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Effect of Monoclonal Antibody to Human Zona Pellucida 3 in Luteineizing Hormone Receptor Expression, Estradiol Levels and de Graaf Follicles Quantity in Mice

İnsan Zona Pellusidasına Karşı Oluşturulmuş Antikorların Farelerde Luteinizan Hormon Reseptörü İfadesi, Estradiol Seviyesi ve De Graaf Folikülleri Sayısına Etkisi

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Abstract

Objective: This research aimed to evaluate effect of monoclonal antibody to human Zona Pellucida 3 (Mab-*h*ZP3) on luteineizing hormone (LH) receptor expression, estradiol levels and de Graaf follicles quantity of *mus musculus* mice ovaries.

Materials and Methods: True experiment post test only control group design was used as research method. Treatments used were control (adjuvant in Tris HCl), and Mab-*h*ZP3 with dosages of 20 µg, 40 µg and 60 µg which tested on day 10, 15 and 20. Luteineizing hormone (LH) receptor, estradiol level and the number of de Graaf follicles measurement were done by immunohistochemical test, ELISA method, and hematoxilin eosin staining, respectively.

Results: The results showed that there was no significant difference found on Mab-*h*ZP3 interaction between 20 µg-60 µg dosages and 10–20 days of observation time towards LH receptor expression, estradiol level and and the number of de Graaf follicles. This was related to specificity of monoclonal antibody used.

Conclusion: Mab-*h*ZP3 did not lower LH receptor expression, estradiol level and the number of de Graaf follicles quantity, which means that Mab-*h*ZP3 did not interfere at folliculogenesis and not altering hormones profile. It can be concluded that Mab-*h*ZP3 has the potential to be a safe immnocontraception material.

Keywords: de Graff follicles, estradiol, immunocontraception, luteineizing hormone, Mab-*h*ZP3.

Öz

Giriş: Bu çalışmanın amacı, insan zona pellucidasına karşın oluşturulmuş antikorların (Mab-*h*ZP3), luteinizan hormon (LH) reseptörü, estradiol seviyesi ve mus musculus farelerinde de Graaf foliküllerinin sayısına olan etkisini araştırmaktır.

Gereçler ve Yöntemler: Çalışma, kontrol grubunun olduğu, işlem sonrası ölçümlerin yapıldığı yöntem ile yapıldı. Kontrol grubuna Tris HCl içinde adjuvan verilir iken, çalışma gruplarına sırası ile 20 µg, 40 µg ve 60 µg dozlarında Mab-*h*ZP3 verildi ve 10 ve 20. günlerde ölçüm yapıldı. LH reseptörü, estradiol seviyesi ve de Graaf foliküllerinin sayıları sırası ile immünohistokimyasal test ile ELISA metodu kullanılarak ve hemotoksilen-eozin boyaması ile saptandı.

Bulgular: Ölçümler, 20 ila 60 µg arasındaki dozlarda uygulanan Mab-*h*ZP3'ün 10-20 günlük gözlemlerde LH reseptörü ifadesini, estradiol seviyelerini ve de Graaf folikülü sayılarını değiştirmediğini gösterdi. Bu durum, kullanılan monoklonal antikorun özgüllüğü ile ilişkili idi.

Sonuç: Mab-*h*ZP3 LH reseptörü, estradiol seviyelerini ve de Graaf folikülü sayılarını düşürmez ve böylece folikül oluşumu ve hormon düzeylerini etkilemez. Bu bulgulara göre, Mab-*h*ZP3'ün güvenilir bir bağışıklık temelli doğum kontrolü yöntemi olduğu belirtilebilir.

Anahtar Kelimeler: de Graaf folikülleri, estradiol, bağışıklık temelli doğum kontrolü, lüteinizan hormon, Mab-*h*ZP3

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Introduction

World population increases for an average of 5 million people each year, it has exceeded for 6.43 × 10^9 and increases 1 × 10^9 for every 12 years.[1] In Indonesia, family planning programs have been implemented to overcome the high rate of population growth. It has been found that 38.25% of married couples in reproductive age, do not use contraception either traditional or modern with variety of reasons, such as discomfort and fear of the contraceptives side effects available.[2]

For some women, hormonal methods and IUD choice may be contraindicated due to medical conditions and undesirable side effects such as decreased bone density, cardiovascular disorders, ovarian cancer, breast cancer and pelvic infections. While barrier method requires consistency and commitment usage to prevent pregnancy.[2] To overcome this problem, some researchers develop new contraceptive method based on natural immune system in reproductive tract which called immunocontraception.

One of the immunocontraceptives has utilized zona pellucida (ZP), an extra-cellular matrix, consists of glycoproteins, which surrounds the mammalian oocyte. This matrix is responsible for cell-to cell communication between oocyte and follicle, protect the development of oocytes and embryos as well as managing fertilization process when sperm binding to oocyte for their specific glycoprotein on its surface that acts as receptor for interaction with spermatozoa, which causes the egg not to be recognized by it.[1,3] Zona Pellucida 3 plays an important role in fertilization process as the primary receptor and induces acrosome reaction in spermatozoa.[4] Based on ZP3 role, researchers continued to develop glycoprotein and anti-ZP3 antibodies to assess its effectiveness as candidates for contraception in preventing fertilization and its influence on ovaries.[5]

Earlier research about immunocontraceptive potential of anti-ZP3 still raises some debate to identify the side effects of anti-goat ZP3 on immunocontraception and ovarian histological structure of mice. The results showed that there were no difference in ovarian structure between control and treatment groups, which indicated that anti-ZP3 immunization does not interfere with ovary.[6] Unlike other research which aimed to evaluate mice follicle that were pre-antrally cultured with anti-ZP3 antibody, these antibody were able to interfere mice follicle development and oocytes in vitro, it also decreased the formation of antral follicles, granulosa cells development, oocyte maturation.[7] Prolonged immunization with ZP3 antigen resulted in an increase of anti-ZP antibody titre which has proved to cause abnormal hormonal profile and resulting in estrous cycle change in rabbits, dogs, horses, and changes in primates menstrual cycle.[8] Hormonal changes caused by long-term immunization can affect ovarian function and it may lead to infertility.[8]

Related data about human anti-ZP3 antibody as well as different findings regarding human oocytes during immunocontraception[9] led to isolate human ZP3 to develop anti-human ZP3 (Mab-*h*ZP3).[10]

Methods

Research Design

The research design used was true experimental posttest only with control group.

