 55% of reported endometriosis cases (Jenkins *et al.*, 1986). Most cases are treated conservatively since endometrioma is commonly diagnosed in women of reproductive age (Giudice and Kao, 2004). Currently, laparoscopic excision is considered to be the 'gold standard' for the conservative treatment of endometrioma (Beretta *et al.*, 1998) and the European Society of Human Reproduction and Embryology (ESHRE) guidelines recommend that laparoscopic ovarian cystectomy can be considered if an ovarian endometrioma >3 cm in diameter is present (to confirm the diagnosis histologically) and for the purpose of reducing the risk of infection, improving access to follicles and possibly improving ovarian response (Kennedy *et al.*, 2005).

However, a frustrating aspect of laparoscopic excision of endometrioma is the disease recurrence after surgery. We have previously reported as high as 30.4% recurrence within 2 years after surgery (Koga *et al.*, 2006). Although the definition of recurrence can vary, other recent studies also indicate a 30% recurrence rate after 3–5 years of follow-up (Kikuchi *et al.*, 2006; Liu *et al.*, 2007), which is higher than previously believed.

Whereas laparoscopic excision is known to improve fertility (Chapron *et al.*, 2002), recurrence and repeated surgery can cause significant ovarian damage, thus reducing future reproductive

performance. Given that the majority of women who undergo this operation seek pregnancy in the future, a dilemma arises when a physician plans laparoscopy, with the expectation that fertility should improve but with concerns that future recurrence can place fertility at risk. It is therefore crucial to prevent recurrence after laparoscopic excision in order to maintain 'improved' fertility for long as possible.

Despite the necessity, a gold standard treatment modality of preventing recurrence does not yet exist. According to ESHRE guidelines (Kennedy *et al.*, 2005) and the Cochrane library (Yap *et al.*, 2004), post-operative hormonal treatment does not significantly reduce symptoms at 12 or 24 months, and has no effect on disease recurrence. However, the Cochrane library added 'there is insufficient evidence to conclude that hormonal treatment in association with surgery is associated with a significant benefit' (Yap *et al.*, 2004). As a result, it has remained controversial as to whether post-operative medical treatment or different drug treatments can reduce disease recurrence.

Previously, we analyzed risk factors that are associated with the recurrence of endometrioma after laparoscopic excision and identified post-operative pregnancy as a favorable prognostic factor (Koga *et al.*, 2006). The benefit of pregnancy on recurrence has also been observed in other reports (Busacca *et al.*, 2006; Porpora *et al.*, 2008). This prompted us to hypothesize that post-operative administration of oral contraceptive (OC) pills, which inhibit ovulation and create a pregnancy-like hormonal condition, may reduce the incidence of endometriosis recurrence. When we completed our previous study, only one randomized controlled trial had attempted to evaluate the efficacy of post-operative OC use for reducing recurrence (Muzii *et al.*, 2000). However, the duration of OC administration in this study (Muzii *et al.*, 2000) was limited to 6 months, consequently the long-term effect of OC on recurrence has been remained unclear.

Given that OC may reduce the recurrence after laparoscopic endometrioma excision, in May 2005, our clinic introduced a 'post-operative OC recommendation' for patients who underwent a laparoscopic excision of endometrioma. That is, at the time of the operation, we provided each patient with information about OC, and known and possible benefits and risks and let her decide whether or not to take OC. In this study, we conducted two analyses, a 'retrospective cohort study' and a 'before–after study'. In the retrospective cohort study, we compared recurrences between OC users and non-users to evaluate the 'effect' of OC use on endometriosis recurrences. On the other hand, the before–after study is a historical study which compares recurrences between before and after the introduction of the 'post-operative OC recommendation' in a clinical setting to define the efficiency of the 'post-operative OC recommendation' for the overall population.

Materials and Methods

Subjects

This investigation consists of two studies (i) a retrospective cohort study and (ii) a before–after study. For the retrospective cohort study, a total of 137 patients who underwent a laparoscopic excision of ovarian endometrioma performed at the University of Tokyo Hospital between May 2005 and August 2006 were enrolled. Of the 137 subjects originally enrolled, a total of 87 women who had a minimum of 24 months of post-operative follow-up were studied. The clinical characteristics of these patients are summarized in Table I.

For the before–after study, we included another 332 patients who underwent the same procedures in the same hospital but between January 1995 and December 2002 (before the introduction of 'post-operative OC recommendation'). Of the 332 subjects originally enrolled, a total of 224 who had a minimum of 24 months of post-operative follow-up were compared with the above-mentioned 87 patients (after the introduction of 'post-operative OC recommendation').

We confirmed that there was no significant difference in clinical characteristics between patients who were lost to follow-up and who were not, both before and after the introduction of the 'post-operative OC recommendation'. Institutional Review Board approval was not requested because laparoscopic excision is a standard procedure for the treatment of ovarian endometrioma. All patients gave written informed consent before surgery.

Surgery and the 'post-operative OC recommendation'

The method of laparoscopic excision of ovarian endometrioma was performed as previously described (Koga *et al.*, 2006). Briefly, after the ovary was freed from any adhesion, the capsule of the cyst was completely stripped away from the normal ovarian tissue. Endometriotic peritoneal implants were excised with scissors or coagulated with bipolar electro coagulation.

In May 2005, we introduced the 'post-operative OC recommendation' for patients treated with laparoscopic excision of endometrioma, who were under the age of 40 and not seeking pregnancy. At the time of the laparoscopy, every patient was routinely provided with the following information about OC: (i) known side-effects (nausea etc.) and risks (thrombosis etc.) and their likelihoods, (ii) known benefits supported by conclusive evidence (reducing pain etc.) and (iii) the possible benefits of reducing or delaying recurrence, not supported by conclusive data but the theoretical explanations. We also provided the recurrence rate and risk factors that we analyzed in our previous study (Koga *et al.*, 2006). The decision to take OC was left up to the individual women. Women who chose to take OC, were given a cyclic, monophasic OC containing ethinyl-estradiol (0.035 mg) and norethisterone (1.0 mg) (Ortho-M 21®, Mochida, Tokyo, Japan), starting in Days 1–5 of the first menstrual cycle after the laparoscopy.

Table I Characteristics of the 87patients after the 'OC recommendation'

Factors	Number of cases (%)
Age (years)	33.4 ± 4.6[a]
Infertility	11 (12.6)
Pain	40 (46.0)
Previous medical treatment of endometriosis	35 (40.2)
Previous surgery of ovarian endometrioma	7 (7.9)
Largest cyst diameter (cm)	5.3 ± 2.1[a]
Bilateral involvement	32 (36.8)
Revised ASRM score	59 ± 37[a]
Post-operative medical treatment	59 (67.8)
Post-operative pregnancy	9 (10.3)

ASRM, American Society for Reproductive Medicine.
[a]Mean ± SD.

Post-operative follow-up and diagnosis of recurrence

Post-operative follow-up was performed by transvaginal ultrasonography. The initial check up was conducted on Day 4 and Day 30 after the operation, to exclude residual cysts. Patients were then followed-up every 3 months for 24 months following the laparoscopy. The diagnosis of endometrioma recurrence was made as described in our previous study (Koga *et al.*, 2006). Briefly, a recurrence was defined as the presence of cysts with a typical aspect detected by transvaginal ultrasonography (Exacoustos *et al.*, 2003) more than 2 cm in diameter. The diagnosis of recurrence endometrioma was made when the cyst was indistinguishable from a corpus luteum cyst or intraovarian hematoma, and the cyst had not disappeared after several successive examinations. Recurrence was defined by the diagnosis of ovarian endometrioma at anytime within 24 months. Pain recurrence was defined as requiring analgesia at least once a month for dysmenorrhea or chronic pelvic pain at 24 months after the laparoscopy.

Statistical analysis

For the retrospective cohort study, we compared patients' characteristics between OC users and non-users by Mann–Whitney *U*-test and Fisher's exact test. Relative risk of OC use for endometrioma recurrence was calculated. Ten variables: age, presence of infertility, pain, previous medical treatment of endometriosis, previous surgery for ovarian endometrioma, the diameter of the largest cyst, unilateral or bilateral involvement, revised American Society of Reproductive Medicine (revised ASRM) score, post-operative OC use and post-operative pregnancy were evaluated to assess their independent effects on the recurrence rate using univariate analysis followed by a step-wise selection and logistic regression analysis.

For the before–after study, we used the Mann–Whitney *U*-test and Fisher's exact test to compare patients' characteristics between patients who underwent laparoscopy before and after the introduction of the 'post-operative OC recommendation'. The relative risk of the introduction of 'post-operative OC recommendation' for endometrioma recurrence was calculated.

Statistical analyses were performed by Statview for windows Ver. 5.0 (SAS Institute Inc., Cary, NC, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Retrospective cohort study

Fig. 1 shows the flowchart of the 87 patients who underwent laparoscopic excision of endmetrioma after May 2005. All received the 'post-operative OC recommendation'. Of the 87 patients, 48 started to take OCs, but 39 did not. The reasons for not commencing OC were seeking pregnancy ($n = 25$) and age over 40 ($n = 6$) although 8 women chose not to take OC without a particular reason. Of the 48 patients who had started OC, 34 continued OC for the entire study period (24 months), whereas 14 discontinued. The reasons for discontinuing OC were desire for pregnancy ($n = 5$), and side effects ($n = 9$). This group suffered minor side effects e.g. headache and nausea; major complications such as thrombosis did not occur. The mean length of OC taken in this group was 9.7 ± 6.1 months.

