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The Effect of Human Pellucid Zone 3 Monoclonal Antibody on Expression of Bcl-2 and Bax in Follicle Granulosa Cells of Mice Ovary

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ABSTRACT

Pellucid zone 3 (ZP3) involves in fertilization mechanism. Moreover, an antibody of ZP3 can develop to inhibit egg and sperm interaction. This study aims to determine the effect of *h*ZP3 (mab-*h*ZP3) monoclonal antibody on the expression of Bcl-2 and Bax in follicle granulosa cells of the mice ovary. Female BALB/c mice were divided into 12 groups which consisted of a control and experimental treatment group. Each group was added with 30% of total mice as error sample (1 mice). Each group was treated differently: 50 µL adjuvant $Al(OH)_3$ in 50 µL Tris HCl, 20 µg Mab-hZP3, 40 µg Mab- *h*ZP3, and 60 µg Mab-*h*ZP3. Each group was then dissected at day 10, 15 and 20. Measurement of Bcl-2 and Bax was performed with immunohistochemistry. Data were then analyzed by Two-Way ANOVA. The result showed that there was no significant effect of Mab-*h*ZP3 administration in various doses on Bcl-2 ($p = 0.0825$), and Bax ($p = 0.836$). There was no significant effect of administration of Mab-*h*ZP3 in time ($p = 0.807$) on Bcl-2 expression ($p = 0.088$) but the significance difference was found in Bax level ($p = 0.031$). The lowest Bcl-2 level was found in a dose of 60 µg in day 15. There was no significant effect of Mab-*h*ZP3 in various doses and time ($p = 0.691$), neither to Bcl-2 and Bax. The results obtained due to the specificity of a monoclonal antibody that recognizes a specific antigen. Mab-*h*ZP3 is proposed as immunocontraception for women causing no disturbance of folliculogenesis.

Keywords: *Antibody, Bax, Bcl-2, contraception, Pellucid zone 3 (ZP3)*

INTRODUCTION

Contraception is required to control population's growth rate which increased gradually each year, mostly in developing countries. However, hormonal contraception that commonly used causes side effects both temporarily and permanently [1]. For that matter, contraception is required to be safe, reliable, effective, and reversible to reduce side effects, one of which is immunocontraception [2].

Immunocontraception is a candidate derived from pellucid zone inhibiting the interaction between sperm and eggs, and it is currently being developed. Pellucid zone 3 (ZP3) involves in fertilization control. Therefore, administration of ZP3 antibody can inhibit the interaction between sperm and eggs [3]. Aulanni'am and Sumitro found that ZP3 antibody was an effective inhibiting interaction between sperm and eggs *in vivo* fertilization by disrupting ZP3 binding to its ligand [4]. ZP3-antibody based-contraception is also permanent and reversible [5]. However, studies regarding its side effects remain elusive.

A side effect of immunocontraception on folliculogenesis remains controversial. Study of Calongos *et al.*, using ovary follicle of mice with administration of ZP3 antibody, showed smaller follicles and antrum, and it is also found in follicle culture with ZP3-antibody which granulosa did not attach to oocyte properly that leads to oocyte extrusion [6]. Paterson reported the exposure of

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*H*ZP3 recombinant in Marmoset, caused disturbance of folliculogenesis and decreased primordial follicle [7]. In contrary, Mustofa found that there were no abnormality of ovary and follicles found after treatment of goat ZP3 (*g*ZP3) to the number of primordial, primary, secondary, and De Graff follicles [5]. A similar finding was also reported by Bagavant *et al.,* that *Macaca* with three-fold immunization of *porcine* pellucid zone showed normal ovulation and follicle development [8].

The administration of ZP antibody affects the structure of zona pellucida and folliculogenesis. It also disrupts *gap junction* among granulosa cells and oocyte leading to disturbance of follicle development which will be smaller than normal. Disturbance of *gap junction* between oocyte and granulosa cells causes *Premature Ovarium Failure* (POF) [6].

