Research Article

Monoclonal antibodies of human zona pellucida 3 (Mab-*h*ZP3) as immunocontraception candidate on connexin expression 43 (Cx43) in granulosa ovarian cell and luteinizing hormone level in the blood serum of mice (*Mus musculus*)

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ABSTRAC T

Objective: To prove that the use of Mab-*h*ZP3 in various doses and observation time does not influence connexin expression 43 in granulosa ovarian cell and luteinizing Hormone (LH) rate on blood serum of mice (*Mus musculus*). **Materials and Methods:** The type of research method that is used is true experiment post-test only control group design. This research uses 48 mice that are classified into 12. They are control (adjuvant) and treatment groups (Mab *h*ZP3 20 µg, 40 µg, and 60 µg). The measurement of connexin 43 (Cx43) is conducted by applying immunohistochemistry method and LH rate with enzyme-linked immunosorbent assay. Data processing technique is using analysis of variance. **Results:** The giving of Mab *h*ZP3 with a dose range 20 µg–60 µg to Cx43 expression and LH rate does not show significant difference. **Conclusion:** The administration of Mab-*h*ZP3 various doses and observation time does not contribute any effect to Cx43 expression and LH concentration of serum mice (*M. musculus*).

KEY WORDS: Connexin 43, Immunocontraception, Luteinizing hormone, Monoclonal antibody, Population control

INTRODUCTION

Indonesia is one of developing countries contributing to the increase of population of the world. Based on the projection of statistical center Badan Pusat Statistik, the number of Indonesian people in the next 20 years is going to increase from 238.5 million in 2010 to 305.6 million in 2035.[1] Total fertility rate in Indonesia is also increase, is 2.60 in 2012 from 2.40 the previous year.[2] The globally high population will give negative impacts that will influence the quality of people's life. Environmental damage, depletion of natural resources, lack of water supply, and food availability, deterioration of health, and global warming are the impact of an increase of the population.

Population growth rate is significantly essential to be controlled; therefore, the role of a contraception is highly required. Ideal contraception should be safe, effective, practical, and reversible and has minimal side effects. The common contraception being used in Indonesia is hormonal contraception including combination of pill contraception and depot medroxyprogesterone acetate (DMPA) injection.[3] Unfortunately, the contraception has some weakness relating with the side effects. Combination of pill contraception, for example, has side effects of nausea, spotting bleeding, amenorrhea,[4] dizziness,[5] and the appearance of pimples.[6] Where the side effects of contraception of DMPA injection are irregular menstruation circle, bleeding, amenorrhea, change in body weight, headache, and stomach pain.[7] Those side effects surely cause inconvenience to the acceptors so that it is important to develop contraception method that has minimal side effects.

Alternative contraception method which is intensively developed is immunocontraception. The main focus of development of immunocontraception research by experts is Zona Pellucida (ZP3) because it is the primary receptor of introduction of sperm and

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oocyte.[8] General mechanism of immunocontraception with ZP as the major target is distracting or blocking carbohydrate and protein receptor on ZP surface so that it can cause closure of bonding site between sperm and ZP and complete comformational changes in the peptide chain resulting in receptors in the egg cell cannot be recognized by sperm.[9]

Immunocontraception effectiveness with ZP3 target has been tested by various researches both *in vitro* and *in vivo*. Paterson *et al*. have proved that antibody ZP3 can block the bonding between sperm and human ovum until 60%.[10] Bagavant *et al*. have also proved that female bonnet ape (*Macaca radiata*) that has been injected antibody ZP3 becomes infertile along with the increase of titer antibody anti-ZP3.[11] The research of Lou *et al*. and Millar *et al*. has also shown the same thing in which the giving of antibody ZP3 can cause infertility to mice.[12,13] However, the research dealing with immunocontraception has not produced a product that is ready to launch to the market. The main cause is the controversy of the safety and the side effects of using it. Calongos *et al*. reported that to likely preantral of mice cultured with anti-ZP3 can cause folliculogenesis disturbance.[14] Different result is stated by Bagavant *et al*. who concludes that female bonnet ape (*M. radiata*) that has gotten antibody ZP3 injection does not experience menstruation cicle disturbance. Furthermore, from laparoscopy diagnose, it can be seen that the ovary is still normal and there is a change of the number of follicles on every step of its development.[11]

The development of the immunocontraception method has used various kinds of antibody ZP3 of mammals both native and recombinant has been frequently conducted and has been proved that there is a reaction of interspecific antibody to ZP3 on mammals.[15] On the other hand, there is still limited data dealing with antibody ZP3 of human-caused by the limited availability of human oocyte for research.[16] Therefore, Mab-*h*ZP3 derived from human blood serum has been developed by Mubarakati *et al*.[17] As potential immunocontraception candidate. Moreover, so far there has not been any research about the side effect of administering Mab-*h*ZP3. Therefore, further research to find out the safety of administering Mab-hZP3 on folliculogenesis and hormone profile as a contraception candidate with various doses and observation time.

