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Effect of Monoclonal Antibody to Human Zone Pellucida 3 on Bone Morphogenetic Protein 15 Expression and Number of Preantral and Antral Follicles in Ovary of Mice

İnsan Zona Pellusida 3'e Karşı Oluşturulan Monoklonal Antikorun Kemik Şekillendirici Protein 15 ve Fare overinde Preantral ve Antral Folikül Sayısına Olan Etkisi

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Abstract

Introduction: Immunocontraception of zone pellucide (ZP3) is known causing disturbance of gap junction which inhibits transport growth factor such as bone morphogenetic protein (BMP-15). Decreased BMP-15 generates disturbance of proliferation and differentiation of follicle, granulosa, and techa cells. Anti-Human ZP3 monoclonal antibody with high specificity is being developed to prevent such effects. This study aimed to evaluate the effect of Mab-*h*ZP3 on the expression of BMP-15 and number of preantral and antral follicles in the ovary of mice.

Materials and Methods: This was true experiment post-test only control group design. Subjects involved were 48 mice (Mus musculus) that were divided into control (adjuvant) and treatment group (Mab-*h*ZP3 with dose of 20 µg, 40 µg, and 60 µg). In each group, a fraction of mice were killed at day 10, 15 and 20. Measurement of BMP-15 was performed with immunohistochemical, assessment, and a number of preantral and antral follicles was measured with hematoxylin and eosin.

Results: Overall, results showed that there was no significant difference in the effects of Mab-*h*ZP3 in a dose range of 20–60 µg on the expression of BMP-15 and number of preantral and antral follicles. A similar finding was also observed in the period of 10–20 days. Such results are suggested to be associated with the specificity of the monoclonal antibody.

Conclusions: Anti-Human ZP3 monoclonal antibody has no effect on decreasing expression of BMP-15 and number of preantral and antral follicles. It is considered as effective and safe immunocontraception.

Keywords: Antral follicle, BMP-15, monoclonal antibody, preantral follicle

Öz

Giriş: Zona Pellusida'ya (ZP) yönelik olarak bağışıklık sistemi ile yapılan doğum kontrolünde, şekillendirici (morfojenik) protein 15 (ŞP-15) gibi taşıma-büyüme faktörünün baskılanması ile gap-kavşaklarının bozulduğu gösterilmiştir. Düşük ŞP-15 foliküllerin, granüloza ve teka hücrelerinin çoğalmasının ve farklılaşmasının bozulmasına neden olur. İnsan ZP3'üne karşı oluşturulan ve yüksek özgüllüğü olan antikorlar, bu etkilerin oluşmasını engeller. Bu çalışma, insan ZP3'üne (iZP3) karşı oluşturulan monoklonal antikorların ŞP-15 ifadesine ve faredeki preantral ve antral foliküllerin oluşumuna etkisine olan etkisini araştırmayı amaçlamaktadır.

Gereçler ve Yöntemler: Bu çalışmada, gerçek deneyde, tetkikler sonrası sadece kontrol grubunun irdelemenmesi amaçlanmıştır. Denekler, 48 adet mus musculus ırkı fareler idi. Denekler, kontrol grubu (adjuvan) ve tedavi grubu olarak iZP3'üne karşı 20, 40 ve 60 µg dozlarda yüksek özgüllükte antikorun verildiği gruplar olarak ayrıldı. Her bir grupta bir grup denek, 10, 15 ve 20. günlerde öldürüldü ŞP-15 ölçümü, immünohistokimyasal yöntem ile yapıldı ve preantral ile antral foliküller, hematoksilen-eozin ile boyandıktan sonra sayıldı.

Bulgular: Bulgularımız, iZP3'üne karşı oluşturulmuş antikorun 20 ila 60 µg dozlarda verilmesinin ŞP-15 ifadesine, preantral ve antral foliküllerin sayısına herhangi bir etkisi olmadığını gösterdi. On-20 günlük süreler sonrasında da benzer sonuçlar elde edildi. Bu bulguların monoklonal antikorların özgüllüğü ile ilgili olduğu düşünülebilir.

Sonuçlar: İnsan ZP'sine karşı oluşturulmuş antikorların verilmesinin ŞP-15 ifadesi, preantral ve antral foliküllerin sayısına olan etkisinin olmadığı izlenmiştir. Bunun, etkin ve güvenli bir bağışıklık temelli doğum kontrolü sağladığı düşünülebilir.