Follicles viability is determined by its diameter where 2 – 5 mm is considered normal and healthy. It further developed into dominant follicles of 5 – 18 mm and then ovulated. If selected follicles change to non-dominant follicles sized 0.5 – 10 mm, follicles will undergo atresia via apoptosis [9, 10]. Both follicles growth and atresia involve Bcl-2 protein which is responsible for viability and apoptosis of granulosa cells.

The Bcl-2 family consisted of anti-apoptosis (Bcl-2 and Bcl-x) and pro-apoptosis (Bax and Bid), involved in regulating apoptosis on the intrinsic pathway, by promoting cytochrome C release in mitochondria. In Bcl-2-deficient mice, oocyte and primordial follicles reduced. The increased Bcl-2 can reduce apoptosis of granulosa cells during folliculogenesis. Bax is highly expressed in granulosa cells of follicles which undergo atresia, compared to healthy follicles in human [11].

Development of immunocontraception of pellucid zone among mammals, both native and recombinant, is widely developed from cows, goats, or rabbits. It shows the interspecific reaction of antibody on ZP3 [4]. Mubarakati *et al.* produced ZP3 antibody from human blood (mab-*h*ZP3) as a potential candidate for immunocontraception, but its side effect on folliculogenesis still need to be further studied [12]. Thus, this study aimed to determine side effects of monoclonal antibody on folliculogenesis, specifically on the expression level of Bcl-2 and Bax.

MATERIALS AND METHOD

Research design and animal model

The research design used was a true experiment using Post Only Control Group Design approach. The study was conducted in Animal Model Unit Laboratory, Faculty of Medicine, Airlangga University, Surabaya, Indonesia. Forty-eight female BALB/c mice *(Mus musculus)* were divided into 12 groups of experimental treatment (3 mice of each group). Each group was added by 30% (1 mice) of total mice required as error sample. Groups were then divided into 3 control groups and 9 experiment group. The independent variables were human pellucid zone-3 monoclonal antibodies in various doses and time, and dependent variable were Bcl-2 and Bax expression of follicle granulosa in mice ovary.

Treatment of adjuvant and Mab hZP3

The control mice groups were injected subcutaneously in back feet with a syringe containing adjuvant of 20 µL. The experiment groups mice were also injected subcutaneously with *h*ZP3 monoclonal antibody in $AI(OH)_3$ (*Alumina Hydrogel*) by various doses of 20 µg, 40 µg, and 60 µg.

According to Ma *et al.*, and Dahlberg *et al.*, antibody injected into the subcutaneous region or intramuscular way, was absorbed through lymphatic tissue. Absorption takes 2 – 8 days to peak its plasma concentration (t-max) with bioavailability between 50 – 100% [13, 14].

Analysis histopathology of the ovary

Histopathology visualization of the ovary was performed with fixation with *Paraformaldehyde (PFA)* 10%. Afterward, the organ was dehydrated, cleared, impregnated, and embedded. Organ cutting and staining were performed immunohistochemically.

Measurement of Bcl-2 and Bax expression

Measurement of Bcl-2 and Bax expression in granulosa cells was performed immunohistochemically according to BIOSS protocol.

Statistical analysis

Data analysis was Two-Way ANOVA with confident level 95%. This aimed to compare mean among groups (control and experimental treatments) in various doses and time. Data obtained from Bcl-2 and Bax measurement was tabulated based on groups and analyzed statistically. The dataset was previously tested its parametric condition.

RESULTS AND DISCUSSION

Measurement of Bcl-2 and Bax expression

Vaginal swab during estrus period in mice is shown in Figure 1. The histopathology visualization was shown

