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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

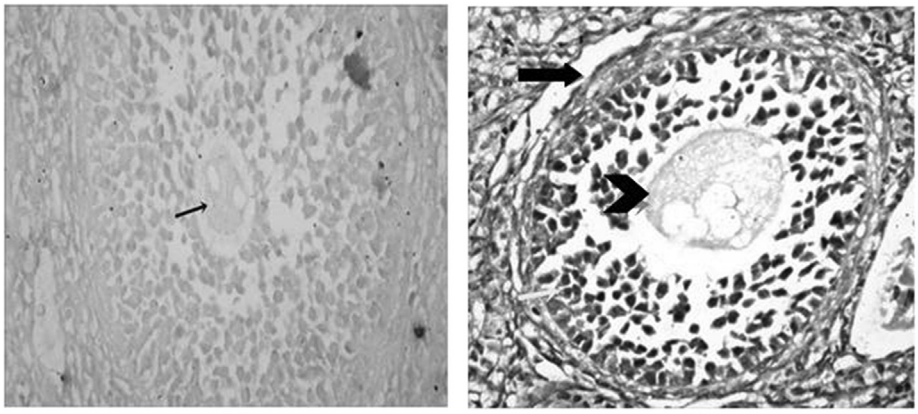


Figure 1: (a) Expression of GDF-9 as a brown chromogen in an oocyte (blue arrow); (b) A secondary follicle containing theca cells (arrow) and proliferating granulosa cells (arrow head).

group on day 10. Moreover, comparison among all mAb *h*ZP3 treatment groups at different observation times showed there was no significant difference in average GDF-9 expression (Figure 2).

Characteristics of the theca cells in secondary follicles

The characteristics of the theca cells and granulosa proliferation cells is shown in Figure 1b.

Effect of mAb hZP3 on the number of theca cells

ANOVA showed there was no interaction effect between the various doses of mAb *h*ZP3 and observation times on the number of theca cells on days 10, 15, and 20 ($p = 0.713$). As shown in Figure 2, the average number of theca cells in the control group was similar to that in all mAb *h*ZP3 treatment groups. Average number of theca cells was lowest in the control on day 10. However, although there was an increase in the number of theca cells following exposure to mAb *h*ZP3 at various doses, the difference was not significant (Figure 2).

Correlation between the number of theca cells and GDF-9 expression

Pearson correlation analysis showed that there was no significant correlation between the number of theca cells and GDF-9 expression in oocytes treated with various doses of mAb *h*ZP3 (correlation coefficient = −0.024; $p = 0.869$; Table 2).

Discussion

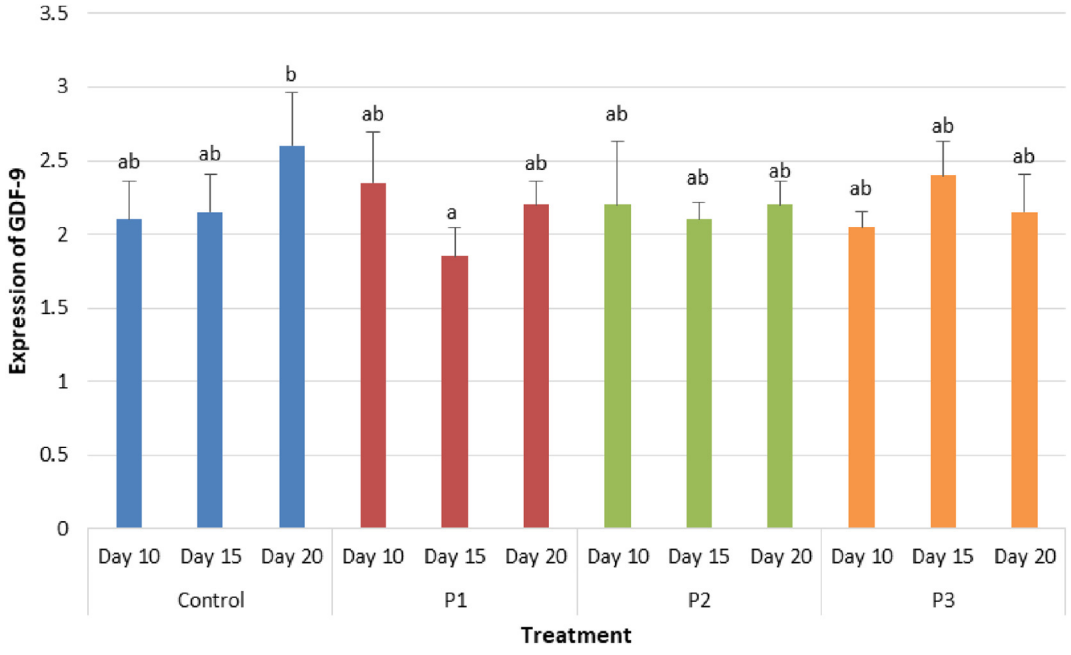
Effect of mAb hZP3 at various doses on the expression of GDF-9