Endometrioma recurrence was detected in 20 out of 87 subjects (23.0%) in all study groups. The mean diameter of the recurrent cyst at diagnosis was 33.1 ± 12.0 (SD) mm. Among patients who had recurrence, one underwent second-line surgery due to the large size of the recurrent cyst.

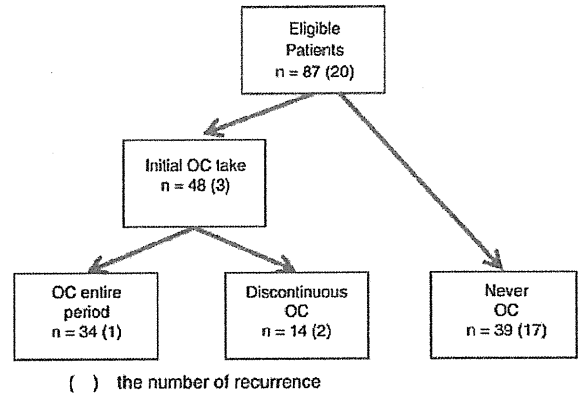

Figure 1 Flowchart of the patients who underwent laparoscopic excision of endometrioma after May 2005, for the retrospective cohort study.

A total of 87 patients were followed up for 24 months. Of the 87 patients, 48 started to take OCs, but 39 did not. Of the 48 patients who had started OC, 34 continued OC for the entire study period (24 months), whereas 14 discontinued. Recurrence was detected in 20 out of 87 subjects (23.0%) in all study groups. To determine the effect of OC use on recurrence, we compared recurrence rates with patients who used OC for the entire study period (entire period OC group), patients who discontinued OC use (discontinuous OC group) and patients who never used OC (never OC group). The recurrence rate was 2.9% (1/34), 14.3% (2/14) and 43.5% (17/39) in the entire period OC, discontinuous OC and never OC groups, respectively.

We then calculated recurrence rates with patients who used OC for the entire study period (entire period OC group), patients who discontinued OC (discontinuous OC group) and patients who never used OC (never OC group). The recurrence rates were 2.9% (1/34), 14.3% (2/14) and 43.5% (17/39) for the entire period OC, discontinuous OC and never OC groups, respectively.

We then combined the discontinuous OC group and never OC group together ('others') and compared that with the entire period OC group ('entire period OC group'). Table II shows the comparison of patients' characteristics between these two groups. Age, percentages of patients who have pain, previous medical treatment of endometriosis, previous surgery of ovarian endometrioma, the diameters of the largest cyst at laparoscopy, the percentage of patients who had bilateral involvement and the revised ASRM scores were not significantly different between groups. The percentage of patients who were infertile and patients who achieved pregnancy in the post-operative period were significantly lower in the 'entire period OC group' ($P < 0.05$ for both).

We then compared the recurrence rate between the groups. The recurrence rate in the 'entire period OC group' was significantly lower than in the 'others' (2.9 versus 35.8%, relative risk 0.082, 95% CI 0.012–0.58, $P < 0.001$). This indicates that taking OC without cessation reduces the risk of endometrioma recurrence by a factor of 12.

In order to confirm whether post-operative OC use is independently associated with the lower recurrence rate, we further performed univariate analysis followed by logistic regression analysis

Table II Comparison of characteristics between those who used OC for the entire period and the others who did not

Factors	OC entire period ($n = 34$)	Others ($n = 53$)	*P*-value
Age (years)	33.4 ± 4.8[a]	33.4 ± 4.3[a]	NS
Infertility	0%	11.0%	<0.05
Pain	55.9%	39.6%	NS
Previous medical treatment of endometriosis	38.0%	28.0%	NS
Previous surgery of ovarian endometrioma	3.0%	11.0%	NS
Largest cyst diameter (cm)	5.2 ± 2.4[a]	5.4 ± 2.8[a]	NS
Unilateral involvement	59.0%	66.0%	NS
Revised ASRM score	61 ± 37[a]	56 ± 38[a]	NS
Post-operative pregnancy	0%	17.0%	<0.05

ASRM, American Society for Reproductive Medicine; NS, not significant.
[a]Mean ± SD.

Table III Univariate and logistic regression analysis of factors related to the recurrence of ovarian endometrioma

Factors	Univariate analysis *P*-values	Logistic regression analysis *P*-values	Odds ratio (95% confidence interval)
Age (years)	NS	NS	0.951 (0.853–1.061)
Infertility	NS		
Pain	NS		
Previous medical treatment of endometriosis	NS		
Previous surgery of ovarian endometrioma	NS		
Largest cyst diameter (cm)	NS		
Bilateral involvement	NS		
Revised ASRM score	<0.05	<0.005	1.018 (1.003–1.034)
Post-operative OC for the entire study period	<0.01	<0.0001	0.054 (0.007–0.429)
Post-operative pregnancy	NS		

ASRM, American Society for Reproductive Medicine; NS, not significant.

(Table III). Ten variables were evaluated by univariate analysis and three variables (age, revised ASRM score and post-operative use of OC) were selected for logistic regression analysis by a forward stepwise variable selection. Post-operative use of OC was determined as an independent variable which is associated with lower recurrence (OR 0.054, 95% CI 0.007–0.429, $P < 0.001$). A higher revised ASRM score was significantly associated with higher recurrence (OR 1.018, 95% CI 1.003–1.034, $P < 0.01$). Neither infertility nor post-operative pregnancy influenced the rate of recurrence.

Before–after study

To evaluate the impact of the 'post-operative OC recommendation' on the endometrioma recurrence rate and pain recurrence rate in the overall population who underwent laparoscopic excision, we performed a historical study, the before–after study. Among patients who had a minimum of 24 months of post-operative follow-up, patients who were over the age of 40 and/or were infertile were excluded since these patients would not take OC regardless the recommendation. In total, 133 received laparoscopy before the introduction of 'post-operative OC recommendation' and 70 underwent laparoscopy after the introduction of the 'post-operative OC recommendation'. As indicated in Table IV, clinical characteristics of both groups were not different except for the patient's age. Among patients who received laparoscopy before the introduction of the 'OC recommendation', 15.8% actually used post-operative medical therapy including OC, GnRH analogue etc., whereas among patients who underwent laparoscopy after the introduction of 'OC recommendation', 78.6% started post-operative medical therapy, and most of the cases in this group was OC.

We then compared the recurrence rate between the groups. The overall endometrioma recurrence rate in patients who underwent laparoscopy after the introduction of the 'post-operative OC recommendation' was significantly lower than that in patients who received laparoscopy before the 'post-operative OC recommendation' (18.6 versus 33.1%, relative risk 0.56, 95% CI 0.32–0.97, $P < 0.05$). As for pain recurrence, the recurrence rate was not significantly different between the groups (58.7 versus 53.1% in before versus after the introduction of the 'post-operative OC recommendation', relative risk 0.90, 95% CI 0.62–1.31, $P = 0.6739$).

Discussion

In this study, we demonstrated the 'effect' of post-operative OC use on the recurrence of ovarian endometrioma following laparoscopic excision in a retrospective cohort study. Furthermore, we showed the 'efficiency' of our intervention; the introduction of the 'post-operative OC recommendation', for reducing the overall recurrence rate in a before–after study.

In the retrospective cohort study, we demonstrated that post-operative OC use significantly reduces the recurrence of ovarian endometrioma within 24 months following laparoscopic excision. The recurrence rate after surgery in patients who used OC for the entire study period (24 months), in patients who discontinued OC and in patients who never used OC was 2.9, 14.3 and 43.5%, respectively. The relative risk of recurrence was 0.082 in patients who used OC for the entire study period compared with patients who did not, in other words, patients who took post-operative OC without cessation were12 times less likely to have recurrence. We further showed that post-operative use of OC is an independent variable

Table IV Comparison between patients' characteristics before and after the 'OC recommendation'

Factors	Before 'OC recommendation' ($n = 133$)	After 'OC recommendation' ($n = 70$)	P-value
Age (years)	30.7 ± 4.8[a]	32.7 ± 4.3[a]	<0.01
Pain	60.2%	47.1%	NS
Previous medical treatment of endometriosis	47.4%	41.4%	NS
Previous surgery of ovarian endometrioma	9.0%	4.3%	NS
Largest cyst diameter (cm)	5.8 ± 1.8[a]	5.5 ± 1.9[a]	NS
Bilateral involvement	33.8%	35.7%	NS
Revised ASRM score	52 ± 30[a]	55 ± 31[a]	NS
Post-operative medical treatment of endometriosis	15.8%	78.6%	<0.001
Post-operative pregnancy	14.3%	8.6%	NS

ASRM, American Society for Reproductive Medicine; NS, not significant.
[a]Mean ± SD.

that is associated with lower recurrence. Subsequent to our previous study (Koga *et al.*, 2006) and commencement of the 'post-operative OC recommendation' in our clinic in 2005, two studies have been conducted to investigate the efficacy of post-operative OC in preventing endometrioma recurrence following laparoscopic excision. Vercellini *et al.* (2008a, b) evaluated the recurrence within 36 months after excision and reported that 94% were recurrence-free in the always OC users compared with 51% in the never users, with an incidence rate ratio of 0.10. Furthermore, in a study comparing cyclic and continuous OC use versus non-use, the recurrence rates within 24 months were 14.7, 8.2 and 29%, respectively (Seracchioli *et al.*, 2008). Our current results corroborate with these observations and strongly support the proposal that post-operative OC administration reduces the endometrioma recurrence rate after laparoscopic excision.