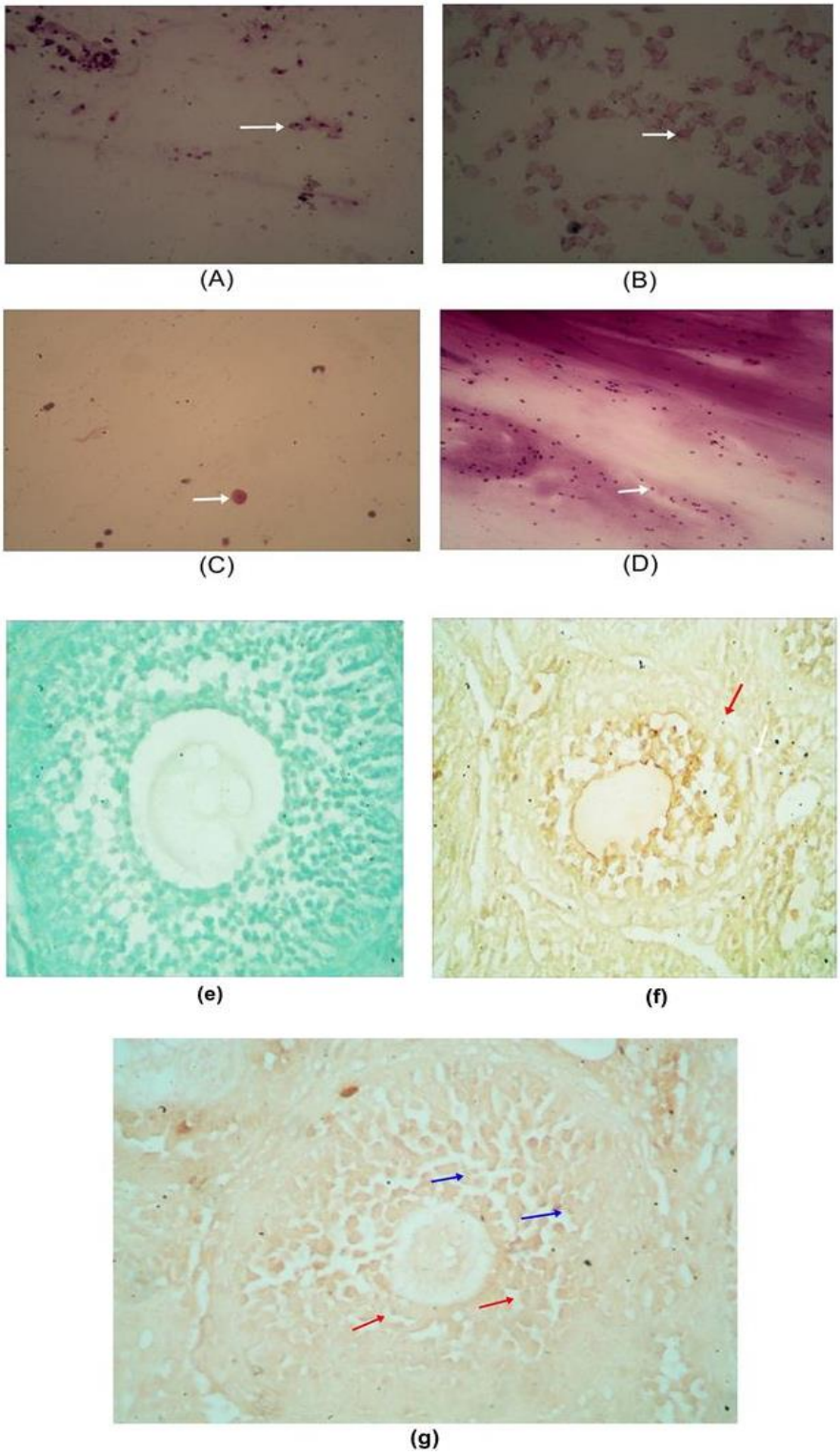

Figure 1. Vaginal swab on estrus cycle: (A) proestrus; (B) estrus; (C) metestrus; (D) diestrus. Visualization of Bcl-2 expression in secondary follicle granulosa: (E) there was no Bcl-2 expression in secondary follicle granulosa; F) expression of Bcl-2 in granulosa indicated by chromogen brown (white arrow) in granulosa (400×). (G) Bax visualization in secondary follicle granulosa, Red arrow shows there was no Bax expression in secondary follicle granulosa and blue arrow shows Bax expression in granulosa indicated by brown chromogen in granulosa

with light Nikon H600L facilitated by digital camera DS Fi2 300 megapixel and image processing software, Nikkon Image System to measure Bcl-2 and Bax expression. Visualization of Bcl-2 expression in secondary follicle granulosa is shown in Figure 1.