Folliculogenesis is closely related to Gap junction (GJ). GJ in ovary is mainly composed of connexin 43 (Cx43), which has important role in the growth and development of follicle and oocyte.[18] Interaction among cell facilitated by GJ affect hormone production and expression of growth factor on each follicle compartment (oocyte, granulosa, and theca cell).[19] Luteinizing hormone (LH) is one of important hormones in providing androgen substrate for synthetic estrogen which, in turn, will contribute to oocyte maturation and ovulation.

The different results of research about the effects of immunocontraception and the limited research developing Mab-*h*ZP3 have led to a thought about whether the administration of Mab-*h*ZP3 of various doses and observation time affects Cx43 expression and LH rate of mice (*Mus musculus*).

MATERIALS AND METHODS

Experimental Animal

Mice that had ever given birth weighing 20–25 g were acclimatized and synchronized for 3 weeks. They were randomly classified into 12 groups; control group (injected with 50 µL adjuvant aluminum hydroxide [Al(OH)3] + 50 µL Tris Hcl) and treatment group injected with Mab-*h*ZP3 doses 20 µg, 40 µg, and 60 µg using SC and terminated on the 8th day, 12th day, and 16th day on proestrus phase.

Measurement of Expression Cx43

Expression Cx43 was observed from granulosa cells of ovarian follicles then procedures of immunohistochemistry were performed with primary antibody catalog (Bioss, bs-0651R). The assessment of expression Cx43 refers to the assessment of semiquantitative of Remmele score.

Measurement of LH Levels

Measurement of LH level on blood serum of mice (*M. musculus*) was performed using the method of enzyme-linked immunosorbent assay (ELISA) with the kit used is mouse LH ELISA kit (ELABSCIENCE, USA).

Data Processing

The data processing technique used was an analysis of variance (ANOVA) in which first the normality and homogeneity were tested. All calculations are performed with software of SPSS for Windows 15.0.

RESULTS

Expression Cx43 on Granulosa Cells

Cx43 expression was observed in the cells of the ovary secondary granulosa follicles, and immunohistochemistry procedures were applied with primary antibody catalog (Bioss, bs-0651R) [Figure 1].

The Effect of Mab-*h*ZP3 Various Doses to Cx43 Expression

Based on the result of the analysis using ANOVA, $P = 0.125$ is greater than $\alpha = 0.05$, so that from this test, it can be concluded that there is no significant effect of administering Mab-*h*ZP3 various doses to Cx43 expression [Table 1].

The Effect of Mab-*h*ZP3 Various Times to Cx43 Expression

Based on the result of analysis using ANOVA, $P = 0.147$ is greater than $\alpha = 0.05$, so that from this test, it can be concluded that there is no significant effect of observation time of Cx43 expression on administering Mab-*h*ZP3 [Table 2].

The Effect of Mab-*h*ZP3 Various Doses and Observation Time to Cx43 Expression

Based on the histogram of the effect of Mab-*h*ZP3 various doses and observation time to Cx43 expression on picture 5.4 above, it shows that the control group has the lowest average of Cx43 expression, especially on the 12th day. Mab-*h*ZP3 administration of various doses increases Cx43 expression in which the highest average of Cx43 expression is obtained on a group of Mab-*h*ZP3 doses 20 µg (P1) administration with 16 days of observation; however, it is not a significant increase [Figure 2].

Measurement of LH Levels

Measurement of LH level on blood serum of mice (*M. musculus*) was conducted by using ELISA using mouse LH Elisa kit (ELABSCIENCE, USA). Based on the result of the analysis using ANOVA, $P = 0.101$ is greater than $\alpha = 0.05$, so that from this test, it can be concluded that there is no significant effect on giving Mab-*h*ZP3 various doses to LH level [Table 3].