Experimental Diets and Animal Treatment

This study was conducted in Laboratory of Experimental Animal Cage Unit of Faculty of Veterinary Medicine, Airlangga University Surabaya and Physiology Laboratory of Faculty of Medicine, Brawijaya University Malang, Indonesia. The in vivo experiment was conducted on 3–3.5 week old Balb/c non-pregnant female mice (weighted at least 20–25 gr) which obtained from Animal Husbandry Department, East Java Province and maintained in pathogen-free facility They were fed *ad libitum*. Forty-eight mice were then divided into 12 treatment groups:

1. The first group (K1) was a control group in which mice were injected with 50 µL adjuvant Al $(OH)_3$ (aluminum hydroxide) solution in 50 µL Tris HCl and then were sacrificed on the day 10.

2. The second group (K2) was a control group which injected with 50 µL adjuvant Al $(OH)_3$ (aluminum hydroxide) solution in 50 µL Tris HCl and then they were killed on day 15.

3. The third group (K3) was a control group. Mice were injected with 50 µL adjuvant Al $(OH)_3$ solution in 50 µL Tris HCl and then they were killed on the day 20.

4. In the Forth group(K4) mice were given monoclonal antibody 20 µg Mab-hZP3 then were terminated on the day 10.

5. In Group K5 mice were given Mab-hZP3 at a dose of 20 µg. These mice were sacrificed on the 15th day.

6. K6: Mice were injected 20 µg MabhZP3. Group was terminated on day 20.

7. K7: 40 µg of Mab-hZ3 was injected. Mice were killed on 10th day.

8. K8: 40 µg Mab-hZP3 was given to mice. The group was sacrificed on 15th day.

9. K9: Mice were given Mab-hZP3 at a dose of 40 µg. They were killed on 20th day of the experiment.

10. K10: 60 µg Mab-hZP3 was injected. Mice were sacrificed on day 10.

11. The eleventh group (K11) was the treatment group in which the mice were injected with Mab-hZP3 at a dose of 60 µg and then they were terminated on the day 15.

12. K12: 60 µg of Mab-hZP3 was given. The mice were sacrificed on 20th day.

The experimental protocol was approved by Research Ethics Committee (Animal Care and Use Committee) of University of Brawijaya. No. 606/EC/KEPK/12/2015.

The sacrification of mice and blood collection were performed on day 10, 15 and 20 of the proestrus phase to measure the levels of estradiol. Ovaries were also isolated on day 10, 15 and 20 to measure the expression of LH receptor and de Graaf follicle numbers.

LH receptor expression measurement

LH receptor expression measurement was done tertiary follicles, ovarian theca cells and they were analyzed using immunohistochemical procedures. Assesment of LH receptor expression was performed according to Remmele semiquantitative assessment score.

Estradiol level measurement

Blood samples were collected from ventricle and serum was separated using centrifugation at a rate of 3000 rpm for 15 minutes at room temperature. Estradiol levels were determined by ELISA method.

The number de Graaf follicles

Mature and dominant follicles quantity which are ready to be ovulated on antral phase were measured using haematoxylin and eosin staining followed by histopathology examination.

Data Analysis

One-way ANOVA was used to analyze the data. The differences between groups were considered significant when p value was smaller than 0.05. All results were presented as the mean ± standard deviation (SD) values of 3 mice in each group. This was followed by a post-hoc Tukey's test.

Results

LH Recep Expression in Different Doses of Mab-zZP3

Control group has the lowest average value of LH receptor expression. However, there was no statistically significant difference (p=0.290) in terms of LH receptor expression in different doses. However, the expression increased to some extent (Table 1).

Table 1. Effect of Mab-*h*ZP3 dosage variety against LH receptor expression

Mab-*h*ZP3	Mean ± SD	*p*-value
Control (K1, K2, K3)	4.18±2.56	0.290
20 µg of Mab-hZP3 (K4, K5, K6)	5.54±2.36	
40 µg of Mab-hZP3 (K7, K8, K9)	5.32±1.12	
60 µg of Mab-hZP3 (K10, K11, K12)	4.72±1.65	

ZP3 immunocontraception significantly affected histology of the ovarium (Figure 1, 2 and 3). Zona pellucida (ZP) appeared thin, transparent, loose, swollen, and partially dissolved, there was gap obtained between ZP-oocyte, ZP-granulosa cells or between granulosa cells.

In addition, decrease in expression of GDF-9, NOBOX and Gjal with rMCMV *m*ZP3 immunization will produce abnormal granulosa cells with increased expression of Kit Ligand and failed to recruit theca cells.[12,13] Oocytes cells, granulosa cells and theca cells is a major component of follicle functional and interaction among them plays an important role in steroidogenesis, follicular development, and atresia.[14] Disturbance of cell proliferation and differentiation of granulosa and theca cells will decrease expression of LH receptors.[15]

LH receptor expression which did not differ significantly with various doses of Mab-*h*ZP3 might probably not bind to all structural protein in zona pellucida but only bind to ZP3.[16]