The measurement of Bcl-2 and Bax expression in various doses and time was analyzed with ANOVA. As shown in Table 1, *p*-value on Bcl-2 and Bax expression was more than $\alpha = 0.05$. Those results explained that data variable of Bcl-2 and Bax expression was distributed normally. Homogeneity test was conducted with Levene test ($p > 0.05$)

p-value on Bcl-2 expression was more than $\alpha = 0.05$ ($p > 0.05$) which indicates variable of Bcl-2 expression was homogeneous (Table 1). *p*-value of Bax expression was less than $\alpha = 0.05$ ($p < 0.05$). Therefore, data transformation was further required. Logarithm transformation was used by converting data to the logarithm (Log (10Y)). In variable of Bax, there was observation measured as 0 (Y=0), thus, the transformation was further performed log transformation Log (10(Y+0.5)). These are results of homogeneity test transformed. Bax expression variable is shown in Table 3. *p*-value < 0.05 indicates variable was not homogeneous even after through log transformation. Thus, the test was performed non-parametric by transforming data with Rank Transform method and further analyzed with ANOVA. Bax expression has coefficient 2.957 with *p*-value 0.007 and it assumes that not homogenous.

Effect of Mab-hZP3 in various doses on Bcl-2 and Bax expression

ANOVA analysis showed p-value was 0.825, higher than $\alpha = 0.05$ ($p > 0.05$) which concluded there was no significant effect of Mab-*hZP3* on Bcl-2 expression. Effect of mab-*hZP3* in various doses is shown in Figure 2(a). As shown in the histogram, an average of Bcl-2 expression was highest in the control group. It was presented that Bcl-2 level decreased due to the administration of Mab-*hZP3* in various doses. However, the effect of Mab-*hZP3* on Bcl-2 expression was not statistically significant.

Effect of Mab-*hZP3* in various doses on Bax expression was also analyzed with ANOVA. ANOVA results showed *p*-value was 0.505 ($p > 0.05$), which concluded there was no significant effect of Mab-*hZP3* on Bax expression. The effect of Mab-*hZP3* in various doses is shown in Figure 2(d). As shown in the histogram, an average of Bax expression is lowest in the control group. It was also found increasing Bax level in the administration of Mab-*hZP3* in various doses, although it was not significant.

Results showed Bcl-2 expression in the treatment group was lower than control, whereas Bax expression was higher in the treatment group than control, although statistically, effect of Mab-*h*ZP3 was not significant.

Decreased Bcl-2 and increased Bax occurs due to Mab-*h*ZP3 administration disturbs cells where Bcl-2 and Bax expressed. Borillo *et al.* [15] stated that the administration of ZP3 causes disturbance of ovarium formation [15]. ZP3 administration on preantral follicles will disrupt ZP3 protein synthesis and secretion. In microscopic observation, ZP seems thin, transparent, loosen, swollen, and half dissolved, and slot emerged between ZP-oocyte, ZP-granulosa cell, and among granulosa cells. The slot formed due to F171 dysfunction affecting gap junction formation which decreases the amount of gap junction between oocyte and granulosa.

In vitro study of Calongos *et al.*, found that preantral follicles cultured with ZP2/ZP3-antibody did not develop to antral follicles on day 4, and granulosa differentiation and antrum were also absent [6]. Results of day 7 showed incomplete follicles development and granulosa attachment to the oocyte, causing oocyte extraction and granulosa degeneration.

In Bcl-2-deficient mice, decreased oocyte and primordial follicles are present due to the low level of Bcl-2 which is responsible reducing apoptosis in granulosa, whereas a high level of Bax is present in granulosa of follicles which undergo atresia [11]. In contrary, Mustofa observed the effect of *gZP3* antibody in various doses of 20 µg and 40 µg in two treatment groups and results showed there was no disturbance of follicles in mice ovary and abnormality of ovarium [5]. Study of East *et al.*, showed *m*ZP3 monoclonal antibody in microgram quantity both in vitro and in vivo, inhibits fertilization and also has no effect on follicle and embryo development [16, 17]. Study of Bagavant *et al.*, also reported *p*ZP3 immunization on monkey has no effect on decreasing follicle and increasing atresia follicles [18].