Anahtar Kelimeler: Antral folikül, şekillendirici protein 15, ŞP-15, monoklonal antikor, preantral folikül

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Introduction

Population's growth rate is very a important factor to be controlled. Therefore, contraception is proposed to prevent its effects. Contraception is an attempt to regulate number and frequency of desired siblings. Furthermore, several alternatives are developed to prevent pregnancy. Contraception is expected to be reversible, safe, and effective. According to National Family Planning Coordinating Board (BKKBN), general contraception widely used nowadays is hormonal contraception, consisting of combination pills and Depot Medroxyprogesterone Acetate injections.[1]

Immunocontraception utilizing glycoprotein and zone pellucida 3 (ZP3) of mammals, in the presence of specific antibody binding to ZP3 antigen, is expected to inhibit fertilization and prevent damage.[2] The target of contraception is conception prevention between sperm and egg, called fertilization.[3] There are several major points in the process, one of which is sperm binding to zone pellucida (ZP) inducing acrosome reaction. Rankin et al. describe ZP to play a major role in the development of oocyte, fertilization, and early embryo development.[4] ZP is also responsible as a primary receptor to recognize sperm, and makes it a foundation in the development of antibody to be further studied and developed as contraception vaccine.

ZP3 antibody binds to the surface of oocyte ZP glucose causing sperm receptor closed which promotes early cortical reaction leading to toughening ZP and inhibition of sperm penetration into oocyte.[5] ZP3-antibody is previously reported effective to repress fertilization in several animals such as mice, rats, cats, and rabbits.[6–8]

Study of Pantiwati found that administration of passive immunization of anti *b*ZP3 in mice resulted in highest antibody titer in mice incubated for 63 days repress fertilization.[9] Mustofa conducted the administration of ZP3 from goat (*g*ZP3) resulting no abnormality in ovary and follicles between control and treatment group, as shown in primordial, primary, secondary, and graff follicles.[10] In contrary, Calongos et al. reported anti-ZP3 antibody resulting smaller follicle and antrum than normal.[11] In follicle culture with the presence of anti ZP3, attachment of granulosa to oocyte is found to be incomplete causing oocyte extrusion. Similarly, in a study of Paterson et al., it was found that active immunization of *h*ZP3 in Marmoset (*Callithrix jaccus*) caused ovary dysfunction indicated by inhibition of folliculogenesis and acceleration in decreased irreversible primordial follicles.[12]

However, data regarding ZP3 antigen in human remained low due to the limitation of oocyte used in the study.[13] Thus, Mubarakati et al. developed *human* Zone Pellucida 3 (Mab-*h*ZP3) monoclonal antibody derived from human blood serum as a potential candidate of immunocontraception.[14]

BMP-15, a growth factor, is responsible for the development of follicle. BMP-15 or GDF-9B is a family member of transformation growth factor (TGF-β) superfamily which plays a role in normal fertilization of mammals.[15] BMP-15 contributes in oocyte maturation and follicle development as homodimer and forming heterodimer along with GDF-9. BMP-15 expressed in the primary follicle of ovary shows BMP-15 transcription is translated in the early stage of folliculogenesis. BMP-15 expressed in follicles plays an important role in the recruitment of initial follicle, stimulating granulosa proliferation in preantral follicles through FSH-dependent mechanism.[16]

The monoclonal antibody of zone pellucida has been developed 30 years ago; yet studies regarding its effect on folliculogenesis are rarely done. Thus, further studies regarding the effect of Mab-*h*ZP3 expression of on BMP-15, and number of preantral and antral follicles in the ovary of mice are encouraged.

Materials and Methods

Animal preparation and treatment

The Mus musculus mice with a weight of 20–25 grams were acclimated and synchronized for 3 weeks. Mice were randomly chosen as 12 in the control group (injection of 50 µL Adjuvant Al (OH)3 + 50 µL Tris-HCl), and treatment group (injection of mAb anti-human-hZP3 with dose of 20 µg, 40 µg, and 60 µg). Each group was killed based on proestrus cycle 2, 3, and 4.

Measurement of BMP-15 expression

Measurement of BMP-15 expression on ovary refers to the semi-quantitative assessment of immunohistochemical evaluation according to Remmele score.