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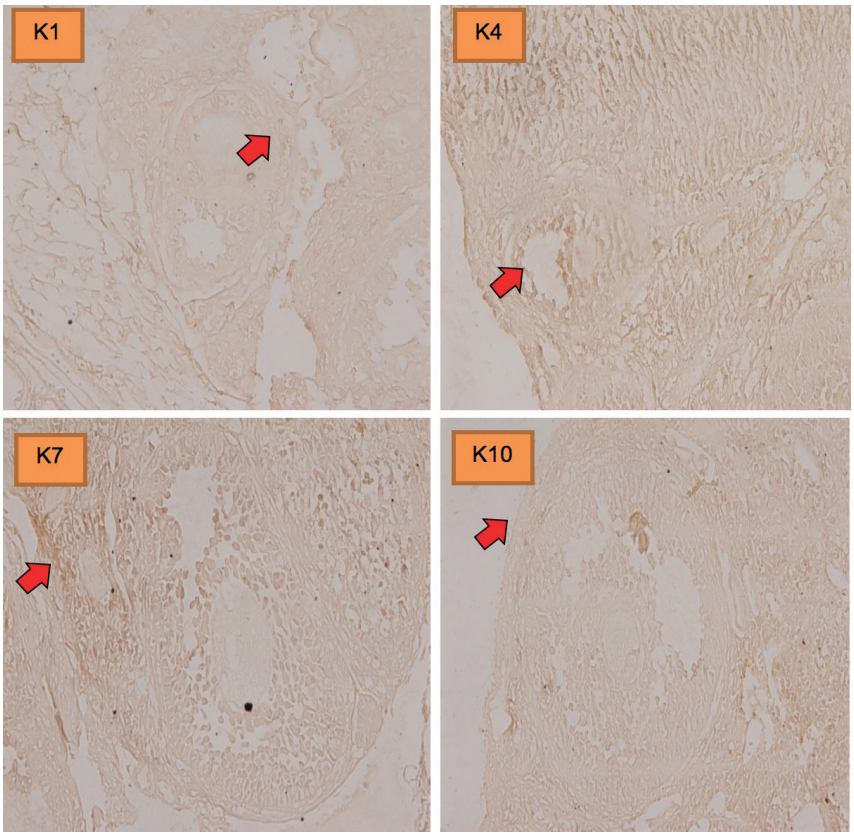

Figure 1. LH receptor expression on tertiary follicular theca cells on day 10. (K1=control group exposed to Al $(OH)_3$ 50 µl in Tris HCl 50 µl, K4=the treatment of Mab-hZP3 at a dose of 20 µg, K7=the treatment of Mab-hZP3 at a dose of 40 µg, K10=the treatment of Mab-hZP3 at a dose of 60 µg). Expression of LH receptor was characterized by chromogenic brown (arrow) on tertiary follicular theca cells. The results showed that the Immuno Reactive Score (IRS) expression of LH receptors on tertiary follicular theca cells in the K1 (control) group was lower than that of the IRS of the K4, K7 and K10 treatment groups. LH receptor expression was noted using immunohistochemical staining with 400x magnification using Nikon H600L microscope equipped with Fi2 300 megapixel DS camera.

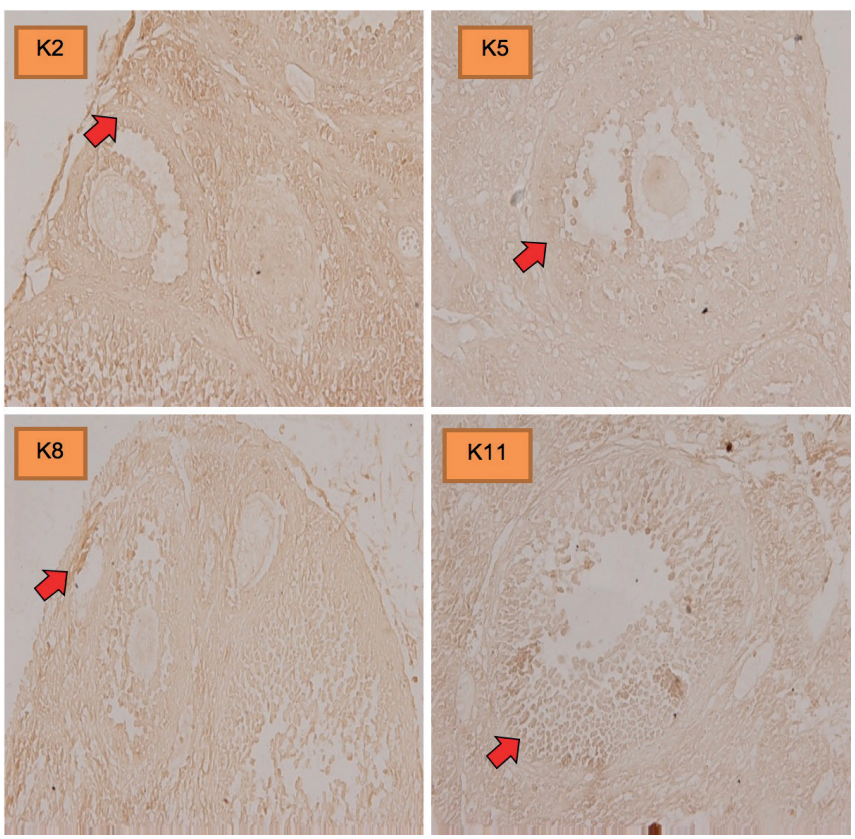

Figure 2. LH receptor expression on tertiary follicular theca cells which observed on day 15. (K2=control group exposed to Al $(OH)_3$ 50 µl in Tris HCl 50 µl, K5=the treatment of Mab-hZP3 at a dose of 20 µg, K8=the treatment of Mab-hZP3 at a dose of 40 µg, K11=the treatment of Mab-hZP3 at a dose of 60 µg). Expression of LH receptor was characterized by chromogenic brown (arrow) on tertiary follicular theca cells. The results showed that the Immuno Reactive Score (IRS) expression of LH receptors on tertiary follicular theca cells in the K2 (control) group was lower than that of the IRS of the K5, K8 and K11 treatment groups. LH receptor expression was noted using immunohistochemical staining with 400x magnification using Nikon H600L microscope equipped with Fi2 300 megapixel DS camera.