In addition to the 'effect' of post-operative OC use, we further demonstrated the 'efficiency' of the introduction of the 'post-operative OC recommendation' for reducing the endometrioma recurrence rate in the overall population who underwent laparoscopic excision, as demonstrated by the before–after study. The overall recurrence rate declined significantly from before to after the introduction of 'post-operative OC recommendation'. This is a unique observation that showed the impact of 'post-operative OC recommendation' in a daily clinical context. As a consequence of informing patients of the 'possible' benefits of OC in preventing recurrence and leaving the decision whether to take and continue OC to individuals, not many patients chose OC. However, the introduction of a 'post-operative OC recommendation' significantly contributed to the reduction of recurrence among the overall population who underwent laparoscopic excision. This encourages us to continue the 'post-operative OC recommendation' with further emphasis of its benefit in reducing endometrioma recurrence.

In contrast to the efficiency of the OC recommendation for reducing endometrioma recurrence, the intervention did not decrease pain recurrence. One explanation would be that the pain does not necessarily reflect the presence of endometriosis. It is also possible that our pain evaluation did not detect a subtle change of pain severity because it was not quantitative. Further studies are needed to determine the effect of OC on pain recurrence.

This study also suggests that OC can provide a better option in terms of safety and tolerability. Despite the small study group, no patients developed major complications and all side effects reported were minor, which is consistent with a previous report which used OC for dysmenorrhea (Harada *et al.*, 2008). Moreover, although the recommendation was not absolute, most women continued OC, indicating a good tolerability that yielded high compliance. This is in contrast to other hormonal drugs for endometriosis such as GnRH analogue and danazol, which are known to cause various side effects (Vercellini *et al.*, 2008a, b).

The mechanism by which OC reduces endometrioma recurrence has not been elucidated. Previously, we found that post-operative pregnancy was associated with a low recurrence rate (Koga *et al.*, 2006), and consequently we hypothesized that pregnancy or a pregnancy-mimicking hormonal condition may reduce the risk of recurrence. Indeed, several epidemiological studies have shown that OC use reduces the risk of endometriosis development (Vercellini *et al.*, 1993; Vessey *et al.*, 1993; Missmer *et al.*, 2004). One possible explanation is the effect of OC on the eutopic endometrium. OC intake has been reported to increase apoptosis and decrease cell proliferation in the eutopic endometrium (Meresman *et al.*, 2002), which could result in lowering *de novo* endometriosis development. It is also possible that OC may decrease the risk of endometrioma development by inhibiting ovulation since ovarian endometrioma can develop from ovarian follicles (Jain and Dalton, 1999) and corpus luteum (Vercellini *et al.*, 2009). Additionally, OC intake diminishes the amount of retrograde menstruation and reduces the chance of recurrence in the same manner as endometrial ablation can prevent endometriosis recurrence (Bulletti *et al.*, 2001; Osuga, 2008). If this is the case, it would be interesting to compare the effect of OC between cyclic and continuous administration since the latter yields less frequent bleeding. Intriguingly, Seracchioli *et al.* (2008) addressed this point and reported that the recurrence rate was slightly lower in continuous OC users than in cyclic users, although the difference was not statistically significant. Further studies will be needed to confirm a benefit of continuous OC.

Our study arouses debate in regard to the benefits of OC administration; whether it 'prevents' recurrence or 'delays' it. The first study

to evaluate the efficacy of post-operative OC demonstrated that using OC for a period of 6 months reduced the recurrence rate at 12 months, compared with controls, but then by 24 months this benefit was eroded and recurrence was similar to non-user controls (Muzii *et al.*, 2000). Consequently this group concluded that post-operative OC does not affect the long-term recurrence rate but achieves a delay in recurrence. Focusing on the disease recurrence after the cessation of OC, this observation seems consistent with ours and others (Vercellini *et al.*, 2008a, b), that OC users have an extremely small chance of recurrence (2.9% in our study, 6% in others) whereas patients who discontinued OC were at higher risk. These findings suggest that OC administration may only 'delay' the chance of recurrence rather than 'prevent'. From the clinical point of view, however, this 'delay' provides a significant benefit especially for patients who seek pregnancy several years after the laparoscopy.

There are several limitations in the current study. Firstly, the numbers of patients in both the retrospective cohort study and the before–after study were not sufficient enough to generalize the results to the general population, although the difference in each study was statistically significant. Secondly, since the retrospective cohort study was designed in a clinical context rather than in an experimental context, the allocation of subjects to OC groups was not random but based on patient preference. Therefore, the result might be affected by selection bias. This may explain why the magnitude of OC-use benefit in our study was comparable with the one designed in a clinical context (Vercellini *et al.*, 2008a, b), but was slightly different from the one conducted in more experimental context (Seracchioli *et al.*, 2008). Lastly, we cannot exclude the presence of confounding factors in our before–after study, such as surgical technique and surgeons' experience, although we tried to minimize them. Further studies will be necessary to overcome these limitations.

Several questions remain unanswered. Firstly, how long should patients continue using OC? Given that most of studies set the OC administration period at <3 years, it will be interesting to see whether OC can yield 'preventive' effects if the period of treatment is prolonged. Secondly, up to what age should OC is recommended? In the current study, we did not offer 'post-operative OC recommendation' for patients over the age of 40, since OC is considered to be relatively contraindicated for women over the age 40, in regard to promoting thrombotic disease (1981). However, given that an increasing number of women seek pregnancy in their early 40s and that the presence of endometriosis in the 40s has a high risk of cancer development (Kobayashi *et al.*, 2008), we must weigh the benefits of OC in preventing the recurrence against the possible adverse effects of OC. Further evidence is necessary to evaluate both efficacy and safety.

In summary, this study demonstrates that post-operative OC use reduces the risk of ovarian endometrioma recurrence after laparoscopic excision. Having this information will help gynecologists and patients plan pre- and post-operative management appropriately to prevent recurrence and consequently to maintain fertility for long as possible.

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Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin β subunits, and anti-Müllerian hormone in human granulosa cells

Immunohistochemical staining using human normal ovaries showed that bone morphogenetic protein-6 (BMP-6) was abundantly present in the granulosa cells (GC) of healthy tertiary follicles but not in atretic follicles. An in vitro study showed that BMP-6 induced gene expression of FSH receptor, inhibin/activin β subunits, and anti-Müllerian hormone (AMH) in human GCs, suggesting that BMP-6 is an important mediator to support healthy follicle growth in the human ovary. (Fertil Steril® 2009;92:1794–8. ©2009 by American Society for Reproductive Medicine.)

Folliculogenesis is the process by which primordial follicles grow and develop to the ovulatory follicle stage. Through this process, one healthy follicle is usually selected for maturation in the spontaneous menstrual cycle. It has been reported that granulosa cells (GCs) of healthy follicles express activins and FSH receptor (1, 2). Activins support GC survival and cell proliferation and maintain the functional FSH receptor (3). Activation of FSH receptor triggers cytodifferentiation and proliferation of GCs (3). Although activins and FSH receptor are recognized as important factors during folliculogenesis, the precise mechanism of activin and FSH receptor expression is poorly understood.

A growing body of evidence indicates that the bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, play a key role in female fertility in mammals (4, 5). Among the BMPs, BMP-15, GDF-9, BMP-7, and BMP-6 are expressed in the ovary. These four molecules maintain folliculogenesis by inhibiting early luteinization (4). In addition, each BMP has a specific role in folliculogenesis. BMP-15 and GDF-9 have been of particular interest in the study of mammalian reproduction. Mutation of the GDF-9 gene leads to arrested folliculogenesis in mice, ewes, and humans (4, 6, 7). In contrast, the mutation of BMP-15 leads to arrested folliculogenesis in monovulatory species, ewes, and humans, but not in mice (4). Furthermore, an in vitro transfection system of 293 human embryonic kidney and Chinese hamster ovary cell lines demonstrated that recombinant GDF-9 proteins of mice, ewes, and humans are readily processed. Although human and sheep BMP-15 are processed in this system, mouse BMP-15 is not produced (4). Thus, it has been proposed that GDF-9 protein is essential for early folliculogenesis in mammals, whereas the role of BMP-15 in folliculogenesis is different between species (4). Like BMP-15, the function of BMP-6 in folliculogenesis appears to differ in ruminants versus rodents (8, 9). BMP-6 decreased FSH receptor, inhibin α, inhibin/activin β subunits messenger RNA (mRNA) expressions in GC of rats (8), whereas BMP-6 enhanced inhibin-A and activin-A production in bovine GC (9). Regulation of activins and FSH receptor is crucial in folliculogenesis (1, 2), thus these findings prompted us to investigate the roles of BMP-6 in the human ovary. We studied the localization of BMP-6 in the ovary, and then examined the effects of BMP-6 on folliculogenesis-related molecules in GC. We also investigated the regulator of BMP-6 mRNA expression.