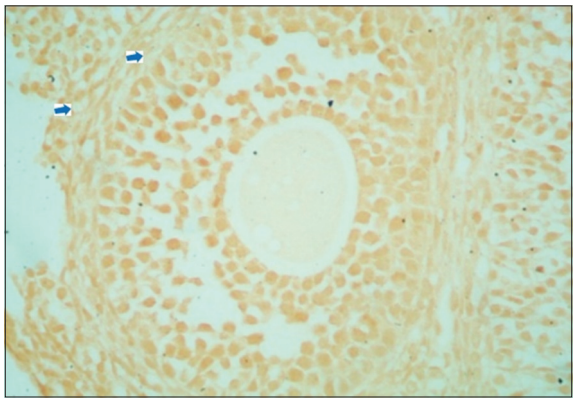

Figure 1: Connexin 43 expression is indicated by the presence of brown chromogen on granulose cells (blue arrow)

Table 1: The effect of Mab-*h*ZP3 various doses to C×43 expression

Mab-*h*ZP3	Mean±SD	*P*-value
Control	4.03±2.92	0.125
P1	6.63±2.64	
P2	6.32±3.24	
P3	5.97±2.05	

Table 2: The effect of Mab-*h*ZP3 various times to C×43 expression

Time	Mean±SD	*P*-value
8 days	4.89±2.78	0.147
12 days	5.48±2.6	
16 days	6.85±2.97	

The Effect of Observation Time to LH level

Based on the result of analysis using ANOVA, $P = 0.978$ is greater than $\alpha = 0.05$, so that from this test, it can be concluded that there is no significant effect of observation time to LH level on administering Mab-*h*ZP3 [Table 4].

The Effect of Mab-*h*ZP3 Various Doses and Observation Time to LH level

Based on the histogram of the effect Mab-*h*ZP3 interaction of various doses and observation time to LH level on picture 3, it shows that the control group has relatively the same average of LH level as all groups administering Mab-*h*ZP3 of various doses. The administration of Mab-*h*ZP3 of doses 40 µg (P2) and 60 µg (P3) decrease LH level and the lowest LH level is obtained on the group of Mab-*h*ZP3 doses 60 µg (P3) administration in 12 days of observation time; however statistically, it is not a significant decrease [Figure 3].

DISCUSSION

The Effect of Mab-*h*ZP3 Various Doses and Observation Time to Cx43 Expression

The result of the research shows that there is no significant effect of Mab-*hZP*3 of various doses and observation time to Cx43 expression. The control group has the lowest average of Cx43 expression, and the administration of Mab-*h*ZP3 various doses increases Cx43 expression in which the highest average is obtained on the administration group of Mab-*h*ZP3 doses 20 µg (P1) in 16 days of observation; however, it is not a significant increase statistically.

The result of this research is different from the result of research conducted by O'Leary *et al*. to female mice which have been immunized by cytomegalovirus mZP3 (mCMV-ZP3) in which there is a significant effect on the deterioration of mRNA Cx43 expression the condition causes the decrease of granulose cells and mature follicles followed by the increase of the number of immature follicles.[20] Calongos *et al*. in his research stated that preantral follicles of mice cultured with anti-ZP3 antibody, morphologically the ZP is thinner compared with the control group.[14] Therefore, it can be concluded that the anti-ZP3 antibody is suspected to have direct side effect to ZP structure so that it can ruin the development of normal GJ between oocyte and granulosa cells. Consequently, there is two-way communication damage required abnormal folliculogenesis. The same result has been obtained by Borillo *et al*. Peptida used to produce ZP3 antibody was injected to mice both *in vitro* and *in vivo*; it

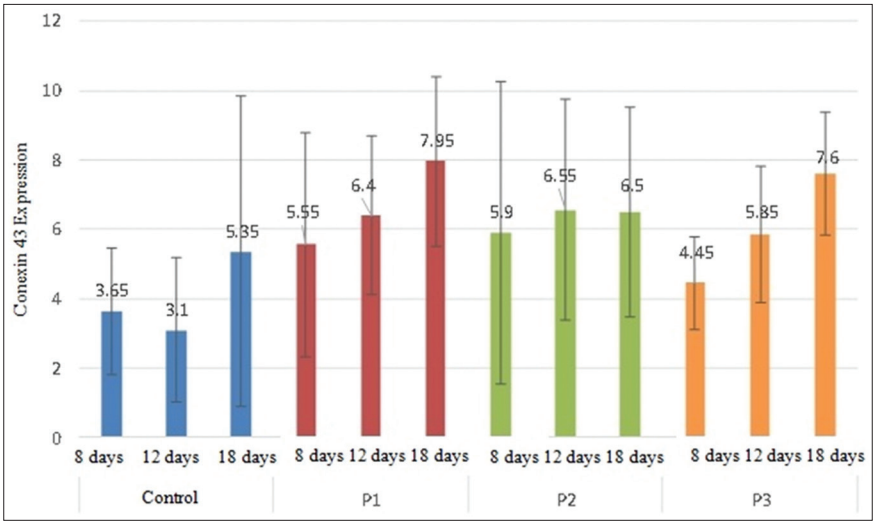

Figure 2: The effect of Mab-*h*ZP3 various doses and observation time to Cx43 expression