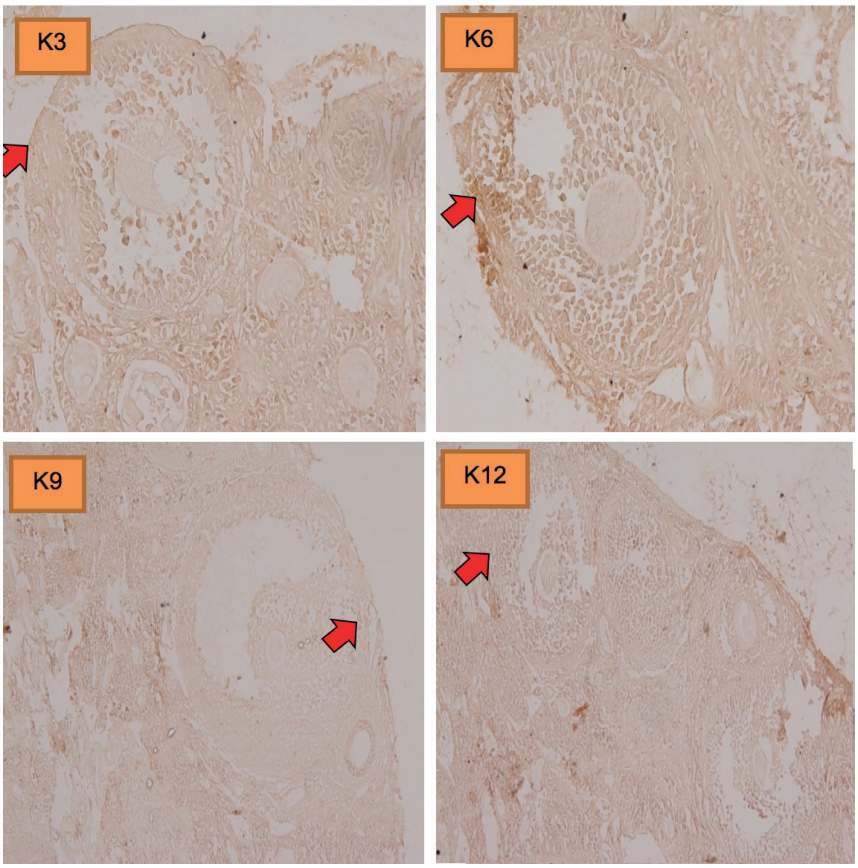

Figure 3. LH receptor expression on tertiary follicular theca cells which observed on day 20. (K3=control group exposed to Al $(OH)_3$ 50 µl in Tris HCl 50 µl, K6=the treatment of Mab-hZP3 at a dose of 20 µg, K9=the treatment of Mab-hZP3 at a dose of 40 µg, K12=the treatment of Mab-hZP3 at a dose of 60 µg). Expression of LH receptor was characterized by chromogenic brown (arrow) on tertiary follicular theca cells. The results showed that the Immuno Reactive Score (IRS) expression of LH receptors on tertiary follicular theca cells in the K3 (control) group was lower than that of the IRS of the K6, K9 and K12 treatment groups. LH receptor expression was observed using immunohistochemical staining with 400x magnification using Nikon H600L microscope equipped with Fi2 300 megapixel DS camera.

Effect of Mab-*h*ZP3 on Detection Time of LH Receptor Expression

Lowest level of LH receptor expression was found to be increased at day 20 (p=0.039) (Table 2).

Table 2. Effect of Mab-*h*ZP3 observation time against LH receptor expression

Day	Mean ± SD	p-value
10	4.4±1.6 [ab]	0.039
15	4.5±1.4 [a]	
20	5.9±2.6 [b]	

The lowest LH receptor expression was seen in day 10 of observation, but it did not differ significantly from that of day 15.

Dose Dependent Effects of Mab-hZP3 on LH Receptor Expression

Mab-hZP3 slightly increased the LH receptor expression (p>0.05). Different doses (i.e., 40 and 60 µg) did not statistically significantly increased the expression (Table 3).

Table 3. Effect of different doses of Mab-hZP3 on LH-Receptor Expression at Different Days

Mab-*h*ZP3 treatment	Observation Time	Mean ± SD	*p*-value
K1	Day 10	3.2±1.7	0.663
K2	Day 15	3.7±1.0	
K3	Day 20	5.7±3.9	
K4	Day 10	5.3±0.6	
K5	Day 15	4.3±2.0	
K6	Day 20	7.0±3.3	
K7	Day 10	4.9±0.6	
K8	Day 15	4.7±1.1	
K9	Day 20	6.4±0.8	
K10	Day 10	4.3±2.4	
K11	Day 15	5.4±1.2	
K12	Day 20	4.5±1.4	

Effects of different Mab-hZP3 doses on Estradiol levels

The administration of Mab-*h*ZP3 at a dose of 20 µg (P1) slightly decreased estradiol levels (p=0.065). (P1) decreased the estradiol levels (Table 4). Different doses of antibody did not change estradiol levels statistically significantly.

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Table 4. Effects of different Mab-hZP3 doses on Estradiol levels

Mab-*h*ZP3	Mean ± SD	*p*-value
Control (K1, K2, K3)	96.92±94.53	0.065
20 µg of Mab-hZP3 (K4, K5, K6)	85.21±35.36	
40 µg of Mab-hZP3 (K7, K8, K9)	104.86±52.09	
60 µg of Mab-hZP3 (K10, K11, K12)	100.79±34.42	

The Effects of Mab-*h*ZP3 on Estradiol Levels at Different days

Mab-*h*ZP3 caused peak level of estradiol on day 10. However, this increase was not statistically significant (p=0.334) (Table 5).

Table 5. The Effects of Mab-*h*ZP3 on Estradiol Levels at different days

Day	Mean ± SD	*p*-value
10	124.05±87.91	0.334
15	78.41±23.55	
20	88.37±31.45	

Effects of Different Doses of Mab hZP3 on Estradiol Levels at Different Days

Estradiol reached the lowest level on day 15 (Table 6). On day 15 and 20 of observation, the levels of estradiol did not statistically significantly decreased (p>0.05).