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MATERIALS AND METHODS

Except where indicated, all reagents were purchased from Sigma (St. Louis, MO). Recombinant human BMP-6 and BMP-7 were purchased from R&D Systems (Minneapolis, MN). A monoclonal antibody against BMP-6 was purchased from Chemicon International (Billerica, MA). Recombinant human FSH and activin-A were kindly provided by Nippon Organon (Tokyo, Japan) and Dr. Shunichi Shimasaki (University of California, San Diego, CA), respectively.

Collection of Ovarian Tissues and Immunohistochemistry

Tissue specimens of human ovaries were obtained under signed informed consent from eight women (age range, 28–40 years old) who underwent salpingo-oophorectomy for the treatment of

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uterine cervical cancer. All patients had normal ovarian cycles before surgery and any histologic abnormalities and malignant lesions were not observed in ovarian tissues. The experimental procedure was approved by the institutional review board (IRB). Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks, and 6-μm sections were prepared. Antigen retrieval was performed using sodium citrate buffer (10 mM, pH 6.0) (10). The sections were stained with 2 μg/mL anti-BMP-6 antibody or mouse IgG as negative control using an Envision+ System/HRP Mouse (DAB+) kit (Dako, Tokyo, Japan). Healthy and atretic follicles were identified on the basis of classic histologic features (11). Briefly, the healthy follicles had multiple intact layers of GC, which lined the entire circumference of the basal lamina. The atretic follicles displayed a variety of degenerative changes; in atretic follicles, sheets of GC had dislodged and were floating free in the antral cavity.

Cell Culture of Human Granulosa Cells

Granulosa cells were obtained from patients undergoing ovarian stimulation for IVF. The method to purify and culture human GC was described previously (12). The experimental procedures were approved by the IRB, and signed informed consent for use of GC was obtained from each patient. The collected human GC were cultured in Dulbecco's minimum essential medium (DMEM)/F12 containing 5% fetal bovine serum and antibiotics in 12-well plates at a density of 2×10^5 cells/mL. To evaluate the effects of BMP-6, human GC were cultured with or without BMP-6 (100 ng/mL) for 24 hours. To investigate the regulation of BMP-6, GC were cultured with BMP-6 (100 ng/mL), BMP-7 (100 ng/mL), E_2 (10 ng/mL), activin-A (100 ng/mL), FSH (0.5 IU/mL), or 8-bromo-cyclic adenosine 3′:5′ monophosphate (cAMP) (1 mM).

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA extraction from GC, the primer sequences and real-time polymerase chain reaction (PCR) conditions were describe elsewhere (12), except the primer sequence of anti-Müllerian hormone (AMH) (NM_000479: 619-638 and 820-801) and BMP-6 (NM_001718: 420-441 and 839-820). All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. Data were analyzed by Student's *t*-test for paired comparison and one-way analysis of variance (ANOVA) with post hoc test for multiple comparisons. A P value less than .05 was considered statistically significant.

RESULTS

Localization of BMP-6 in Human Ovaries

The expression of BMP-6 in human ovaries was examined by immunohistochemistry using normal human ovaries. As shown in Figure 1a,c, BMP-6 expression was clearly detected in the oocytes of primordial and primary follicles. BMP-6 was also detected in GC. The intensity of the staining of BMP-6 in GC was barely detected in primordial follicle, and low in GC of primary and secondary follicles (Fig. 1a,c,d), whereas it was high in GC of healthy antral follicles (Fig. 1e,g). In contrast, BMP-6 staining was very weak in GC of atretic follicles (Fig. 1f,h).

The Effect of BMP-6 on Gene Expression of Folliculogenesis Factors

Incubation of GC with BMP-6 (100 ng/mL) for 24 hours significantly increased the gene expression of inhibin/activin βA and βB subunits, but exerted no effect on inhibin α subunit mRNA (Fig. 2a–c). Notably, BMP-6 caused a nearly threefold increase in FSH receptor mRNA levels (Fig. 2d). BMP-6 also significantly increased AMH mRNA levels (Fig. 2e).

Regulation of BMP-6 in Granulosa Cells

To investigate the regulation of BMP-6 gene expression, human GCs were cultured with various stimuli. Because the growth of follicles is highly influenced by intraovarian factors (i.e., activins and BMP-7), we checked whether these factors could induce BMP-6 expression. As shown in Figure 2f, activin-A (100 ng/mL) and BMP-7 (100 ng/mL) increased the mRNA level of BMP-6, whereas BMP-6 (100 ng/mL) itself had no effect. In addition, 8-bromo-cAMP, FSH, and E_2 did not alter BMP-6 mRNA levels (data not shown).

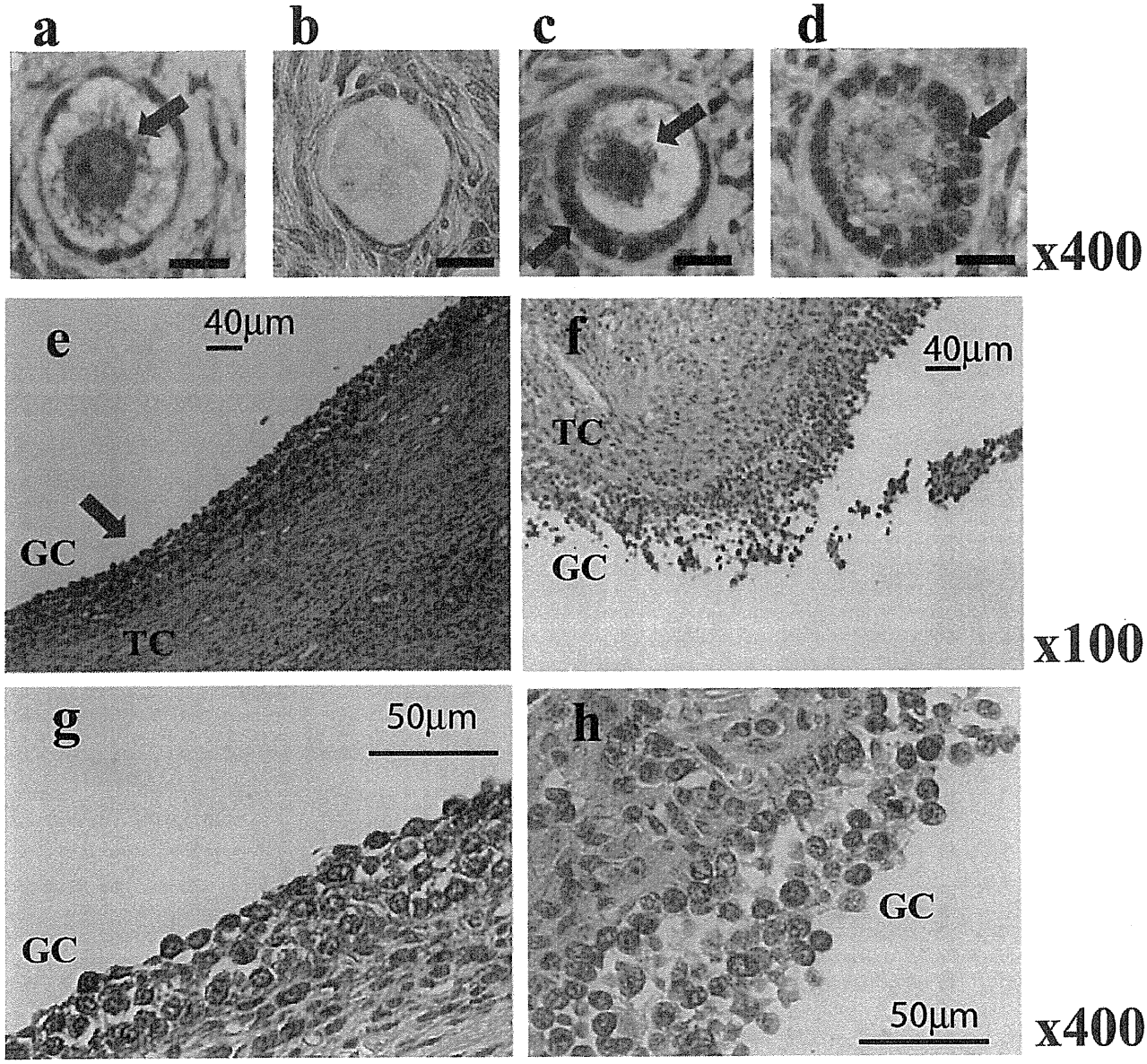
DISCUSSION

In the present study, we demonstrated that BMP-6 is strongly expressed in GC of tertiary follicles and oocytes. In cultured human GC, BMP-6 stimulated gene expression of the inhibin/activin βA and βB subunits, but not the inhibin α subunit. BMP-6 also stimulated mRNA expression of FSH receptor and AMH in cultured human GC. In addition, BMP-6 gene expression in cultured human GC was increased by activin-A and BMP-7.