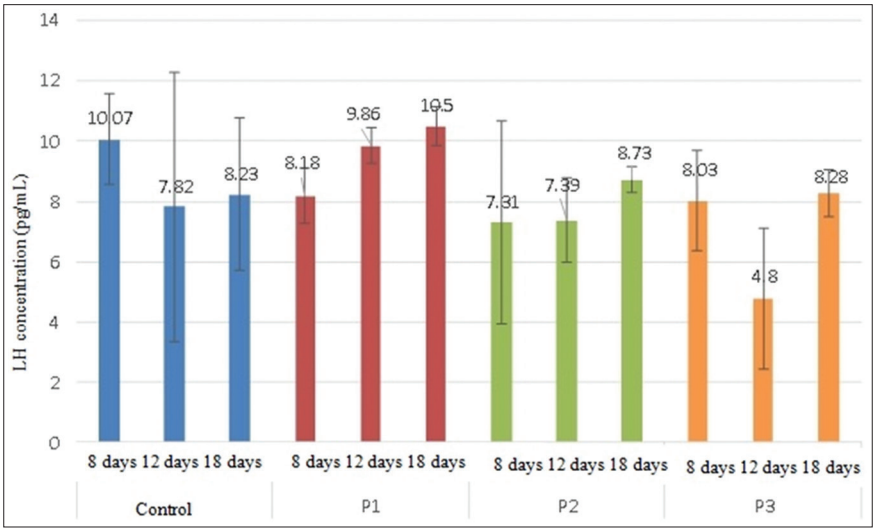

Figure 3: The effect of Mab-*h*ZP3 various doses and observation time to luteinizing hormone level

Table 3: The effect of Mab-*h*ZP3 various doses on luteinizing hormone level

Mab-hZP3	Mean±SD	*P*-value
Control	8.71±2.98	0.101
P1	9.51±1.22	
P2	7.81±2.02	
P3	7.04±2.27	

Table 4: The effect of observation time to luteinizing hormone level

Time	Mean±SD	*P*-value
8 days	8.4±2.12	0.978
12 days	7.47±3.00	
16 days	8.93±1.56	

shows that the number of GJ formed between oocyte and granulosa cells is less than that in the control group.[21] The same impact takes place in the process of transzona formation which has a role in GJ formation between oocytes and granulosa cells. The research of Lloyd *et al*. concludes that recombinant vaccine ZP3 given to female mice causes deterioration of transzona concentration and follicle damage.[22]

GJis intercellular membrane channel that specifically joins a group consisting of 10–10.000 plaques on the surface of the membrane[23] formed by the interaction of two opposite hemichannel (individual connection), in which each connection is formed by six protein connection.[24] Therefore, the damage and the deterioration of the number of connection expressions are Cx43. It is because connection expressions are regulated in various levels, including by degradation of GJ.[23] Cx43 also known as Gja1 is expressed in the compartment of granulose cells. The number of Cx43 GJ in each granulose cells increases along with the increase of follicle phase, reaching maximal number in the final follicular phase. The location of subcellular connection 43 in the space between oocytes and around cumulus cells indicates

that Cx43 GJ takes part in connecting transzona projections.[25]

The result of this research is different from other research above in which in this research the administration of Mab-*h*ZP3 various doses and observation time does not give any effect to Cx43 expression, in other words, the administration of Mab-*h*ZP3 does not give good effect on both the number and the damage of GJ so that ZP remains normal and undistracted. The basic materials used in the research are the same as those in the various researches above, which is ZP3. The difference is the process of the production in which in this research monoclonal *h*ZP3 are used whereas other research above use antigen and polyclonal antibodies. Although all research could prove the presence of infertility on mice immunized with the immunocontraception, the impact is significant enough both on folliculogenesis disturbance and ovary damage. Therefore, the risk of permanent infertility is feared.