Effect of Mab-hZP3 at various times on Bcl-2 and Bax expression

The effect of Mab-*hZP3* at various times on Bcl-2 expression was analyzed with ANOVA. Analysis of ANOVA obtained the p-value equal to 0.807 ($p > 0.05$), which considered there was no significant effect on the administration of Mab-*hZP3* at various time on Bcl-2 expression. The effect of Mab-*hZP3* at various times is

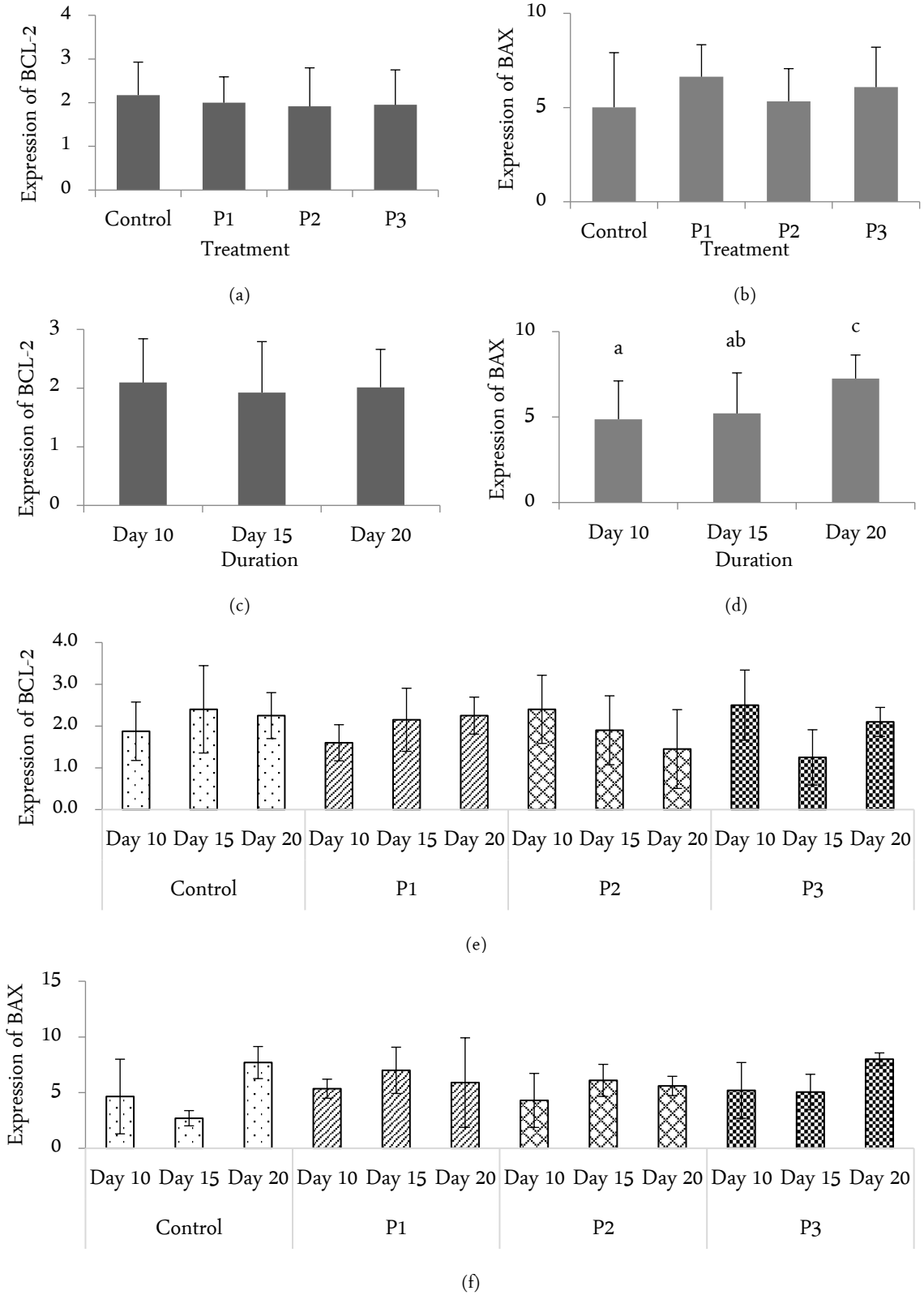

Figure 2. Effect of Mab-hZP3 in (A) various doses, (B) various time and in (C) various doses and time on BCL-2 expression. Effect of Mab-hZP3 in (D) various doses, (E) various time and in (F) various doses and time on Bax expression (P1 = Mab-hZP3 20 µg, P2 = Mab-hZP3 40 µg, and P3 = Mab-hZP3 60 µg). Data are mean of ± standard deviation values of three mice in each group with p-value <0.05. Different letters indicate significant difference based on Tukey's high significant differences test at a 95% significance level. While figures with no letters indicate that there is no significant different.

shown in Figure 2(b). As shown in Figure 2(b), the highest average of Bcl-2 expression was 2.09 found on day 10. Furthermore, decreasing Bcl-2 level occurred on day 15 and 20. Decreased Bcl-2 at its lowest level was obtained on day 15, although it was not significant.

Effect of Mab-*hZP3* at various times on Bax expression analyzed with ANOVA is shown in Figure 2(e). ANOVA results showed that *p*-value equal to 0.031 ($p<0.05$), which considered to have significant effect in various time on Bax expression. As shown in Figure 2, the lowest average of Bax expression was 4.88 in day 10 followed by increasing its level in day 15 and 20. The highest increasing level of Bax is on day 20 which considered significant compared to day 10.

If administration time is associated with the effect of a compound including immunoglobulin (antibody), a monoclonal antibody can reach to 20 days of its effectiveness. According to Keizer, IgG is a big molecule since it is not carried out through urine [19]. Longest IgG period is 21 days.