Our immunohistochemical study revealed that BMP-6 protein was strongly expressed in GC of healthy tertiary follicles (Fig. 1e,g). In contrast, BMP-6 protein was only weakly expressed in GC of atretic follicles (Fig. 1f,h). Because BMP-6 increased mRNA expression of inhibin/activin β subunits in GC, the observed expression pattern of BMP-6 in GC is consistent with the finding that inhibin/activin β subunits are expressed in healthy follicles, but not in the similarly sized atretic follicles (1). Serum FSH concentration decreases in the latter half of the follicular phase. Therefore, the sensitivity of follicles to FSH during this period is critical and determines whether follicles become atretic or dominant (2). In view of the present finding that BMP-6 increased the expression of FSH receptor, follicles with high BMP-6 expression may be more likely to survive the decrease in serum FSH, thus increasing the chances of surviving to the dominant follicle stage. This notion is also supported by the strong expression of BMP-6 in GC of healthy tertiary follicles. In contrast to our immunohistochemical findings in human ovaries, the mRNA levels of BMP-6 in GC were found to decrease at the time of dominant follicle selection in rats (13). Furthermore, our findings on the in vitro effects of BMP-6 in cultured human GC are the opposite of that observed in rats, in which BMP-6 decreased FSH-induced expression of FSH receptor and inhibin/activin β subunits (8). One possible explanation may be that the different expression pattern of BMP-6 in the follicle between human and rat in vivo is due to the different effect of BMP-6 on folliculogenesis between the species. Glister et al. (9) also reported that in bovine GC, the effect of BMP-6 is different from rat GC. Because there is a growing evidence that the expression pattern of BMP-15, another BMP family cytokine, is different from in mono-ovulatory and polyovulatory species, leading to the

FIGURE 1

Localization of bone morphogenetic protein-6 (BMP-6) expression in human ovaries. The BMP-6 expression in normal human ovaries was investigated by immunohistochemistry. Primordial follicle (**a, b**), primary follicle (**c**), secondary follicle (**d**), healthy tertiary follicle (**e, g,**) and unhealthy tertiary follicle (**f, h**). (**b**) Negative control. Arrows indicate positive BMP-6 signal. GC = granulosa cells; TC = theca cells.



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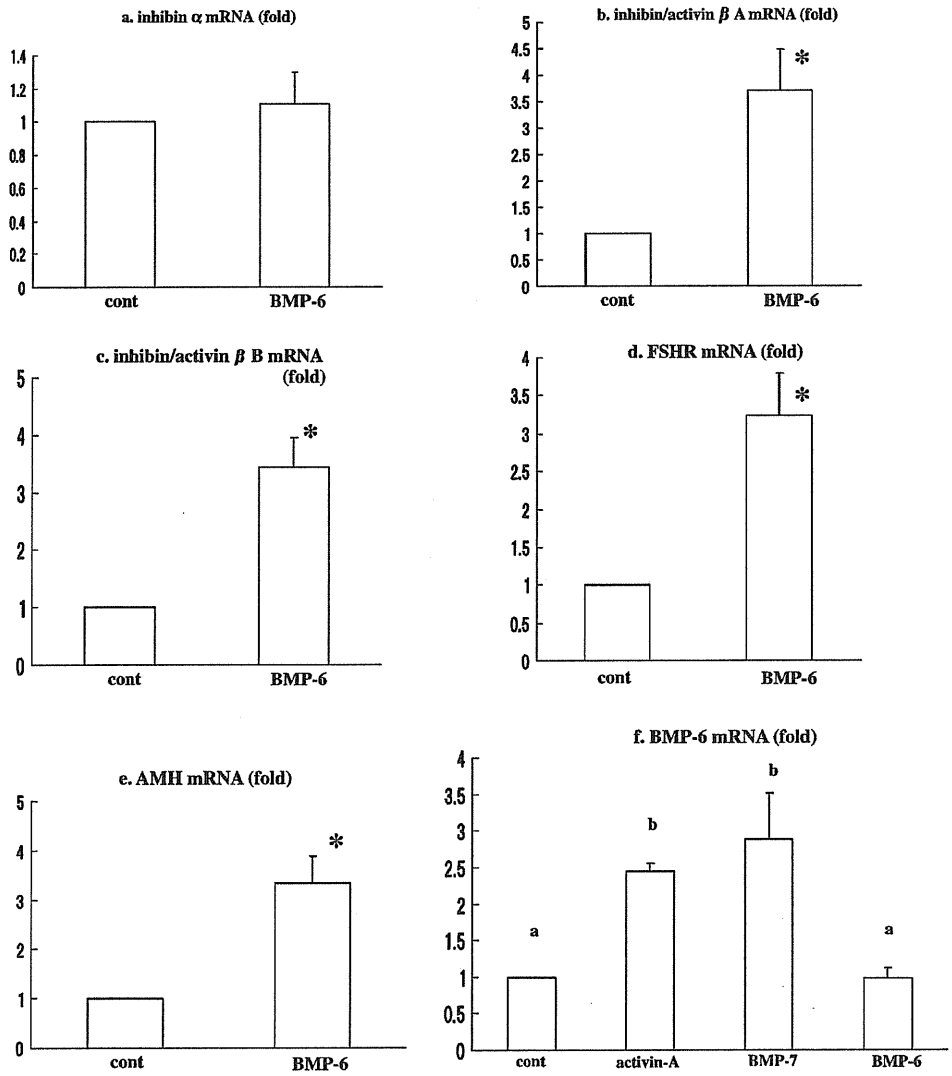
concept that BMP-15 governs ovulation quota, mono, and polyovulation (4), it is possible that the differences in BMP-6 expression and functions between species might also be related to ovulation quota.

Activin-A and BMP-7 increased mRNA level of BMP-6 in cultured human GC. Activins and BMP-7 are derived from GCs and theca cells, respectively (5), thus, an autocrine or paracrine mechanism might be working to regulate BMP-6 expression in the follicle. Interestingly, activin-A and BMP-7 are both known to induce FSH receptor (12). In view of the present finding that BMP-6 induced FSH receptor mRNA in GC, activin-A and BMP-7 might induce FSH receptor partially by up-regulation of BMP-6 expression in GC.

Our immunohistochemical study also revealed that oocytes of primordial and primary follicles strongly expressed BMP-6 protein (Fig. 1a,c). In primordial and primary follicles, which do not express FSH receptor, activins are known to be important factors for follicle growth (3), but regulation of activins in the follicles of this stage is not well understood. Given that BMP-6 secreted from oocytes of primordial and primary follicles could act on

FIGURE 2

(a–e) Effect of bone morphogenetic protein-6 (BMP-6) on inhibin α (a), inhibin/activin βA (b), inhibin/activin βB (c), FSH receptor (d), and anti-Müllerian hormone (AMH) (e) messenger RNA (mRNA) expression. The granulosa cells (GC) were cultured with BMP-6 (100 ng/mL) for 24 hours. (f) Effect of various stimuli on BMP-6 mRNA expression. The GCs were cultured with activin-A, BMP-7, or BMP-6 (100 ng/mL) for 24 hours. Total RNA was extracted from the GCs and subjected to real-time polymerase chain reaction (PCR) to determine the mRNA levels. Data were normalized to GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. *$P<.05$ (vs. control). Bars with different letters indicate a significant difference at $P<.05$.



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GC, activins, rather than inhibins, might be induced preferentially in early follicles that do not express the inhibin α subunit abundantly. Namely, BMP-6 derived from oocytes might be an inducer of activins in the primordial and primary stages of folliculogenesis. We also found that activin-A induced BMP-6 expression (Fig. 2f), thus BMP-6 and activins might have a reciprocal effect on inducing one another, especially in the early follicles.

Recently, AMH has been demonstrated to play an important role in ovarian function with its inhibitory effect on follicle recruitment (14). In addition, in humans, AMH has been found to be a marker of ovarian reserve (14). Although it is reported that FSH and E_2 down-regulate AMH expression in the GC (15), no AMH up-regulators have been identified to date. The present study provided the evidence that BMP-6 increased expression of AMH. Thus, we hypothesize that BMP-6 in the healthy growing follicles up-regulates AMH expression, which, in turn, suppresses growth of the surrounding primordial follicles, thereby preserving the ovarian reserve.

In summary, BMP-6 is expressed in the GC of healthy, growing follicles, but not in atretic follicles. BMP-6 increased gene

expression of FSH receptor, inhibin/activin β subunits, and AMH, contributing to the survival of healthy growing follicles and suppressing depletion of the primordial follicle reserve. Cultured GCs used in this study may not represent the stages of growing follicles and further studies are needed. However, our findings that BMP-6 regulates folliculogenesis-related genes in human GCs are novel, and open new insights into our understanding of ovarian physiology.