Growth Differentiation Factor-9 (GDF-9), a member of the transforming growth factor β (TGF-β) family, is an oocyte-secreted factor.[22,23] GDF-9 is found in most species, exclusively in the oocyte at nearly every stage of follicle development, except in the primordial oocyte, and at ovulation. After fertilization, GDF-9 mRNA levels decrease, and at 1.5 days postfertilization, it is undetected.[24] The role of GDF-9 was demonstrated in previous studies; it is a strong mitogen that stimulates the proliferation of granulosa and theca cells, the oocyte, and embryo development.

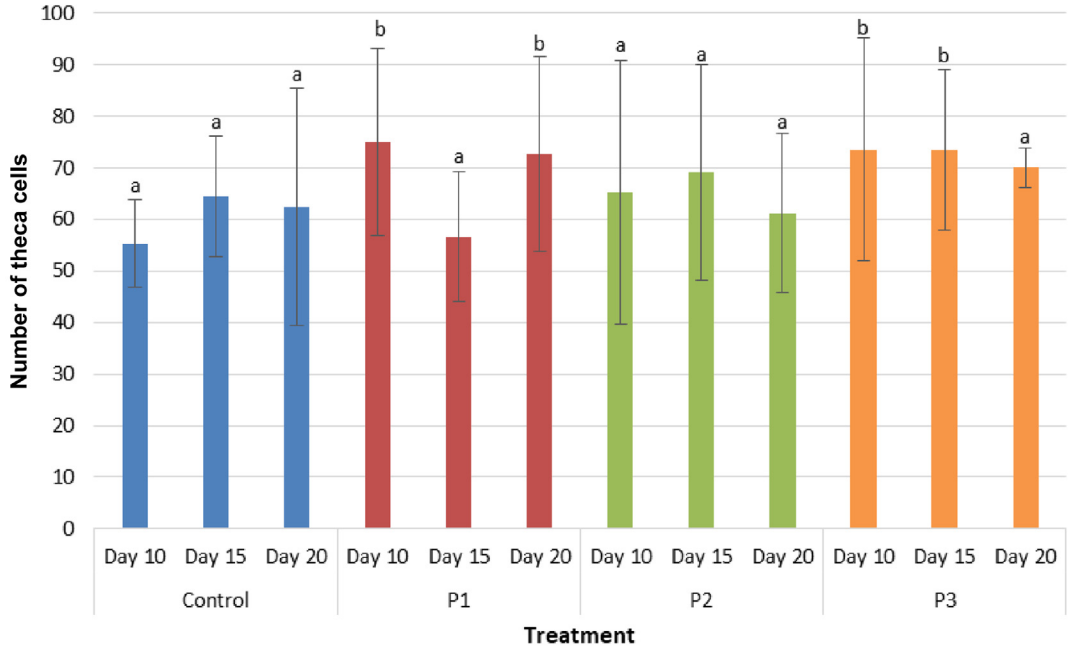
In this study, we showed that treatment with mAb *h*ZP3 did not affect the expression of GDF-9. According to Anifandis et al., the variable domain of ZP3 IgG binds to the surface of the ZP epitope and promotes the early cortical reaction, causing ZP hardening, which leads to inhibition of sperm penetration.[25] Such an alteration might be caused by the monospecificity of mAb *h*ZP3. A variety of amino acids in the variable domain of the ZP3 antibody, which is produced by a single specific B-cell clone, only recognizes a single ZP3 epitope. Abbas et al. reported that an antibody with high specificity can recognize different antigens, even in the presence of a different amino acid.[26] Simply put, antibody specificity is determined by the amount of binding epitope that least recognizable epitope is associated with higher specificity. This indicates that ZP3 antibody ability in mice did not disrupt the ZP synthesis. Stabilization of the ZP is associated with gap junctions, and an intact ZP is associated with properly functioning gap junctions. Therefore, there is no disturbance of gap junctions, as indicated by proper transport of GDF-9 from the oocyte to granulosa cells, leading to proper folliculogenesis. Borillo et al. reported that the alteration of the conformation of the ZP depends on antibody binding during follicle development.[11] Antibody binding to the oocyte in the antral phase and ovulation maintain the structure of the ZP and disrupt the synthesis and secretion of ZP protein when it binds in the preantral phase.