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Measurement of preantral and antral follicles number

The ovary was was stained and histopathological assessment was performed using hematoxylin and eosin (HE). Theca cells were counted manually.[17]

Results

Results of parametric precondition

There is a comparison result between normality and homogeneity (Table 1).

Expression of BMP-15 in mice oocyte

Figure 1 shows the expression of BMP-15 in the oocytes of *Mus musculus* mice.

Effect of Mab hZP3 on expression of BMP-15

The result showed that there was no significant effect of Mab-*h*ZP3 with observation time on BMP-15 expression (p=0.425, p>0.05). Shortly, there was no significant difference in expression of BMP-15 as the response of Mab-*h*ZP3 in various dose and time.

As shown in Figure 2, treatment group of Mab-*h*ZP3 with a dose of 60 µg (P3) resulted in the lowest average of BMP-15 expression, mostly in day 10. Administration of Mab-*h*ZP3 in various dose and time has no effect on expression of BMP-15 statistically significantly.

Table 1. Results of normality and homogeneity

Variable	Shapiro-Wilk		Levene	
	Coefficient	p-value	Coefficient	p-value
Expression of GDF-9	0.983	0.690	1.379	0.225
Theca cell number	0.964	0.152	1.243	0.296

Shapiro-Wilk and Levene test showed p>0.05 indicating data was normally distributed and homogeneous.

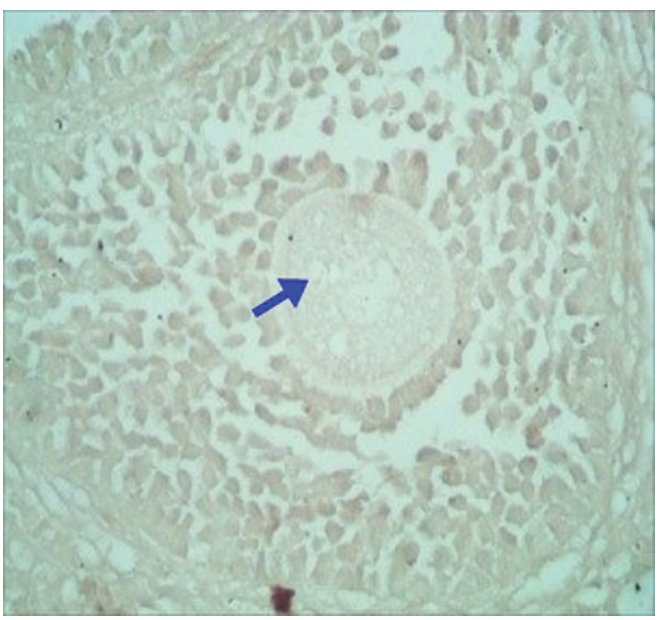
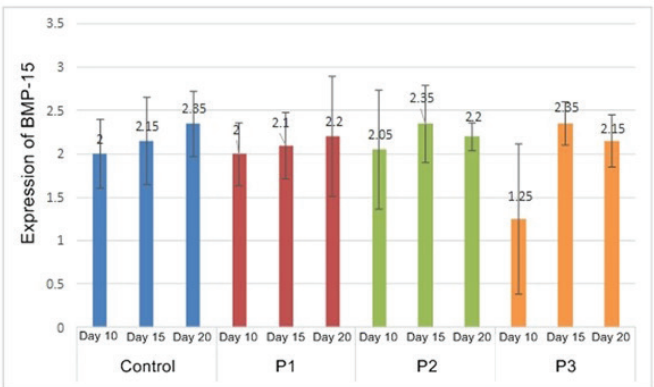
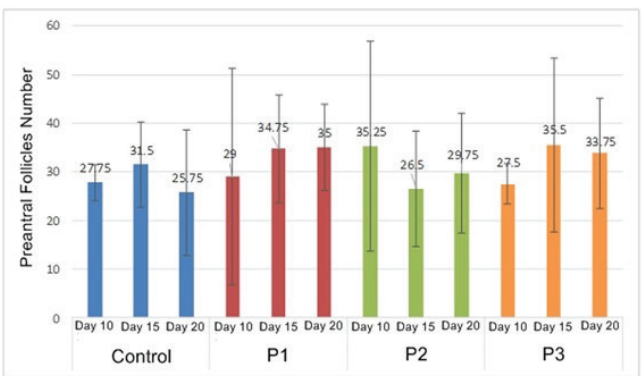