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Bone morphogenetic protein 7 (BMP-7) increases the expression of follicle-stimulating hormone (FSH) receptor in human granulosa cells

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Objective: To examine the effect of bone morphogenetic protein 7 (BMP-7) on FSH receptor (FSHR) expression in human granulosa cells.
Design: Laboratory study using human samples.
Setting: University hospital.
Patient(s): Human granulosa cells were obtained from 60 women undergoing oocyte retrieval for IVF.
Intervention(s): Human granulosa cells (GCs) were cultured with recombinant BMP-7, followed by RNA extraction.
Main Outcome Measure(s): mRNA levels of GCs were measured by real-time reverse-transcription polymerase chain reaction.
Result(s): Bone morphogenetic protein 7 increased FSHR gene expression in human luteinized granulosa cells, whereas it decreased LH receptor gene expression. Bone morphogenetic protein 7 also increased FSH-induced cyclic adenosine monophosphate production in GCs, indicating up-regulation of the cellular response to FSH. Although BMP-7 increased gene expression of activin-βA and -βB in GCs, inhibition of activin function did not affect the BMP-7–induced FSHR gene expression.
Conclusion(s): These findings provide new insight into the biologic function of BMP-7 in the human ovary and demonstrate its unique mechanism of regulating FSHR action. (Fertil Steril® 2010;93:1273–9. ©2010 by American Society for Reproductive Medicine.)

Key Words: BMP, FSH receptor, ovary, folliculogenesis, female fertility

The pituitary gonadotropin FSH is a key hormone in the regulation of folliculogenesis and female fertility (1, 2). In the ovary, FSH triggers cytodifferentiation and proliferation of granulosa cells (GCs), ultimately resulting in the development of preovulatory follicles (3, 4).

Because FSH acts on the ovary in an endocrine manner, the expression of FSH receptor (FSHR) on target cells is essential for modulation of ovarian function by FSH. It is well known that FSHR is not expressed until midway through follicle development. In mature follicles, maintenance of FSHR expression is required to avoid death by atresia (5–7). Therefore, it is important to elucidate the mechanism responsible for regulation of FSHR expression to better understand the process of folliculogenesis.

Several factors, such as activins (8, 9), FSH (10), cyclic adenosine monophosphate (cAMP) stimulants and cAMP analogs (11), are known to modulate the synthesis of FSHR mRNA in GCs. Recently, the bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-β superfamily, have emerged as important players in ovarian physiology and female fertility (12, 13). There is growing evidence that BMP-7 can modulate steroidogenesis in a way that promotes estrogen production while inhibiting progesterone biosynthesis in many species (14–16). Bone morphogenetic protein 7 increases FSH mRNA levels in cultured mouse ovaries (18). However, no information is available on the effect of BMP-7 on human primary GCs, although Abir et al. (17) reported that BMP-7 and its receptors are expressed in human ovarian follicles. In view of the finding that BMP-7 is expressed from small follicles (17), we hypothesized that BMP-7 might increase FSHR mRNA levels in human ovarian follicles. In this study, we investigated the effect of BMP-7 on FSHR expression using cultured human luteinized granulosa cells (LGCs), aiming to elucidate possible roles of BMP-7 in the human ovary.

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MATERIALS AND METHODS

Reagents and Materials

Hyaluronidase, fetal bovine serum (FBS), DMEM/F12, and antibiotics (mixture of penicillin, streptomycin, and amphotericin B) were purchased from Sigma (St. Louis, MO), recombinant human BMP-7 was purchased from R&D Systems (Minneapolis, MN), and SB-431542 was from Calbiochem (La Jolla,

CA). Recombinant human FSH and activin-A were kindly provided by Nippon Organon (Tokyo, Japan) and Dr. Shunichi Shimasaki (University of California, San Diego), respectively.

Cell Culture of Human Luteinized Granulosa Cells

The method to obtain and culture human LGCs was described previously (19–21). Briefly, follicular fluid with LGCs was aspirated from 60 patients undergoing ovarian stimulation for in vitro fertilization (IVF). The clinical reasons for IVF in these patients were primarily male factor or tubal factor infertility. Patients with ovarian dysfunction were excluded from the study. The experimental procedures were approved by the institutional review board, and signed informed consent for use of LGCs was obtained from each patient. All of the follicular aspirates from each patient were pooled and centrifuged at 200*g* for 5 min, resuspended in phosphate-buffered saline (PBS) with 0.2% hyaluronidase, and incubated at 37°C for 30 min. The suspension was layered onto Ficoll-Paque and centrifuged at 150*g* for 20 min. The LGCs were collected from the interphase, washed with PBS, and cultured in DMEM/F12 medium supplemented with 5% FBS and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 250 ng/mL amphotericin B) for 15 min at 37°C to remove contaminating macrophage cells from LGCs. Using this procedure, LGCs were isolated in the supernatant and macrophages were attached to the culture dish. The collected human LGCs were cultured in DMEM/F12 containing 5% FBS and antibiotics in 12-well plates at a density of 2×10^5 cells/mL and kept at 37°C in a humidified 5% CO_2/95% air environment for 5 days. With this method, the contamination of monocyte/macrophages and endothelial cells were less than 1 % judged by immunohistochemistry for CD68 and von Willebrand factor, respectively (data not shown). All of the LGCs used for the experiments were precultured for 5 days before treatments to allow the LGCs to regain sensitivity to FSH stimulation (22). The media were changed at 48 hr intervals. To evaluate the effects of BMP-7, human LGCs were cultured with or without BMP-7 (100 ng/mL) for 24 hr. In a dose-response study, LGCs were cultured with increasing concentrations of BMP-7 (0–300 ng/mL) for 24 h. In the time course experiments, LGCs were incubated with or without BMP-7 (100 ng/mL) for 3, 8, 24, and 48 h. To investigate which BMP receptor was used for induction of FSHR by BMP-7, activin receptor–like kinase (ALK) 4, 5, and 7 inhibitor SB-431542 was used before stimulation with BMP-7 or activin-A. Recombinant BMP-7 was dissolved in 0.1% BSA + 4 mmol/L HCl, and SB-431542 was dissolved in dimethylsulfoxide as vehicle. The same amount of vehicle was used for control.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted from LGCs using the RNAeasy minikit (Qiagen, Hilden, Germany). Reverse transcription (RT) was performed using Rever Tra Dash (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a 20-μL volume. For the quantification of various mRNA levels, real-time polymerase chain reaction (PCR) was performed using LightCycler (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. The PCR primers were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosomal DNA contaminants. The primer sequences were as follows, FSHR (NM_000145: 174–196 and 510–492), inhibin-α (NM_002191: 369–388 and 602–582), inhibin/activin-βA (NM_002192: 505–526 and 673–653), inhibin/activin-βB (NM_002193: 1184–1204 and 1325–1305), LH receptor (LHR) (NM_000233:747–767 and 981–962), ALK-6 (NM_001203: 356–375 and 761–742), BMPR-II (NM_001204: 647–666 and 1075–1054), and GAPDH (NM_002046: 628–648 and 1079–1060) (21). The PCR conditions for FSHR, inhibin-α, and inhibin/activin-βA and -βB consisted of 40 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 14 s, followed by melting curve analysis. The PCR conditions for LHR consisted of 40 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 9 s, followed by melting curve analysis. The PCR conditions for GAPDH consisted of 35 cycles at 95°C for 10 s, 64°C for 10 s, and 72°C for 18 s, followed by melting curve analysis. Expression of each mRNA was normalized by GAPDH mRNA. The temperature condition for regular PCR was 94°C for 10 s, 55°C for 4 s, and 72°C for 30 s. The number of PCR cycles was 22 for GAPDH, 25 for inhibin-α and inhibin/activin-βA, 30 for ALK-6 and BMPR-II, and 33 for inhibin/activin-βB, FSHR, and LHR. The PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide.

Measurement of cAMP Levels

To assess the level of cAMP synthesis, LGCs were cultured in 48-well plates with DMEM/F12 containing 1% FBS and antibiotics with or without BMP-7 (100 ng/mL) for 24 h. Then cells were cultured with 0.1 mmol/L IBMX, a phosphodiesterase inhibitor, in the presence or absence of FSH (0.5 IU/mL) for 2 h. Conditioned medium was collected and the extracellular content of cAMP was determined using a cAMP enzyme immunoassay kit (Caymen Chemical Company, Ann Arbor, Michigan).

Statistical Analysis

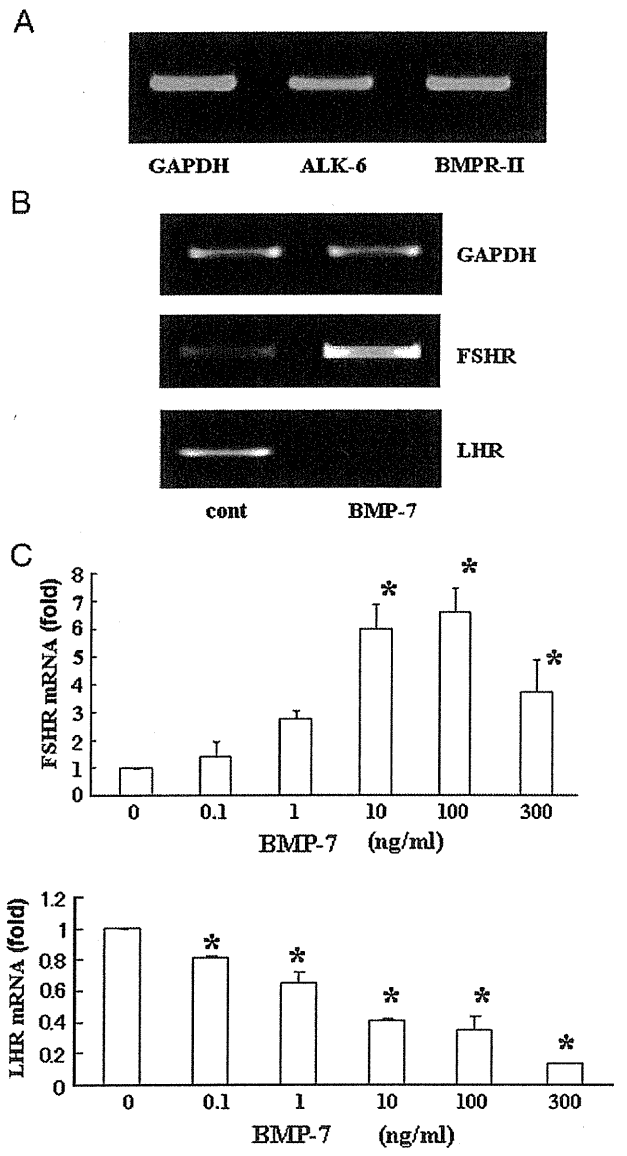
All results are shown as mean ± SD of data from at least three separate experiments, each performed with triplicate samples. Data were analyzed by Student *t* test for paired comparison and one-way analysis of variance with post hoc test for multiple comparisons using Statview software (SAS Institute, Cary, NC). A P value of $< .05$ was considered to be statistically significant.