Table 6. Effect of different doses of Mab-hZP3 on estradiol levels at different days

Mab-hZP3 treatment	Observation Time	Mean ± SD (pg/ml)	*p*-value
K1	Day 10	132.43±165.77	0.157
K2	Day 15	59.07±2.68	
K3	Day 20	99.25±40.96	
K4	Day 10	101.89±48.74	
K5	Day 15	72.89±31.77	
K6	Day 20	80.85±24.51	
K7	Day 10	146.66±68.07	
K8	Day 15	77.55±15.11	
K9	Day 20	90.37±38.54	
K10	Day 10	115.23±51.17	
K11	Day 15	104.14±10.21	
K12	Day 20	83.01±30.10	

Effects of Different Doses of Mab-hZP3 on the Number of de Graaf Folicles

Various doses of Mab-hZP3 decreases the de Graaf follicles. However, the decrease was not found to be statistically significant (p=0.531) (Table 7).

Table 7. Effects of different doses of Mab-hZP3 on the number of de Graaf Folicles

Mab-*h*ZP3	Mean ± SD	p-value
Control (K1, K2, K3)	0.58±0.90	0.531
20 µg of Mab-hZP3 (K4, K5, K6)	0.42±1.00	
40 µg of Mab-hZP3 (K7, K8, K9)	0.33±0.49	
60 µg of Mab-hZP3 (K10, K11, K12)	0.25±0.87	

Effects of on de Graaf Follicle Numbers at Different Days of Observation

Mab-hZP3 increased the number of follicles on day 10(p=0.189)(Table 8). On day 15 and 20 of observation, number of follicles.

Table 8. Effects of Mab-*h*ZP3 on de Graaf follicle Numbers at different days of observation

Day	Mean ± SD	p-value
10	0.63±1.02	0.189
15	0.44±0.81	
20	0.13±0.50	

Effects of Different Doses of Mab-hZP3 on Follicle Numbers at Different Days of Observation

It was found that, follicle numbers decrease in time without reaching statistical significance (p=0.554) (Table 9). Also, there was no statistically significant change in follicle numbers with different doses of antibody.

Table 9. Effect of Different doses of Mab-hZP3 on follicle numbers at different days of observation

Mab-*h*ZP3 treatment	Observation Time	Mean ± SD	*p*-value
K1	Day 10	1.0±1.2	0.554
K2	Day 15	0.3±0.5	
K3	Day 20	0.5±1.0	
K4	Day 10	0.5±1.0	
K5	Day 15	0.8±1.5	
K6	Day 20	0	
K7	Day 10	0.3±0.5	
K8	Day 15	0.8±0.5	
K9	Day 20	0	
K10	Day 10	0.8±1.5	
K11	Day 15	0	
K12	Day 20	0	

de Graaf Follicles Characteristics

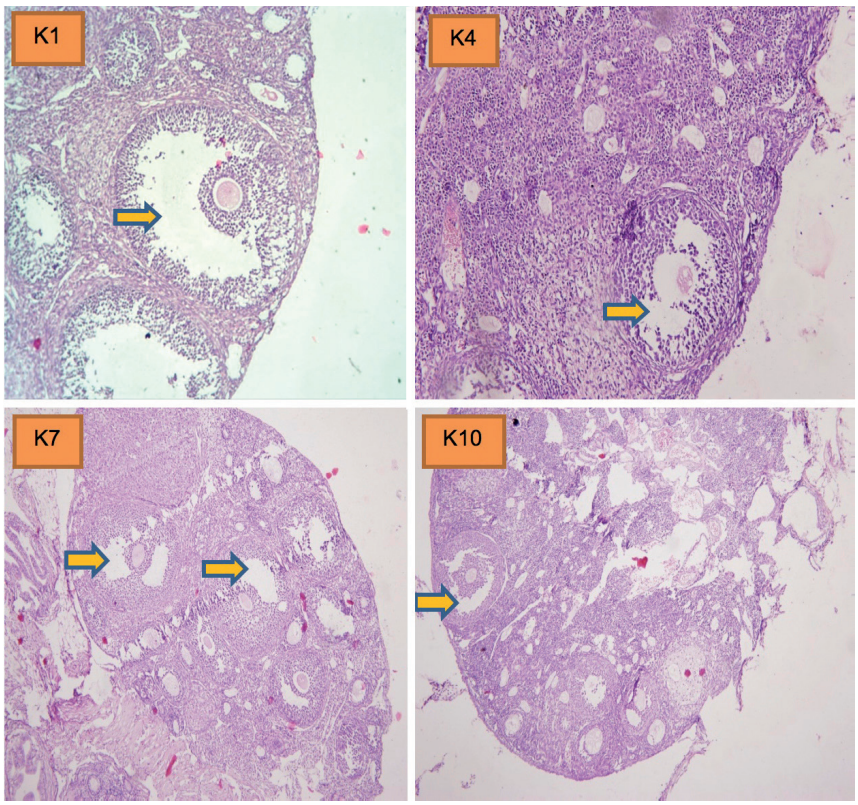

Figure 4. Comparison of number of de Graaf follicles in the ovaries on day 10. (K1=control group exposed to Al $(OH)_3$ 50 µl in Tris HCl 50 µl, K4=the treatment of Mab-hZP3 at a dose of 20 µg, K7=the treatment of Mab-hZP3 at a dose of 40 µg, K10=the treatment of Mab-hZP3 at a dose of 60 µg). The results showed that the de Graaf follicle has a larger folicullar antrum containing liquor foliculi (orange arrow) as showed in K1 (control) and K4, K7, K10 (treatment). Hematoxylin-eosin staining 400x magnification using Optilab Plus 12 Megapixel Digital Camera.