After the antibody injection, the immune system will immediately react after injection and held during IgG lifetime *in vivo* which is approximately 3 week [20]. Results showed that Bcl-2 decreased on day 15 (compared to day 10 and 20) due to the presence of Mab-*hZP3*, whereas Bax level increased in various doses. The results obtained due to the disturbance of granulosa cells as a result of altered ZP caused by a monoclonal antibody. Decreased Bcl-2 in day 15 might be associated with monoclonal antibody absorption temporarily, following its increasing in day 20. The part of the pellucid zone can be found in granulosa intercellular space, therefore, ZP antibody affects granulosa.

Granulosa maintains follicles viability [11, 21]. Bcl-2 and Bax are expressed as antiapoptosis and proapoptosis in granulosa cell. The role of Bcl-2 as anti-apoptosis is to bind and neutralize proapoptotic protein granulosa. Otherwise, Bax as proapoptotic promotes apoptosis in granulosa cell. Low level of Bcl-2 generates atresia on follicle preceded by apoptosis [22, 23].

Expression of Bcl-2 and Bax are also associated with gonadotropin level which increasing gonadotropin and Bcl-2 but decreasing Bax otherwise [24]. Statistically, there was no difference in the effect of Mab-*h*ZP3 in various time on Bcl-2 and Bax due to the monoclonal antibody as a compound of immunocontraception. Monoclonal antibody is known specific and directly acts on a targeted cell, ZP3; it has only one epitope to recognize a specific antigen. Its specificity affects the role of ZP3 as a primary receptor on spermatozoa recognizing [25].

Effect of Mab-hZP3 in various doses and time on Bcl-2 and Bax expression

The effect of Mab-*hZP3* in various doses and time on Bcl-2 expression was analyzed with ANOVA (Figure 2(f)) . Analysis of ANOVA showed the *p*-value was 0.088 ($p > 0.05$) which concluded there was no significant effect of interaction between Mab-*hZP3* in various doses and time on Bcl-2 expression. As shown in the histogram, an average of Bcl-2 expression in the control group was relatively similar to the treatment group of Mab-*hZP3* in various doses. Decreased Bcl-2 at its lowest level occurred in the administration of a Mab-*hZP3* dose of 60 µg (P3) in day 15, although it was not significant.