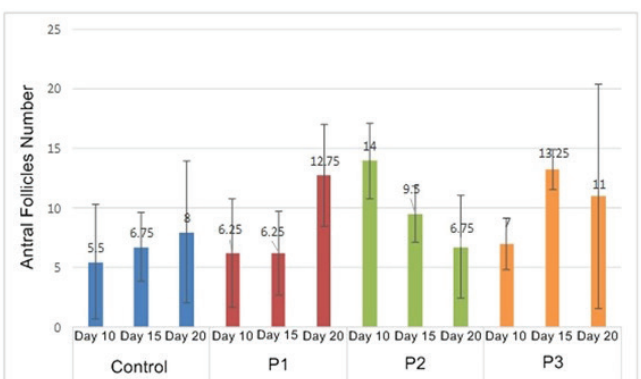
Figure 1. Expression of BMP-15 is shown in the presence of brown chromogen in the oocyte *(blue arrow)*.



(a)



(b)



(c)

Figure 2. a–c. Effect of Mab-*h*ZP3 in various dose and observation time on BMP-15 expression (a), effect of Mab-*h*ZP3 on number preantral follicles (b), and number of antral follicles (c).

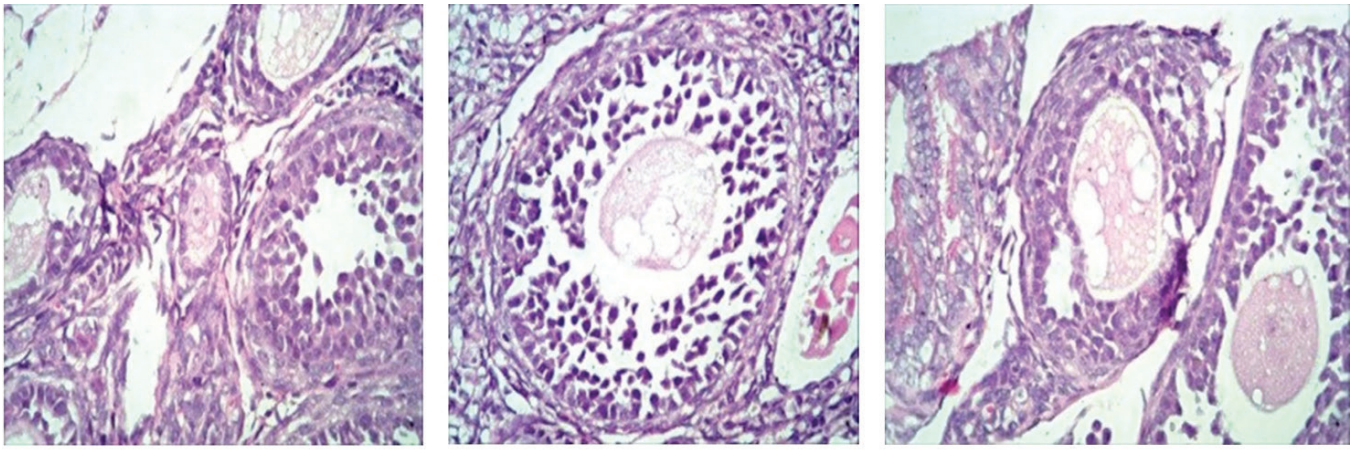


Figure 3. a–c. Characteristics of preantral follicle: primary follicle (a), secondary follicle (b), and antral follicle (c) (Hematoxylin-eosin staining).

Characteristics of preantral follicle

Figure 3 shows that the differentiation characteristics of the primary follicle and secondary follicle.

Effect of Mab-hZP3 on preantral follicles number

There was no significant effect of Mab-*h*ZP3 on observation time of preantral follicles (p=0.884, p>0.05). There was no significant difference in preantral follicles on the administration of Mab-*h*ZP3 in various dose and time. As shown in the histogram, control at day 20 showed the lowest average of preantral follicles. Exposure of Mab-*h*ZP3 in various dose increased a number of preantral follicles which peak at a dose of 60 µg (P3) in day 15. However, such an increase was not significant statistically (Figure 2).

Characteristics of antral follicle

Figure 3 shows the characteristic of the tertiary follicle.