A monoclonal antibody is a specific antibody as it has only one kind of epitope so that it can only identify specific antigen.[26] The high specificity of antibody will only react to one target cell. The monoclonal antibodies directly go to target cell that is ZP3 and affect the function of ZP3 as primary receptor of the introduction between spermatozoa and inducer of acrosome reaction. Polyclonal antibodies have a lot of introductory sites of epitope so that it lacks of specificity.[27] The specificity of monoclonal antibody has been proved by Bukovsky *et al*. who started that murine monoclonal antibodies (Mabs) ZP3 that have been produced show high specificity to human ZP3 protein.[28] Moreover, there is no cross-reaction of *Mabs*-ZP3 to the type of ovary cells and other tissues, such as endometrium, uterus, cervix, fallopian tube, and kidney. Monoclonal antibodies *h*ZP3 only recognize ZP3. The advantage possessed by Mab ZP3 is better specificity so that it is expected to be more effective and safer as one of the requirements in developing material of immunocontraception.

The stability of Cx43 expression on follicle granulose of mice after Mab-*h*PZ3 injection of various doses and observation time matches the explanation stated by Barber and Fayrer-Hosken about the immunocontraception mechanism after the administration of immunocontraception vaccine, there is a direct Immunoglobulin G (IgG) secretion into the follicles that are related to the formation of carbohydrate in the chain of glycans.[9] IgG produced is also related to ZP protein on the surface of pellucid zone. The bond between sperm and ZP which later can cause conformation change so that the sperm cannot recognize the receptor and fertility can be prevented.

Another manifestation of immunocontraception administration is the early induction of cortical reaction that can cause ZP hardening so that ZP becomes resistant toward sperm penetration.[9] The administration of Mab-*h*ZP3 can increase intracellular calcium rate can affect granular exocytosis located under plasma membrane which releases enzyme working on ZP and harden the ZP. East *et al*. in his research conclude that Mab-*m*ZP3 injection as immunocontraception can block fertility by playing a role as "specific block" of territory bonding with sperm on pellucid zone because ZP3 is the main receptor of sperm. Besides, the prevention of sperm penetration into ZP caused by oocyte activation and early cortical reaction occurs.[29]

Greenhouse *et al*. reported that the administration of Mab-*m*ZP3 of 0.8 mg in 0.5 ml liquid ascites can block fertility in 35–80 days after Mab-*m*ZP3 injection.[30] The same result has also been obtained from an experiment conducted by East *et al* that Mab-*m*ZP3 injection at 250 µg can contribute contraception effect in 40–80 days after injection. However, it is stated that the effectiveness is the same as 10 µg of dosage.[29]

The interesting result of this research is that the lowest average of Cx43 expression takes place in the control group and the administration of Mab-*h*ZP3 all doses increase Cx43 expression with the highest Cx43 expression takes place in the group of Mab-*h*ZP3 doses 20 µg (P1) administration with observation time of 16 days; however, statistically it is not a significant increase. The control group in this research was injected with adjuvant $Al(OH)_3$ in Tris HCl, whereas treatment group was injected with Mab-*h*ZP3 in Tris HCl and adjuvant $Al(OH)_3$. Adjuvant basically has a function to assist the effectiveness of antibody administration. The function of adjuvant among others is: (1) To store immunogen, in this case Mab-*h*ZP3 that can make the release runs slower; (2) as a carrier, and as a material that can improve vaccine quality.[27]

Various researches have stated that the use of $Al(OH)_3$ as adjuvant is safe. Bagavant *et al*. on his research to bonnet ape that was immunized with anti-ZP3 antibodies concludes that there is no ovary dysfunction in using alum as adjuvant.[11] The research of Upadhyay *et al*. which compares ovary morphology between the administration of colonization factor antigen and $Al(OH)_3$ as adjuvant, concludes that there is no ovary morphology damage on the ape injected with $Al(OH)_3$.[31] McKee *et al*. in his research of $Al(OH)_3$ conclude that the administration of $Al(OH)_3$ can activate natural immune respond, macrophage becomes active and produce a lot of cytokine proinflammatory.[32] When natural immune respond is stimulated leukocyte proinflammatory will get into ovary and cause apoptosis of granulose cells;[20]

however, the apoptosis can be blocked by extracellular matrix that is responsible to maintain the survival of the cells.[33]

The low average of Cx43 expression on the whole control group compared to the treatment group is possibly caused by the administration of alum independently so that it can activate natural immune response which can reduce Cx43 expression because of the role of macrophage and cytokine. However, the administration of $Al(OH)_3$ on control group does not give big impact because statistically it is not significant.

