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Original Article

Effect of human ZP3 monoclonal antibody on expression of GDF-9 and number of theca cells in ovary of mice (*Mus musculus*)

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الملخص

أهداف البحث: تهدف هذه الدراسة إلى التأكد من تأثير الأجسام المضادة أحادية المنشأ للمنطقة البشرية الشفافة ٣ على التعبير عن ج د ف-٩ وكمية خلايا ثيكا في المبيض لفئران موس العضلية.

طرق البحث: استخدمنا تجربة حقيقية لما بعد الاختبار- تصميم مجموعة التحكم فقط، التي شملت ٤٨ فأرا تم تقسيمهم إلى مجموعة التحكم والمجموعة العلاجية للأجسام المضادة أحادية المنشأ للمنطقة البشرية الشفافة ٣ (٢٠ ميكروغرام،٤٠و ميكروغرام، و٦٠ ميكروغرام). قتلت كل مجموعة من الفئران في اليوم ١٠ و١٥ و٢٠. كما تم إجراء قياس تعبير ج د ف-٩ باستخدام الكيمياء النسيجية المناعية وتم قياس كمية خلايا ثيكا.

النتائج: تحليل تفاعل الأجسام المضادة أحادية المنشأ للمنطقة البشرية الشفافة ٣ عند جرعة ٢٠ ميكروغرام -٦٠ ميكروغرام على التعبير عن ج د ف -٩ وكمية خلايا ثيكا لم يظهر اختلافات كبيرة. ولوحظ اكتشاف مماثل أيضا في الفترة من ١٠-٢٠- يوما. ولم نجد للأجسام المضادة أحادية المنشأ للمنطقة البشرية الشفافة ٣ أي تعبير عن ج د ف -٩ وخلايا ثيكا.

الاستنتاجات: أظهرت هذه الدراسة أن الأجسام المضادة أحادية المنشأ للمنطقة البشرية الشفافة ٣ يمكن اعتبارها طريقة مناعية لمنع الحمل فاعلة وآمنة.

الكلمات المفتاحية: ج د ف-٩؛ عوامل النمو؛ الأجسام المضادة أحادية المنشأ؛ خلايا ثيكا

Abstract

Objectives: This study investigated the effects of a human ZP3 monoclonal antibody (mAb *h*ZP3) on the expression of growth differentiation factor-9 (GDF-9) and number of theca cells in the ovaries of mice (*Mus musculus*).

Methods: Our study employed a true experiment posttest-only control group design of 48 mice that were divided into the control and three mAb *h*ZP3-treatment groups (20, 40, and 60 μg). Mice in each group were terminated on days 10, 15, and 20. GDF-9 expression was measured by immunohistochemistry and the number of theca cells was counted.

Results: Analysis of the effects of mAb *h*ZP3 (at 20–60 μg) on the expression of GDF-9 and amount of theca cells did not show significant differences. Similar findings were observed throughout the study period (at 10–20 days). Therefore, mAb *h*ZP3 had no effect on GDF-9 expression and theca cells.

Conclusion: This study showed that mAb *h*ZP3 can be considered to be an effective and safe immuno-contraception.

Keywords: GDF-9; Growth factors; Monoclonal antibody; Theca cells

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Introduction

The population of Indonesia was estimated to be a quarter billion in 2015. The government has suppressed population growth by establishing *Keluarga Berencana* (KB) throughout Indonesia. Although many kinds of contraception have been used, hormonal contraception is the most common. However, hormonal contraception can have serious side effects, including an increased risk of cardiovascular diseases, such as hypertension, myocardial infarction, and stroke, as well as reproductive cancer.[1]

As an alternative to hormonal contraception, immunocontraception methods are currently being developed, which are considered to be safe, effective, and reversible. The zona pellucida (ZP) is a strong candidate target for immunocontraception due to its glycoprotein membrane, which is required for fertilization. Most species contain three ZP proteins (ZP1, ZP2, and ZP3); however, in humans, there is the additional ZP protein, ZP4.[2–4]