Effect of Mab-hZP3 on antral follicles number

There was no significant effect of Mab-*h*ZP3 with observation time on antral follicles (p=0.563, p>0.05). There was no significant difference in antral follicles on the administration of Mab-*h*ZP3 in various dose and time. As shown in Figure 2, control showed quite a similar average of follicles to the treatment group of Mab-*h*ZP3. It is concluded that administration of Mab-*h*ZP3 in various dose and time has no significant effect on a number of antral follicles.

Correlation between preantral and antral follicles with BMP-15 expression

There was no significant correlation between BMP 15 expression with a number of preantral follicles in the presence of Mab-*h*ZP3 in various doses (correlation coefficient=0.069, p=0.643, p>0.05). There was no significant correlation between BMP 15 expression with number of antral follicles in presence of Mab-*h*ZP3 in various doses, confirmed by Pearson test (correlation coefficient=0.047, p=0.754, p>0.05), as well as its correlation with preantral follicles (coefficient correlation=0.188, p=0.202, p>0.05).

Discussion

BMP-15 or GDF-9B, both a member of transformation growth factor (TGF-β) superfamily, play a role in normal fertilization of mammals.[15] BMP-15 is expressed in oocyte and act through its receptor in granulosa. This protein is responsible promoting follicles growth, cumulus cells expansion, and expression of connexin 43 (Cx43) which are responsible for communication and promoting glycolysis in cumulus cells. The level of BMP-15 in follicles liquid is a potential factor in determining the quality of oocyte, implantation, and pregnancy in human.[15] This study showed that the administration of Mab-*h*ZP3 has no effect on expression of BMP-15. According to Barber et al., variable IgG ZP3 binds to the surface of ZP epitope, further promoting early cortical reaction causing ZP toughened which leads to inhibition of sperm penetration into the oocyte. Such an alteration might be caused by monospecificity of Mab-*h*ZP3.[5] A variety of amino acid sequence of ZP3 domain derived from B cell clone specifically recognizes one ZP3 epitope. Abbas et al.

described antibody with high specificity which recognize a different antigen with only single different amino acid. Shortly, antibody specificity is determined by a number of epitopes that the least recognizable epitope indicates its high specificity.[18]

ZP3-antibody of mice does not disrupt ZP synthesis. Stabilization of ZP is correlated to gap junction. Intact ZP leads to the proper performance of gap junction. Therefore, there is no disturbance of gap junction and GDF-9 transport from oocyte to granulosa promoting established folliculogenesis. Borillo et al. described that the conformation alteration of ZP depends on antibody binding to the development stage of follicles. When the antibody binds to oocyte in antral phase and ovulation, ZP structure remains. Otherwise, synthesis and secretion are disrupted when binding occurs in preantral.[19]

According to Barber et al., a difference of immunocontraception of ZP's effects is basically associated with antigen purity, animal susceptibility, adjuvant, and absence of T cell epitope used as immunogen.[5] Therefore, development of *h*ZP3 monoclonal antibody is proposed to prevent such a problem. In the *in-vivo* study, assessment of effect and period of a compound or drug immunoglobulin (antibody) depends on pharmacodynamic of materials.

Effect of Mab-hZP3 in various dose on preantral follicles

This study reported the effect of Mab-*h*ZP on preantral follicles development. Statistical analysis showed that there was no effect of *h*ZP3 monoclonal antibody on preantral follicles. The total average of preantral follicles in control and treatment group was not significantly different. Antibody specificity has no effect on follicles development. Specificity level does not generate cross reaction which reduces the inflammatory reaction.[18]

The results of the study showed that there was no significant effect of interaction between Mab-*h*ZP3 and time on preantral follicles. Shortly, there was no significant difference in preantral follicles in the administration of Mab-*h*ZP3 in various doses and times. In contrary, previous *in-vitro* study of Calongos et al. showed that the administration of ZP2 and ZP3-antibody in mice follicle culture caused disturbance of antral follicles development.[11] Average of antral follicle development was lower both in the treatment of ZP3-antibody (68.3%) and ZP2-antibody (63.3%), compared to control (93.3%).