The Effect of the Administration of Mab-*h*ZP3 Various Doses and Observation Time to LH Level

The result of the test on the effect of Mab-*h*ZP3 various doses and observation time to LH level using ANOVA is $P = 0.618$, is greater than $\alpha = 0.05$; therefore, from this test, it can be concluded that there is no significant effect of administering Mab-*h*ZP3 various doses and observation time to LH level. The research is similar to the research conducted by Bagavant *et al.* in which it can be concluded that administration of pure anti-ZP3 antibodies to bonnet ape does not influence hormonal profile indicated with ovulation cycle of the experimental animal.[11] Although estradiol concentration of the animal decreases when antibody titer reaches its peak, there is no significant difference compared with the estradiol rate before it was immunized with pure anti-ZP3 antibodies. The hormonal profile which was observed every 2 weeks did not show any significant change. Each animal experienced an ovulatory cycle and infertile, and it did not experience menstrual cycle disorders. Besides, laparoscopy treatment shows that ovary remains normal and follicular development is not disturbed.

The research conducted by Keenan *et al*. shows the same result.[34] The administration of pure porcine ZP3 antigen to female rabbit does not give any negative effect on the endocrine profile in which there is no significant effect on LH rate compared with control of $P = 0.16$.

The research of both Bagavant *et al*. and Keenan *et al*. use the same level of ZP3 immunocontraception and *h*ZP3.[11,34] Mubarakati *et al*. stated that the production of *h*ZP3 has undergone a series of process which is expected to minimize the side effects.[35] Barber and Fayrer-Hosken stated that the side effects caused by immunocontraception depend on several factors, such as purity, adjuvant, sensitivity of experimental animal relating with immunity, and the existence of cell T epitope, and cell B of the ZP used as immunocontraception material.[9] Female dog immunized actively with pZP non purification experiences abnormal menstruation cycle, on the other hand, pZP immunization that has been purified does not cause any effect to menstruation cycle, in other words, the menstruation cycle remains normal.[36]

The ovulatory cycle experienced by experimental animal in Bagavant *et al*. research, indicates normal rates of LH hormone.[11] Physiologically, in the normal cycle of menstruation, follicle-stimulating hormone (FSH) induces LH receptor on pre-ovulation of granulose cells, allows LH to take over the function of FSH in the final phase of follicle maturation. LH receptor might also cause granulose cells become competent to respond LH increase that finally cause ovulation. The ovulation will occur when the threshold of LH concentration can be reached, not before the time. When the number of LH is too high, it can cause early luteinization and atresia follicles might occur leading to failure of reaching digraph follicles that can prevent ovulation. The low rate of LH level also can fail LH increase that can prevent ovulation.[37]

Specificity is very crucial for immunocontraception material besides its purification. The specificity of monoclonal antibodies has also been proved by Widodo and Aulanni'am in their research of the administration of rabbit anti-*b*ZP3.[38] After induction of *b*ZP3 could recognize ZP3 as its antigene. Sumitro *et al*. also stated that *b*ZP3dG monoclonal antibodies have specific characteristics in recognizing *b*ZP3dG molecule.[15] It is the specificity that causes Mab-*h*ZP3 will only react with the target of the ZP3 of mice. It has also been proved by the absence of effects Cx43 GJ compiler of this research.

GJ has a very significant role in the proliferation of granulose cells because it has a function of provider channel for molecular exchange and transport of tiny molecules among granulose cells,[39] including intraovarian factors.[37] Follicle development is characterized by the increase of the number of granulosa cells, starting from one layer in primary follicle phase to several layers in antral follicular phase. The lack of Cx43 number blocks follicle to develop multiplication layers of granulose cells dealing with the deterioration of oocyte development. Oocytes are morphologically abnormal, meiotically incompetent and could not be fertilized.[40] Follicles whose growth is inhibited could not reach certain diameter as qualification to become dominant follicle; thus, LH surge does not occur so that it can cause disturbance of anovulation cycle. This research shows that Mab-*h*ZP3 does not affect Cx43 expression. It is suspected that one of the causes is high specificity so that it could be explained why LH levels are also not directly affected.

CONCLUSION

The administration of Mab-*h*ZP3 various doses and observation time does not contribute any effect to Cx43 expression and LH concentration.

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AUTHORS' CONTRIBUTIONS

Conceptualization: FRI, SA. Data curation: FRI. Methodology: FRI, SA< KK. Writing – original draft: FRI. Writing – review and editing: FRI, SA, and KK.