RESULTS

Effect of BMP-7 on FSHR and LHR mRNA levels in LGCs

The gene expression of receptor for BMP-7, ALK-6, and BMPR-II was confirmed in LGCs by PCR (Fig. 1A). To investigate the effects of BMP-7 on FSHR mRNA induction in human GCs, the cells were cultured with BMP-7 (100 ng/mL) for 24 h (Fig 1B). Notably, BMP-7 significantly increased FSHR mRNA levels. On the other hand, BMP-7 significantly suppressed LHR mRNA expression (Fig 1C). In

FIGURE 1



Shi. BMP-7 increases FSH receptor. Fertil Steril 2010.

FIGURE 1 Continued

Expression of bone morphogenetic protein 7 (BMP-7) receptor (BMPR) in human granulosa cells and effect of BMP-7 on FSH receptor (FSHR) mRNA and LH receptor (LHR) mRNA expression. (**A**) The receptor for BMP-7, activin receptor–like kinase (ALK) 6, and BMPR-II in human luteinized granulosa cells (LGCs) were examined with regular polymerase chain reaction (PCR). (**B**) LGCs were cultured with or without BMP-7 (100 ng/mL) for 24 h followed by regular PCR for GAPDH, FSHR, and LHR. (**C**) LGCs were cultured with the indicated concentrations of BMP-7 for 24 h. For the quantification of FSHR and LHR mRNA, real-time PCR analysis was performed and the expression levels of FSHR and LHR mRNA standardized by GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. *Significant difference at $P<.05$ (vs. control).

a time course study, BMP-7 (100 ng/mL) increased FSHR mRNA levels after 8 h, and the maximal induction of FSHR mRNA expression occurred after 24 h of treatment (Fig. 2). The specificity of BMP-7 was confirmed by the finding that BMP-7–induced FSHR mRNA expression was completely inhibited by an antibody for BMP-7, but not by the control antibody (data not shown).

Induction of FSHR mRNA Expression by BMP-7 was not Via Production of Activins

Activins are known to be a strong inducer of FSHR mRNA expression (9). Therefore, we first examined the mRNA expression levels of inhibin and activin subunits in GCs stimulated with BMP-7. As shown in Fig 3, BMP-7 increased the mRNA levels of inhibin/activin-βA and inhibin/activin-βB, but had no effect on inhibin-α mRNA expression. Based on this finding, we hypothesized that after induction of mRNA for inhibin/activin-β subunits by BMP-7, new activin protein was synthesized, and that this new protein was responsible for inducing FSHR mRNA expression. To determine whether activin or BMP-7 is responsible for the increase in FSHR mRNA, we used SB-431542, an ALK-4, -5, and -7 inhibitor (23) which can selectively inhibit ALK-4, the receptor of activins, but has no effect on ALK-6, the receptor for BMP-7 (12). The LGCs were cultured with BMP-7 (100 ng/mL) or activin-A (100 ng/mL) in the presence or absence of SB-431542 (10 μmol/L). As shown in Figure 4A, SB-431542 significantly suppressed the stimulatory effect of activin-A on FSHR mRNA, whereas SB-431542 had no effect on the up-regulation of FSHR mRNA induced by BMP-7. This finding suggests that although BMP-7 can induce mRNA expression of activin subunits, the BMP-7–induced increase in FSHR mRNA is not mediated by activins. Interestingly, combination of BMP-7 and activin-A did not have an additive effect to increase FSHR mRNA expression, implying some redundancy between the pathways under BMP-7 and activin-A (Fig. 4B).

BMP-7 Treatment Increases Functional FSHR

Cyclic AMP is a well recognized second messenger for activated FSHR. To assess whether FSHR mRNA induction by BMP-7 results in an increase in functional FSHR, LGCs were pretreated with or without BMP-7 (100 ng/mL) for 24 h, and subsequently cultured with 0.1 mmol/L IBMX in the presence or absence of FSH (0.5 IU/mL) for 2 h. As expected, FSH significantly increased cAMP production by LGCs

FIGURE 2

Effect of bone morphogenetic protein 7 (BMP-7) on FSH receptor (FSHR) mRNA expression. Human luteinized granulosa cells (LGCs) were cultured with BMP-7 (100 ng/mL) for different time intervals (3–48 h). Total RNA was then extracted from the LGCs and subjected to real-time polymerase chain reaction to determine the mRNA levels of FSHR. Data were normalized to GAPDH mRNA levels. Solid and open bars represent the relative mRNA levels obtained from the culture in the presence or absence, respectively, of BMP-7. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the each control. *Significant difference at $P<.05$ (vs. control).

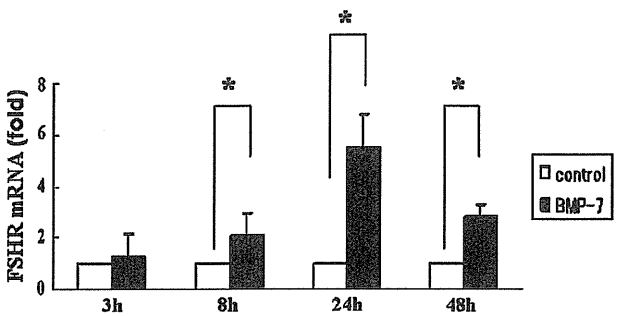


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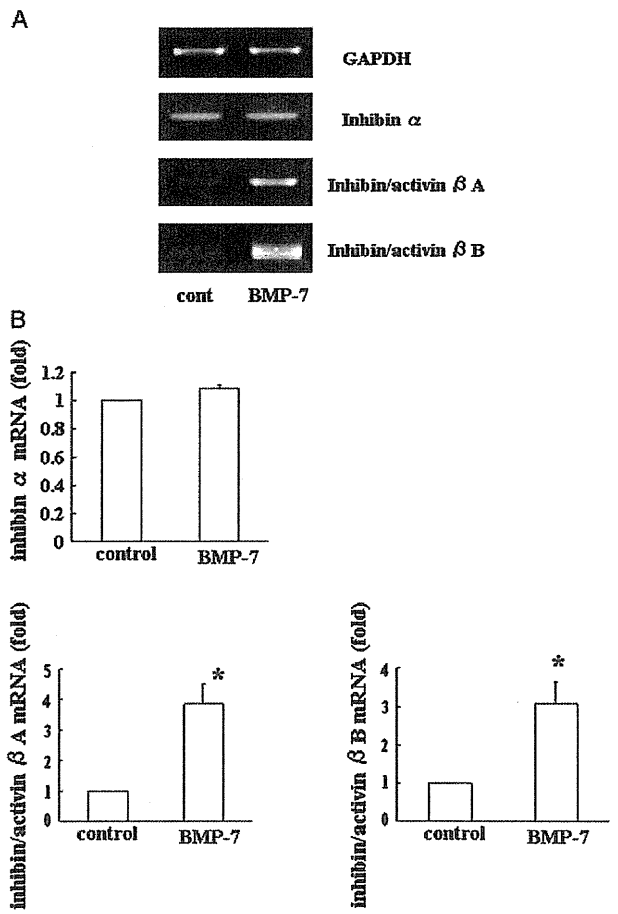
FIGURE 3

Effect of bone morphogenetic protein 7 (BMP-7) on the expression of inhibin/activin-α, inhibin/activin-βA, and inhibin/activin-βB mRNA. Human luteinized granulosa cells (LGCs) were cultured with or without BMP-7 (100 ng/mL) for 24 h. Total RNA was then extracted and subjected to (**A**) regular and (**B**) real-time polymerase chain reaction to determine the mRNA levels of inhibin/activin-α, inhibin/activin-βA, and inhibin/activin-βB. The expression levels of indicated mRNAs were standardized by GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. *Significant difference at $P<0.05$ (vs. control).



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(Fig. 5). Conversely, BMP-7 pretreatment had no effect on the production of cAMP by LGCs. However, BMP-7 pretreatment significantly enhanced the FSH-induced increase in cAMP levels in LGCs compared with nonpretreatment.