The effect of Mab-*hZP3* in various doses and time on Bax expression analyzed with ANOVA is shown in Figure 2(c). ANOVA results showed *p*-value was 0.098 ($p > 0.05$), which considered that there was no significant effect of interaction between Mab-*hZP3* in various doses and time on Bax expression. As shown in the histogram, an average of Bax expression was highest in the control group on day 20. Increasing Bax level in the treatment group was on a dose of 60 µg (P3) in day 20 although it was not significant.

Statistical analysis showed the administration of Mab-*h*ZP3 in various time and time has no significant change in Bcl-2 and Bax expression due to the high specificity of Mab-*h*ZP3. Human ZP3 monoclonal antibody causes damage of ZP loci. Monoclonal antibody directly acts on ZP3 and effects on the ZP3 role as a primary receptor for sperm recognition. *Human* ZP3 as another monoclonal antibody is specific, has one epitope to only identify specific antigen [25]. Bukovsky *et al.* reported *murine* Mabs-*ZP3* monoclonal antibody has high specificity on human ZP3 protein [26]. There is no *cross-reactivity* Mabs-*m*ZP3 found in the ovary and other tissues such as endometrium, uterus, cervix, tuba falopii and kidney.

Study of Sumitro *et al.*, performed with Western Blot, showed that Mab-*b*ZP3 recognizes *b*ZP3 on molecule weight of 79,995±0.051 kDa indicating Mab-*b*ZP3 recognize *b*ZP3 rather than other molecules [27]. It concludes that Mab-*h*ZP3 only recognize ZP3 as a primary receptor of sperm recognition without affecting reproduction organ and folliculogenesis.

The administration of polyclonal antibodies to pZP in *in-vitro* studies using eggs and human spermatozoa has been shown to cause inactivation of spermatozoa receptor sites by interaction with epitope or interaction with sites around spermatozoa receptors. This led to the

prevention of spermatozoa and egg ties up to 95%. The administration of antibodies to pZP3α in intact ZP was significantly capable of inhibiting spermatozoa and egg [28]. The bonding of ZP antibodies with ZP can cause conformational changes and epitope surfaces. This change is similar to ZP-shaped changes in the fertilization process and zone blocking which can lead to premature induction of cortical granular reactions resulting in ZP hardened, and infertile [29]. The HZP3 monoclonal antibody works directly on target cells, giving ZP3 antibodies in mice serum *in-vivo* causing changes in ZP structure in the early stages of antral follicle growth in the form of disruption of gap junction formation between oocytes and granulosa cells [30].

Apoptosis is a programmed cell death which is genetically regulated and involves many physiology and pathology process. Signals activating apoptosis originate from reactive oxygen species (ROS), ceramide, excess Ca^{2+} activation, Bcl-2 protein family [28]. This member of Bcl-2 family consists of antiapoptosis, Bcl-2, and proapoptosis, Bax. The balance of Bcl-2 family is established by controlling the amount of pro-apoptosis and anti-apoptosis which is simultaneously active. Activities such as DNA damage can interfere pro and anti-apoptosis balance which promotes programmed cell death [29]. Although the effect of Mab-*h*ZP3 in various doses and time on Bcl-2 and Bax expression was not significant statistically, decreasing Bcl-2 level was detected in the treatment group with the lowest peak on the dose of 60 µg in day 15 whereas increasing Bax level was on a dose of 60 µg in day 20. Such results occurred due to atresia in follicle selection which only involves one dominant follicle to ovulate.

Follicle atresia includes apoptosis and granulosa release, oocyte autolysis, and pellucid zone collapse. Atresia occurs in each stage of follicle development [30]. Administration of Mab-*h*ZP3 also affects ZP formation by causing depletion which is associated with oocyte *gap junction,* granulosa cells and two-way communication oocyte, granulosa that plays a role in folliculogenesis and oogenesis. Gap junction highly influences growth and development of oocyte and granulosa. ZP antibody causes granulosa dysfunction as well [24, 31].

CONCLUSION

In this study, administration of Mab-HZP3 in various doses and time has no significant effect on Bcl-2 and Bax expression. Mab-HZP3 administration did not decrease Bcl-2 level and increasing Bax level significantly.

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