REFERENCES

1. Statistical Center (BPS). Laporan Laju Pertumbuhan Penduduk Indonesia. Jakarta: Statistical Center; 2014.
2. Indonesian Demographic and Health Survey. Angka Fertilitas Total di Indonesia. Jakarta: Indonesian Demographic and Health Survey; 2012.
3. National Population and Family Planning Board. Profil Kesehatan Indonesia 2012. Jakarta: Indonesian National Family Planning; 2013.
4. Gallo MF, Nanda K, Grimes DA, Lopez LM, Schulz KF 20 microg versus >20 microg estrogen combined oral contraceptives for contraception. Cochrane Database Syst Rev 2008;4:CD003989.
5. Allais G, Gabellari IC, De Lorenzo C, Mana O, Benedetto C. Oral contraceptives in migraine. Expert Rev Neurother 2009;9:381-93.
6. Tyler KH, Zirwas MJ. Contraception and the dermatologist. J Am Acad Dermatol 2013;68:1022-9.
7. Kaunitz AM. Current concepts regarding use of DMPA. J Reprod Med 2002;47:785-9.
8. Gupta SK, Gupta N, Suman P, Choudhury S, Prakash K, Gupta T, *et al.* Zona pellucida-based contraceptive vaccines for human and animal utility. J Reprod Immunol 2011;88:240-6.
9. Barber MR, Fayrer-Hosken RA. Possible mechanisms of mammalian immunocontraception. J Reprod Immunol 2000;46:103-24.
10. Paterson M, Jennings ZA, van Duin M, Aitken RJ. Immunocontraception with zona pellucida proteins. Cells Tissues Organs 2000;166:228-32.
11. Bagavant H, Thillai-Koothan P, Sharma MG, Talwar GP, Gupta SK. Antifertility effects of porcine zona pellucida-3 immunization using permissible adjuvants in female bonnet monkeys (*Macaca radiata*): Reversibility, effect on follicular development and hormonal profiles. J Reprod Fertil 1994;102:17-25.
12. Lou Y, Ang J, Thai H, McElveen F, Tung KS. A zona pellucida 3 peptide vaccine induces antibodies and reversible infertility without ovarian pathology. J Immunol 1995;155:2715-20.
13. Millar SE, Chamow SM, Baur AW, Oliver C, Robey F, Dean J, *et al.* Vaccination with a synthetic zona pellucida peptide produces long-term contraception in female mice. Science 1989;246:935-8.
14. Calongos G, Hasegawa A, Komori S, Koyama K. Harmful effects of anti-zona pellucida antibodies in folliculogenesis, oogenesis, and fertilization. J Reprod Immunol 2009;79:148-55.
15. Sumitro SB, Aulanni'am A, Ciptadi G, Soewarto. Produksi dan uji spesifitas antibodi monoklonal terhadap bzp3 terdeglikosilasi (mab-bzp3dg) untuk pengembangan imunokontrasepsi wanita. J Ilmiah Kedokt Hewan 2011;4:1-4.
16. Chiu PC, Wong BS, Lee CL, Pang RT, Lee KF, Sumitro SB, *et al.* Native human zona pellucida glycoproteins: Purification and binding properties. Hum Reprod 2008;23:1385-93.
17. Mubarakati NJ, Aulanni'am A, Sumitro SB, Ciptadi G. Bovine and human zona pellucida 3 gene glycans site prediction using *in silico* analysis. J Trop Life Sci 2014;4:206-9.
18. Sasseville' M, Gagnon MC, Guillemette C, Sullivan R, Gilchrist RB, Richard FJ, *et al.* Regulation of gap junctions in porcine cumulus-oocyte complexes: Contributions of granulosa cell contact, gonadotropins, and lipid rafts. Mol Endocrinol 2009;23:700-10.
19. Palma GA, Argañaraz ME, Barrera AD, Rodler D, Mutto AÁ, Sinowatz F, *et al.* Biology and biotechnology of follicle development. ScientificWorldJournal 2012;2012:938138.
20. O'Leary S, Lloyd ML, Shellam GR, Robertson SA. Immunization with recombinant murine cytomegalovirus expressing murine zona pellucida 3 causes permanent infertility in BALB/c mice due to follicle depletion and ovulation failure. Biol Reprod 2008;79:849-60.
21. Borillo J, Coonrod SA, Wu J, Zhou C, Lou Y. Antibodies to two ZP3 B cell epitopes affect zona pellucida assembly. J Reprod Immunol 2008;78:149-57.