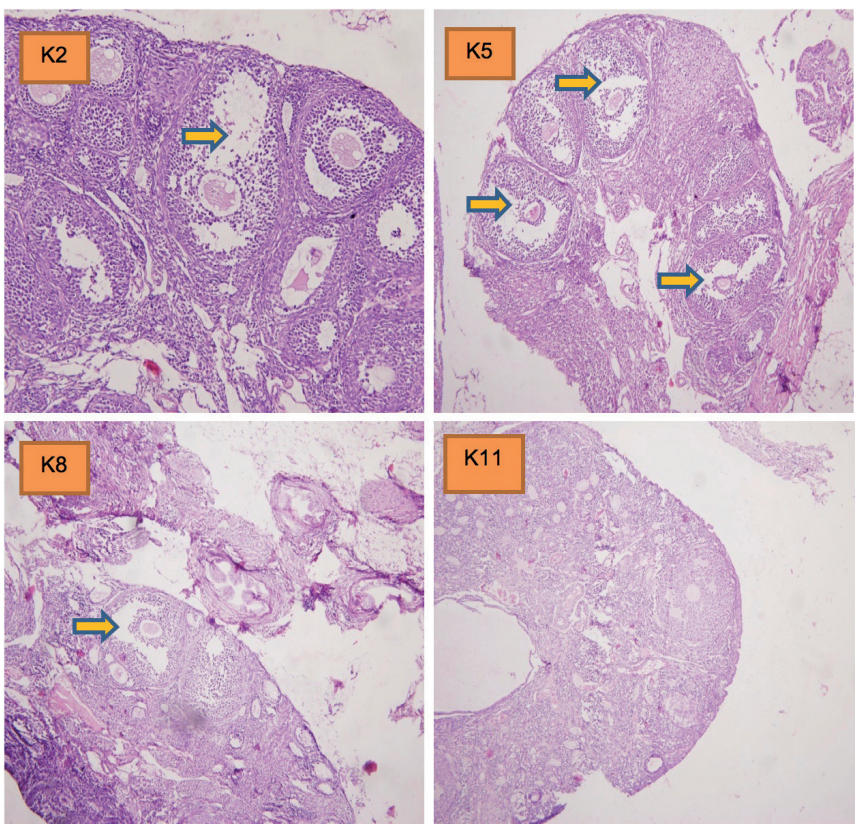

Figure 5. Comparison of number of de Graaf follicles in the ovaries on day 15. (K2=control group exposed to Al $(OH)_3$ 50 µl in Tris HCl 50 µl, K5=the treatment of Mab-hZP3 at a dose of 20 µg, K8=the treatment of Mab-hZP3 at a dose of 40 µg, K11=the treatment of Mab-hZP3 at a dose of 60 µg. The results showed that the de Graaf follicle has a larger folicullar antrum containing liquor foliculii(orange arrow) as showed in K1 (control) and K4, K7, K10 (treatment). Hemotoxylin-eosinm staining, 400x magnification using Optilab Plus 12 Megapixel Digital Camera.

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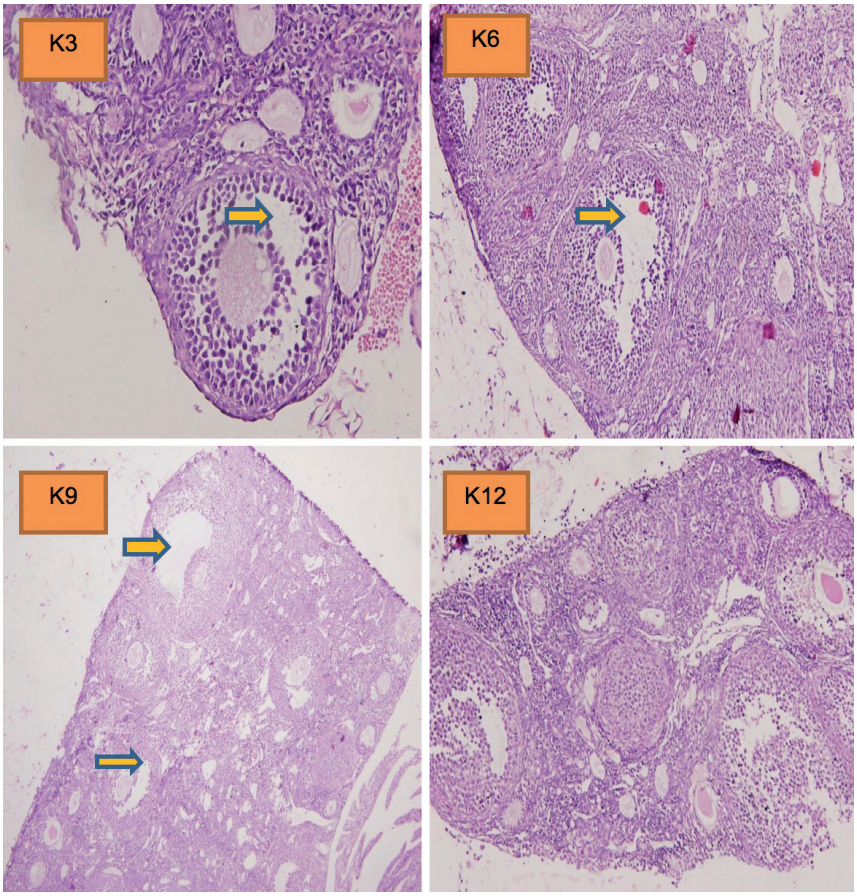

Figure 6. Comparison of number of de Graaf follicles in the ovaries on day 20. (K3=control group exposed to Al $(OH)_3$ 50 µl in Tris HCl 50 µl, K6=the treatment of Mab-hZP3 at a dose of 20 µg, K9=the treatment of Mab-hZP3 at a dose of 40 µg, K12=the treatment of Mab-hZP3 at a dose of 60 µg. The results showed that the de Graaf follicle has a larger folicullar antrum containing liquor foliculi (orange arrow) as showed in K1 (control) and K4, K7, K10 (treatment). Hematoxylin-eosin staining, 400x magnification using Optilab Plus 12 Megapixel Digital Camera.