Anti-ZP3-antibodies have been developed in several species, including rabbit and pig,[5] mouse,[6] cow,[7] and human.[8] In a study by Naz, a human monoclonal ZP3 antibody (mAb *h*ZP3) was produced, that has 73% amino acid similarity to a mAb produced in mice.[9]

This ZP3 antibody binds to glucose on the surface of the ZP oocyte, which is close to the sperm receptor, inducing an early cortical reaction, toughening the ZP, and inhibiting sperm penetration.[10] ZP3 antibody has been confirmed to effectively suppress fertilization in several animals, such as mice, rats, cats, and rabbits.[11–13]

The immunocontraceptive effect of ZP3 has been well studied. In addition, Araujo et al. reported that treatment with a murine ZP antibody had no effect on embryo development and follicle quantity.[6] In contrast, Borillo et al. reported that a treated ZP seemed to be more transparent, swollen, and looser by immunohistochemical assessment, and that the ZP antibody inhibited the synthesis of gap junctions.[11] It has been shown that depletion of the ZP leads to incomplete ZP synthesis, disturbing the establishment of gap junctions and decreasing the number of gap junctions. Altered gap junctions leads to decreased intracellular communication during folliculogenesis.[14,15]

Growth differentiation factor-9 (GDF-9) is a growth factor involved in follicle development. GDF-9-BMPRII binding activates AKT and suppresses pro-apoptotic factors, leading to activation of the follicle and oocyte.[16] Previous studies showed that a deficiency in GDF-9 inhibits granulosa cell proliferation and theca cell recruitment.[17] Theca cells are derived from stromal cells and express LH receptor in the pre-antral stage. In response to LH, theca cells secrete androstenedione, an androgen, which is transferred to the granulosa through the basal lamina, leading to the production of testosterone. Along with aromatase enzyme and FSH, androstenedione is converted to estradiol, further promoting ovulation. Therefore, inhibition of theca cell proliferation decreases androstenedione production.[18–20]

A mAb against the ZP was developed about 30 years ago. However, as studies of *h*ZP3 are limited, additional studies of its effect on folliculogenesis are needed. Thus, we aimed to determine the effect of mAb hZP3 on the expression of GDF-9 and the amount of theca cells in the ovary of mice (*M. musculus*).

Materials and Methods

Mice

Mice weighing 20–25 g that had given birth were acclimated and synchronized for 3 weeks. Mice were randomly divided into the control group (injected with 50 μL of adjuvant $Al(OH)_3$ + 50 μL of Tris–HCl) and three treatment groups (injected with mAb hZP3 at 20 μg [P1], 40 μg [P2], or 60 μg [P3]). Each group was sampled based on proestrus cycle 2, 3, and 4.

Measurement of GDF-9 expression

The ovary was removed, and GDF-9 expression was measured by immunohistochemical staining with a primary antibody against GDF-9 (catalog no. bs-4720R) Bioss Inc., USA. GDF-9 expression was measured via semi-quantitative assessment based on Remmele score.

Measurement of theca cells

The ovary was removed, and the number of theca cells was determined by histopathology assessment of hematoxylin and eosin (HE) staining. The numbers of cells in each layer were counted manually.[21]

Results

Saphiro-Wilk and Levene analyses of the data yielded p values > 0.05, indicating that the data were normally distributed and homogeneous (Table 1).

Expression of GDF-9 in mouse oocytes

Figure 1 shows the expression of GDF-9 as a brown chromogen, which is indicated with a black arrow in panel a.

Effect of mAb hZP3 at various doses on the expression of GDF-9

ANOVA showed that there was a significant interaction effect between mAb *h*ZP3 and time on the expression of GDF-9 ($p = 0.017$). However, based on a histogram of the effect of mAb *h*ZP3 at various doses and times on the expression of GDF-9, the average GDF-9 expression in the control group was similar to that in all mAb *h*ZP3 treatment groups. The only significant difference was between the control group on day 20 and the 20 μg mAb *h*ZP3 treatment

Table 1: Results of normality and homogeneity tests.

Variable	Saphiro-Wilk		Levene	
	Coefficient	p-value	Coefficient	p-value
Expression of GDF-9	0.983	0.690	1.379	0.225
Number of theca cells	0.964	0.152	1.243	0.296