The difference of Mab-*h*ZP3 dosage does not affect a number of preantral follicles in the treatment group. Study of Mustofa et al. demonstrated immunization of *g*ZP3 in mice with doses of 20 µg and 40 µg in Freund's adjuvant with a two-fold booster. Results showed that there was no abnormality detected in ovary and follicles.[10] There was no difference in the primordial, primary, secondary, tertiary, and de Graff follicles in control and treatment group. A similar finding that *m*ZP3 monoclonal antibody with microgram inhibits *in-vivo* and *in-vitro* fertilization was also reported in a study of East et al. The previous study showed that *m*ZP3 monoclonal antibody did not affect follicle and embryo development. Averages of small and medium follicles were similar.[20] Shortly, primary follicles were able to develop to secondary follicles when their recruitment and proliferation occur properly.

Effect of Mab-hZP3 on antral follicles

This study reported the effect of Mab-*h*ZP on antral follicles development. Statistical analysis showed that there was no effect of *h*ZP3 monoclonal antibody on antral follicles. The total average of preantral follicles in control and treatment group was not significantly different. Antibody specificity has no effect on follicles development. Specificity level does not generate cross reaction which reduces inflammatory reaction.[18]

ANOVA results showed that there was no significant effect of interaction between Mab-*h*ZP3 and time on antral follicles. Shortly, there was no significant difference in antral follicles in the administration of Mab-*h*ZP3 in various doses in different times.

As shown in Figure 2, control showed quite similar average of follicles treatment group of Mab-*h*ZP3. It is concluded that administration of Mab-*h*ZP3 in various doses in different times has no significant effect on a number of antral follicles.

In contrary, previous *in-vitro* study of Calongos et al. showed that the administration of ZP2 and ZP3-antibodies in mice follicle culture caused disturbance of antral follicles development. Average of antral follicle development was lower both in the treatment of ZP3-antibody (68.3%) and ZP2-antibody (63.3%), compared to control (93.3%).[11] However, it was found in a study of Mustofa et al. that immunization of *g*ZP3 in mice with dose of 20 µg and 40 µg in Freund's adjuvant with two-fold booster showed that there was no abnormality

detected in ovary and follicles.[10] There was no difference of primordial, primary, secondary, tertiary, and de Graff follicles in control and treatment groups.

Besides, the antral stage also depends on FSH and LH. FSH induces expression of *m*RNA receptor hormone luteinizing (Lhcgr) in mural cells required by follicles to respond LH in promoting ovulation. The absence of rLH inhibits the development of follicles to antral stage whereas FSH plays a role in forming antrum and preventing apoptosis. In the presence of LH, internal theca cells produce androstenedione, an androgen transferred to granulosa through basal lamina producing testosterone. FSH binding to its receptor increases the activity of aromatase causing androstenedione converted to estradiol.[21–23]

Correlation between preantral and antral follicles with BMP-15 expression

Signal of BMP-15 relies on receptor type I and II. BMP receptor type II (BMPRII) is the main receptor expressed in the oocyte, granulosa and theca cells.[24] BMP-15 specifically binds to kinase serine-threonine receptor type I and type II, and generate tetramer formation (2 type I and 2 type II each ligand). Permission of receptor type I by receptor type II to undergo phosphorylation on glycin-serin (GS) domain produces signal transduction. Receptor type I ALK 5 and ALK 6 respectively respond to phosphorylate R-Smads 2/3 and 1/5/8. Complex phosphorylation of R-Smads with Smad-4 (Co-Smads) is translocated to the oocyte. This mechanism activates follicle increasing proliferation, and cell continuity.[8]

Study of Wei et al., on the expression of GDF-9 and BMP-15 in oocyte developed in patients with PCOS, showed that GDF-9 and BMP-15 expression decreased significantly, and makes it unable to reach normal level.[25] Gode et al. found that status of GDF-9 and BMP-15 expression with the maturation of oocyte and embryo quality was significantly correlated.[26] However, there was no significant difference between embryo quality and status of GDF-9 and BMP-15. BMP-15 expressed in primary follicles of ovary shows that the transcription of BMP-15 was translated at the initial stage of folliculogenesis. BMP-15 expressed in follicles plays role in the recruitment of early follicles, stimulating granulosa proliferation in preantral follicles through the FSH-dependent mechanism. Administration of *anti-human* ZP3 monoclonal antibody has no effect on decreasing the expression of BMP-15 and number of preantral and antral follicles.

Statement of potential conflicts of interest
The authors have no conflict of interest to declared.

Ethics
Not applicable.

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