DISCUSSION

The BMP family members are important for folliculogenesis in many species, and there is a growing recognition that BMPs contribute to folliculogenesis by inhibiting luteinization of granulosa cells (12). In the present study, we found that BMP-7 induced FSH receptor (FSHR) mRNA expression in human granulosa cells, suggesting that BMP-7 may contribute to increasing FSH sensitivity of granulosa cells, thus promoting folliculogenesis. Our finding is consistent with that of Lee et al. (18), who, using mouse neonatal ovary, reported that BMP-7 increased FSHR mRNA. On the other hand, BMP-7 treatment inhibited expression of mRNA for LHR, a key factor required by granulosa cells to undergo luteinization (24). Furthermore, we found that the FSHR mRNA induced by BMP-7 resulted in an increase in functional FSHR, as indicated by the finding that FSH stimulated the production of cAMP in BMP-7–primed GCs compared with the control cells.

In the ovary, activins are recognized as important factors in the induction and maintenance of FSHR (7). Our observation that BMP-7 increased mRNA levels of not only FSHR but also inhibin/activin-β subunits (Figs. 1 and 3) led us to examine the possibility that the increase in FSHR mRNA might be mediated by an increase in activin protein synthesis. However, SB-431542, an inhibitor of activins but not BMP-7 signaling (23), failed to suppress BMP-7–induced FSHR mRNA expression (Fig. 4A). A possible explanation for this result

FIGURE 4

(**A**) Effect of SB-431542 on activin-A or bone morphogenetic protein 7 (BMP-7)–induced FSH receptor (FSHR) mRNA expression. Human luteinized granulosa cells (LGCs) were cultured with or without BMP-7 (100 ng/mL) or activin-A (100 ng/mL) in the presence or absence of SB-431542 (10 μmol/L), a selective inhibitor of activin receptor–like kinase 4, 5, and 7, for 24 h. Total RNA was then extracted from the LGCs and subjected to real-time polymerase chain reaction (PCR) to determine the mRNA levels of FSHR. Data were normalized to GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. Bars with different letters indicate a significant difference at $P<.05$. *Significant difference at $P<.05$. NS = not significant. (**B**) LGCs were cultured with BMP-7 (100 ng/mL) and/or activin-A (100 ng/mL) for 24 h, followed by real-time PCR to determine the mRNA levels of FSHR. Data were normalized to GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. Bars with different letters indicate a significant difference at $P\leq.05$ (b vs. c: $P\leq.02$; c vs. d: $P\leq.01$).

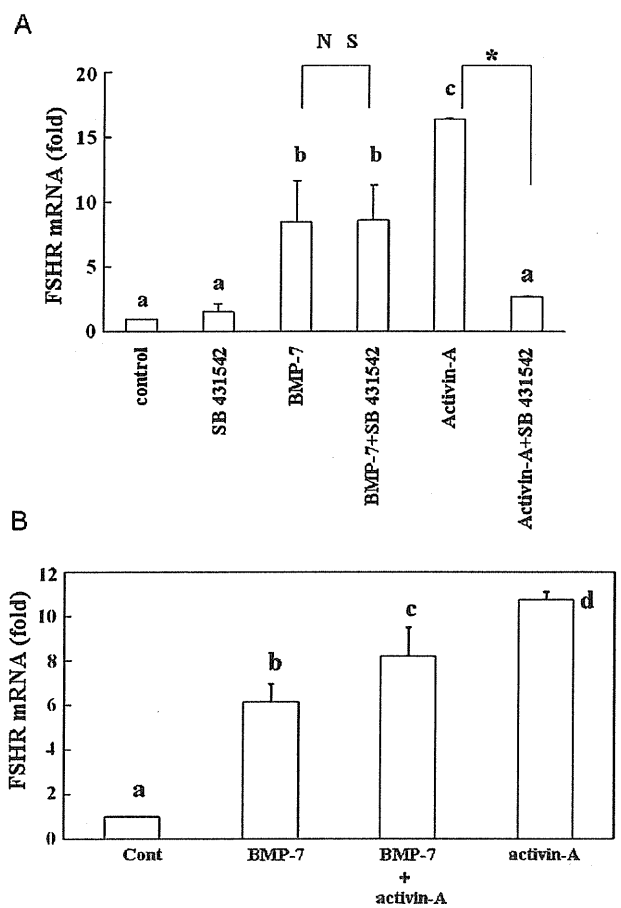

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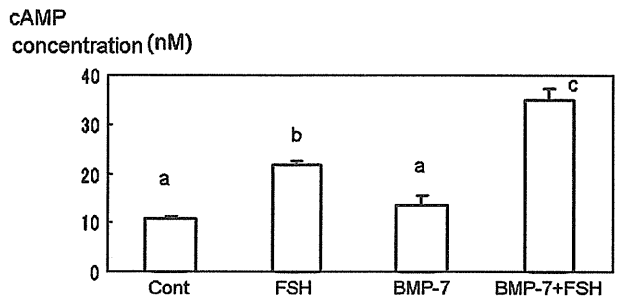
FIGURE 5

Effect of bone morphogenetic protein 7 (BMP-7) and FSH on cyclic adenosine monophosphate (cAMP) concentration. Human luteinized granulosa cells (LGCs) were cultured with or without BMP-7 (100 ng/mL) for 24 h. The cells were then cultured with 0.1 mmol/L IBMX in the presence or absence of FSH (0.5 IU/mL) for 2 h. The cAMP concentration in the supernatant was measured using a cAMP EIA kit. Data from one representative experiment out of three separate experiments was shown. Results shown are mean ± SD values from quadruplet experimental wells. Bars with different letters indicate a significant difference at $P<.01$ (a vs. b: $P\leq.01$; a vs. c: $P\leq.001$; b vs. c: $P\leq.01$).



Shi. BMP-7 increases FSH receptor. Fertil Steril 2010.

could be that BMP-7–induced inhibin/activin-β subunits are preferentially recruited to produce inhibin, dimerizing with an inhibin-α subunit that is known to be abundantly expressed in the mature GCs used in this study (25). Thus, it is speculated that BMP-7 and activin-A act on different receptors to increase FSHR mRNA expression. Interestingly, however, combination of BMP-7 and activin-A did not have an additive effect to increase FSHR mRNA expression. Bone morphogenetic protein 7 and activin-A may use redundant signaling pathways downstream of the point affected by SB431542.

Administration of FSH reagent is a standard method for infertility treatment, but many patients are unresponsive to this therapy. Thus, induction of FSHR in growing follicles would be a desirable therapy for infertility patients. Activins have the potential to serve this purpose, because they are recognized as a strong inducer of FSHR. However, because the majority of circulating activins can be bound and inactivated by follistatin (26–28), it appears to be difficult to stimulate ovarian follicles with exogenously administered activins. On the other hand, the relative affinity of follistatin for BMP-7 is less than 1% compared with activins, and the effect of BMP-7 on SMAD phosphorylation in granulosa cells is not reduced in the presence of high doses of follistatin (15). Therefore, BMP-7 would be more suitable than activins for therapeutic use. Notably, the possibility of BMP-7 administration as the new treatment for renal disease has been evaluated by many laboratories (29).

We also found that BMP-7 suppressed LHR expression. Growth and differentiation factor 9, another transforming growth factor (TGF) β superfamily member, also suppresses LHR expression in GCs (30). The present findings suggest that in human GCs, BMP-7 stimulation and inhibition of FSHR and LHR mRNA expression, respectively, may play a role in the course of follicle growth and maturation, in which the increased FSH sensitivity results in induction of folliculogenesis and the decrease in LH sensitivity results in inhibition of ovulation and luteinization. Pangas et al. (24) reported that in an ovarian conditional mouse knockout of *Smad4*, which is a common SMAD for TGF-β superfamily signaling, GCs undergo premature luteinization and express lower levels of FSHR and higher levels of LHR compared with control. Given that BMP-7 also uses the SMAD signaling pathway, the present data appear to be consistent with the description of the *Smad4* knockout mouse.

Abir et al. (17) reported that BMP-7 mRNA is detected only in theca cells of the human ovary, whereas BMP-7 protein is detected in oocytes, GCs, and theca cells. This discrepancy might be due to the cross-reactivity of BMP-7 antibody for other homologous proteins, such as BMP-6. In the present experiment, BMP-7 mRNA was not detected by PCR in granulosa cells, whereas BMP-6 mRNA was amplified abundantly (data not shown). These findings suggest that in human ovaries, as in sheep and mouse (12), BMP-7 expression is primarily localized to the theca cells.

Cultured LGCs used in the present study may not represent the stages of growing follicles. However, our findings that FSHR mRNA levels are clearly up-regulated by BMP-7 in human LGCs are new and open new insights into our understanding of FSHR regulation in the human ovary.

In summary, the present study demonstrated that BMP-7 increased the expression of FSHR mRNA in human GCs, while decreasing LHR mRNA expression. The effect of BMP-7 on FSHR mRNA expression was found to be independent of the effect of activins on FSHR expression. These findings indicate that BMP-7 may play a role in follicular maturation while inhibiting ovulation and luteinization in human ovary, and they identify BMP-7 as a potential treatment for human infertility in patients with a low response to FSH reagent.

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