22. Lloyd ML, Papadimitriou JM, O'Leary S, Robertson SA, Shellam GR. Immunoglobulin to zona pellucida 3 mediates ovarian damage and infertility after contraceptive vaccination in mice. J Autoimmun 2010;35:77-85.
23. Vinken M, Vanhaecke T, Papeleu P, Snykers S, Henkens T, Rogiers V, *et al.* Connexins and their channels in cell growth and cell death. Cell Signal 2006;18:592-600.
24. Nielsen MS, Axelsen LN, Sorgen PL, Verma V, Delmar M, Holstein-Rathlou NH, *et al.* Gap junctions. Compr Physiol 2012;2:1981-2035.
25. Teilmann SC. Differential expression and localisation of connexin-37 and connexin-43 in follicles of different stages in the 4-week-old mouse ovary. Mol Cell Endocrinol 2005;234:27-35.
26. Putra A. Konsep Elektroforesis-western Blot dan Immunostaining Dalam Pendeteksian Ekspresi Protein Intraseluler. Workshop Laboratory Skill on Immunohistochemistry (IHC/ICC) and Western Blot. Semarang: Unisula Press; 2012. p. 81-104.
27. Howard GC, Bethell DR. Basic Methods in Antibody Production and Characterization. Washington DC: CRC Press; 2001.
28. Bukovsky A, Gupta SK, Bansal P, Chakravarty S, Chaudhary M, Svetlikova M, *et al.* Production of monoclonal antibodies against recombinant human zona pellucida glycoproteins: Utility in immunolocalization of respective zona proteins in ovarian follicles. J Reprod Immunol 2008;78:102-14.
29. East IJ, Gulyas BJ, Dean J. Monoclonal antibodies to the murine zona pellucida protein with sperm receptor activity: Effects on fertilization and early development. Dev Biol 1985;109:268-73.
30. Greenhouse S, Castle PE, Dean J. Antibodies to human P3 induce reversible contraception in transgenic mice with "humanized" zonae pellucidae. J Hum Reprod 1999;14:593-600.
31. Upadhyay SN, Thillaikoothan P, Bamezai A, Jayaraman S, Talwar GP. Role of adjuvants in inhibitory influence of immunization with porcine zona pellucida antigen (ZP-3) on ovarian folliculogenesis in bonnet monkeys: A morphological study. Biol Reprod 1989;41:665-73.
32. McKee AS, Munks MW, MacLeod MK, Fleenor CJ, Van Rooijen N, Kappler JW, *et al.* Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. J Immunol 2009;183:4403-14.
33. Krysko DV, Mussche S, Leybaert L, D'Herde K. Gap junctional communication and connexin43 expression in relation to apoptotic cell death and survival of granulosa cells. J Histochem Cytochem 2004;52:1199-207.
34. Keenan JA, Sacco AG, Subramanian MG, Kruger M, Yurewicz EC, Moghissi KS, *et al.* Endocrine response in rabbits immunized with native versus deglycosylated porcine zona pellucida antigens. Biol Reprod 1991;44:150-6.
35. Mubarakati NJ, Aulanni'am A, Sumitro SB, Ciptadi G. Immunogenicity and characterization of antibodies against to recombinant human zp3 for development woman immunocontraception. J Biosci Biotechnol Res Asia

2015;12:243-8.
36. Bamezai AK, Mahi-Brown CA, Talwar GP. Inhibition of penetration of canine zonae pellucidae by homologous spermatoza *in vitro* using monoclonal antibodies raised against porcine zonae. J Reprod Immunol 1988;13:85-95.
37. Strauss FJ, Williams JC. The ovarian life cycle. In: Strauss JF, Barbieri RL, editors. Yen and Jaffe's Reproductive Endocrinology; Physiology and Clinical Management. 7th ed. Churchill Livingstone: Elsevier; 2014. p. 213-53.
38. Widodo E, Aulanni'am A. Spesifitas antibodi bovine zzona pellusida 3 (anti-bzp3) terhadap zp3 kelinci berbasis bzp3 sebagai antigen kontraseptif. J Hasil Riset 2005;5:182-7.
39. Ackert CL, Gittens JE, O'Brien MJ, Eppig JJ, Kidder GM. Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. Dev Biol 2001;233:258-70.
40. Kidder GM, Mhawi AA. Gap junctions and ovarian folliculogenesis. Reproduction 2002;123:613-20.

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