Discussion

Our results regarding LH receptor expression were different than those were publihed by others indicating that immunization using rMCMV mZP3 decrease GDF-9, NOBOX and Gjal expression.[11]

In addition, decrease in expression of GDF-9, NOBOX and Gjal with rMCMV mZP3 immunization produced abnormal granulosa cells with increased expression of Kit Ligand and failed to recruit theca cells.[12,13] Oocytes cells, granulosa cells and theca cells have been reported to be major components of follicle functional and interaction among them plays an important role in steroidogenesis, follicular development, and atresia.[14] Disturbance of cell proliferation and differentiation of granulosa and theca cells were reported to decrease expression of LH receptors.[15]

Regarding the specificity of Mab-*h*ZP3, our findings are in contrast with some studies in which the immunocontraceptive polyclonal antibodies were used that can bind to many epitopes.[7] Different results related to the effect of ZP3 immunocontraceptive could be basically related to antigen purity, animal susceptibility, presence or absence of adjuvant and epitope of T cells and B cells used as immunogen.[8]

Maximum concentration of monoclonal antibody in the plasma (tmax) can be reached in 2-8 days.[17] In our study average LH receptor expression increased significantly 20 days after injection. It can be explained by expected half-life of the antibody.[18]

Moreover, the increase of LH receptor expression at 20th day of observation was expected as a result of normal hormonal cycle LH receptor expression showed dramatic changes through menstrual cycle as well as FSH and LH levels.[15] FSH induces granulosa cell differentiation and theca cells in dominant follicle which in turn stimulates the expression of LH receptor.[19]

Regarding estradiol levels, our results are almost identical to those of female Macaca radiata used for pZP3 immunization which led to significant reduction in estradiol levels after pZP3 immunization.[20]

In dogs, it may lead to degenerative changes in follicles, changes in estradiol and progesterone levels and in turn it might cause to changed estrous cycle.[21] Immunocontraceptive application alters the conformation of polypeptide chain in zona pellucida.[22]

Conformational ZP3 changes disrupt mutual communication between oocyte and granulosa cells causing interference in gap junction forming.[12] GDF-9 is a paracrine regulator of granulosa cells, proliferation and differentiation of follicular ceells.[23] Mouse growth differentiation factor-9 (GDF-9) causes oocyts to become larger while granulosa cells surrounding the oocyte do not proliferate however, it induces the expression of KL and inhibin-α.[23] GDF9 and BMP 15 along with other growth factors such as IGF-1 and KL, stimulate the differentiation and proliferation of theca cells.[23,24]

Proliferation and differentiation of theca and granulosa cells are associated with the steroidogenesis process.[25] If those important factors decrease, granulosa cells not only lost their functions but also they die which would impact the number of healthy follicles.[26]

Estradiol levels were not affected by different Mab-*h*ZP3 doses in this study because the monoclonal antibody hZP3 given was monoepitope which only binds to specific site, binding to ZP3 alone. The integrity of zona pellucida was not preserved because Mab-*h*ZP3 does not recognized ZP1 which maintains the integrity of zona pellucida. This is consistent with the high specificity of antibody with its immunocontraceptive properties.[5]

Additional assumptions related to this results is that, the antibodies lack glycoprotein to allow them to bind to zona pellucida.[27] Decreased levels of estradiol on day 15 (cycle-3) and day 20 (cycle 4) is consistent with the study conducted in rabbits in which SIZP and ZP3 immunization in pigs showed an increased levels of FSH in blood.[27]

About the slightly changed number of de Graaf follicles by Mab-*h*ZP3, important growth factors in the endocrine system such as FSH and antral follicles did not change significantly.[28]

It was also shown that, Mab-*h*ZP3 did not change oocytes and normal follicular architecture, including secondary, tertiary structures and corpus luteum.[29]

The decline in de Graaf follicle numbers on day 15 (cycle 3) and day 20 (cycle 4) of the study after the administration of Mab-*h*ZP3 was expected because of the increased of FSH hormones since estradiol is the major inhibitor of pituitary FSH secretion. This is consistent with the research in which the rabbits immunized with pig- anti-SIZP and anti-ZP3 antibodies showed an increased FSH levels.[27]

Decreased estrogen levels (estradiol) leads to decreased LH levels and stimulate apoptosis of folliclar and granulosa cells and interfering follicle growth as a result of non occurrence of ovulation and disturbed oocytes maturation marked by numerous follicles undergoing atresia.[23,30]

In our study, we showed that despite the decrease in the number of follicles on the 15th and 20th days, there was no significant difference in terms of number of follicles. Regarding the decrease in follicle numbers with anti-follicle antibody, it was shown that ovarian pathology could be induced by the transfer of $CD4^+$ T cells from immunized mice with ZP into recipient mice, but it did not occur with the antibody transfer.[8] Oophoritis does not occur if it is immunized using B-cell epitope ZP3 peptides resulting low immune response.[31]

MAb-*h*ZP3 did not seem to interfere with folliculogenesis process as proven by our study. In addition, studies have indicated that some monoclonal antibodies can be effective in blocking fertilization.[32] These results may provide further opportunities for Mab-*h*ZP3 to be developed into a better contraceptive agent compared to other conventional contraceptives.

Conclusion

Various dosages of monoclonal antibody to *human* ZP3 had no effect on reducing LH receptor expression, estradiol levels and de Graaf follicle quantity while exerting contraceptive functions.

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References

1. Kaur K, Prabha V. Immunocontraceptive: new approaches to fertility control. BioMed Res Int 2014;2014:1–5. [CrossRef]
2. BKKBN. Profil Kesehatan Indonesia 2014. Jakarta: Kementerian Kesehatan Republik Indonesia 2014. http://www.depkes.go.id/resources/download/pusdatin/profil-kesehatan-indonesia/profil-kesehatan-indonesia-2014.pdf
3. Wassarman PM, Litscher ES. Influence of the zona pellucida of the mouse egg on folliculogenesis and fertility. Int J Dev Biol 2012;56:833–9. [CrossRef]
4. McLaughlin EA, Aitken RJ. Is there a role for immunocontraception? Mol Cel Endocrinol 2011;335:78–88. [CrossRef]
5. Sumitro SB, Aulanni'am A. Zona Pellucida 3 (ZP3) has proper biochemical properties to be considered as candidate antigen for immunocontraceptive vaccine. Reprod Tech 2011;1:51–3.
6. Mustofa I. Identifikasi efek samping imunokontrasepsi zona pelusida-3 kambing pada histologi ovarium mencit (Mus musculus) sebagai model. Media Kedokteran Hewan 2005;21:19–22.
7. 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. [CrossRef]
8. Barber MR, Fayrer-Hosken RA. Possible mechanism of mammalian immunocontraception. J Reprod Immunol 2000;46:103–24. [CrossRef]
9. Chiu PC, Wong BS, Lee CL, Pang RT, Lee KF, Sumitro SB, et al. Native human zona pellusida glycoproteins: purification and binding properties. Hum Reprod 2008;23:1385–93. [CrossRef]
10. Mubarakati NJ, Aulanni'am A, Sumitro SB, Ciptadi G. Immunogenicity and characterization of antibodies against to recombinant human ZP3 for development woman immnucontraception. Biosciences Biotechnology Research 2015.
11. O'Leary S, Lloyd ML, Shellham GR, Robertson SA. Immunization with recombinant murine cytomegalovirus expressing murine zona pellucida 3 cause permanent infertility in BALB/c mice due to follicle depletion anf ovulation failure. Biol Reprod 2008;79:849–60. [CrossRef]
12. Borillo J, Coonrod SA, Wu J, Zhou C, Lou Y. Antibodies to two ZP3 B cell epitopes affect zona pellucida assembly. J Reprod Immuno 2008;78:149–57. [CrossRef]
13. Sanchez F, Smitz J. Molecular Control of Oogenesis. Biochim Biophys Acta 2012;1822:1896–912. [CrossRef]
14. Liu X, Qiao P, Jiang A, Jiang J, Han H, Wang L, Ren C. Paracrine regulation of steroidogenesis in theca cells by granulosa cells derived from mouse preantal follicles. Biomed Res Int 2015;2015:1–8. [CrossRef]
15. Bao B, Kumar N, Karp RM, Garverick HA, Sundaram K. Estrogen receptor-β expression in relation to the expression of luteinizing hormone receptor and cytochrome P450 enzymes in rat ovarian follicles. Biol Reprod 2000;63:1747–55. [CrossRef]
16. 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.
17. Wang W, Wang EQ, Balthasar JP. Monoclonal antibody pharmcokinetics and pharmacodynamics. Clin Pharmacol Ther 2008;84:548–58. [CrossRef]
18. Abbas AK, Lichtman AH, Pillai S. Basic Immunology: functions and disorders of the immune system, 4th ed. Canada: Elsevier Inc; 2014.
19. Rebar RW, Catherino WH. Reproductive Endocrinology and Infertility. Chapter 236. In: Goldman L, Schafer AI, editors. Goldman-Cecil Medicine, 25th ed. Philadelphia, PA: Elsevier Saunders; 2016.
20. Bagavant H, Thillai-Koothan P, Sharma MG, Talwar GP, Gupta SK. Antifertility of porcine zona pellucida-3 immunization using permissible adjuvant in female bonnet monkey (Macaca radiate): reversibility, effect on follicular development and hormonal profile. Reproduction 1994;102:17–25. [CrossRef]
21. Srivastava N, Santhanam R, Sheela P, Mukund S, Thakral SS, Malik BS, Gupta SK. Evaluation of the immunocontraceptive potential of E. Coli-expressed recombinant dog ZP2 and ZP3 in a homologous animal model. Reproduction 2002;123:847–57. [CrossRef]
22. Widodo E, Aulanni'am. Specifity of antibody bovine zona pellucidae 3 (anti-bZP3) to rabbit ZP3 based on bZP3 as contraceptive antigens. Indo J Chem 2005;5:182–7.
23. van den Hurk R, Zhao J. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenolgy 2005;63:1717–51. [CrossRef]
24. Palma GA, Arganaraz ME, Barrera AD, Rodler D, Mutto AA., Sinowatz F. Biology and biotechnology of follicle development. Sci World J 2012;2012:1–14. [CrossRef]
25. Melmed S, Polonsky K, Larsen PR, Kronenberg H. Williams Textbook of Endocrinology, 12th ed. The United State of America: Elsevier; 2012.
26. Matsuda F, Inoue N, Manabe N, Ohkura S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. J Reprod Dev 2012;58:44–50. [CrossRef]
27. Keenan JA, Sacco AG, Subramanian MG, Kruger M, Yurewicz EC, Moghissi KS. Endocrine response in rabbits immunized with native versus deglycosylated porcine zona pellucida antigens. Biol Reprod 1991;44:150–6. [CrossRef]
28. East IJ, Dean J. Monoclonal antibodies as probe of the distribution of ZP-2, the major sulfated glycoprotein of the murine zona pellucida. J Cell Biol 1984;98:795–800. [CrossRef]
29. Greenhouse S, Castle PE, Dean J. Antibodies to human ZP3 induce reversible contraception in transgenic mice with 'humanized' zonae pellucidae. Hum Reprod 1999;14:593–600. [CrossRef]
30. Strauss J, Barbieri R. Yen & Jaffe's Reproductive Endocrinology. China: Elsevier Inc; 2014.
31. Govind CK, Hasegawa A, Koyama K, Gupta SK. Delineation of a conserved B cell epitope on bonnet monkey (Macaca radiata) and human zona pellucida glycoprotein-B by monoclonal antibodies demonstrating inhibition of sperm-egg binding. Biol Reprod 2000;62:67–75. [CrossRef]
32. 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. [CrossRef]