Effect of mAb hZP3 at various doses on the number of theca cells

Theca cells surround the granulosa cells. At the early stage of oocyte differentiation, theca cells are present in a thin layer of polyhedral, lengthwise cells with large nuclei and cytoplasm vacuolization; additional lengthwise cells are then formed, in about 3−5 layers.[27] Data show that mAb *h*ZP3 had no effect



(a)



(b)

Figure 2: The effect of mAb *h*ZP3 at various doses (P1, 20 µg; P2, 40 µg; P3, 60 µg) on the expression of GDF-9 (a) and the number of theca cells (b).

Table 2: Correlation between the number of theca cells and GDF-9 expression.

Correlation variables	Correlation coefficient	p-value	Note
Number of theca cells and expression of GDF-9	−0.024	0.0869	Not significant

on the number of theca cells, as the average numbers of theca cells in the control and treatment groups were not significantly different. As described above, the antibody did not affect follicle development. The high specificity of this antibody means that there is likely no cross reactivity, thus inflammation is avoided.[26] Previous studies also showed that a *m*ZP3 mAb does not disrupt follicle or embryo

development, as the average numbers of small, medium, and large follicles were similar.[28] The primary follicle developed into the secondary follicle, demonstrating proper recruitment and proliferation of theca cells. A similar finding was reported in *p*ZP3-injected monkeys, in which there was no indication of lymphocyte inflammation and infiltration in the ovary, and no increase in follicular atresia.[29] A peroxidase-labeled secondary antibody was used by Greenhouse et al. to characterize the effects of mAb *m*ZP3 and mAb *h*ZP3[30]; the antibody was detected in the cells surrounding the oocyte ZP and the follicles appeared normal, displaying all stages of follicle development, including secondary follicles, tertiary follicles, and the corpus luteum.[11,31] Antibody-ZP binding of a specific, mAb during pre-implantation does not negatively affect embryo development. Thus, the embryo can undergo normal development.[30]

Correlation between the number of theca cells and GDF-9 expression

The GDF-9 signal relies on type 1 and 2 receptors. BMP receptor type II (BMPRII) is the main receptor that is expressed in the oocyte, granulosa cells, and theca cells.[24,32] GDF-9-BMPRII binding mediates activation of the type 1 receptor, ALK 5, followed by phosphorylation of intracellular transcription factors, Smad2, and Smad3.[21,33] This mechanism activates the follicle, causing increased cell proliferation and survival.

Dong et al. analyzed the ovaries of mice with a GDF-9 deficiency, which blocked development at the primary follicle stage. Although oocyte growth and the ZP were normal, the ultrastructure between the oocyte and granulosa was abnormal.[34] In a similar study, it was reported and oocyte growth was faster than normal, while the proliferation and differentiation of theca and granulosa cells was inhibited.[35] In an in vitro study, treatment with GDF-9 increased the amount of secondary follicles and prevented atresia.[37] This is in line with Alves studies, which showed an increase in the diameters of oocytes and follicles in culture medium containing GDF-9.[38] It also describes the role of GDF-9 in triggering the development and survival of the follicle.

This study showed the different results than expected, as the statistical analysis showed there was no significant correlation between GDF-9 and theca cells. Nilsson and Skiner treated mice with GDF-9, and observed no effect on follicle quantity, but only detected in its composition. Besides, GDF-9 also had no effecton the transition from primordial to primary follicle but indirectly stimulated the proliferation of granulosa and theca cells.[36] The GDF-9 signal likely affects growth factor production by increasing expression of KL mRNA. KL promotes the recruitment of theca cells from the stroma and increases their proliferation. In addition to the Kit ligand, IGF, activin, and BMP-15 are also involved in regulating the recruitment and proliferation of theca and granulosa cells and oocyte growth.[16]

Conclusion

In this study, we conclude that treatment of mice with a mAb *h*ZP3 antibody does not affect the expression of GDF-9 or the number of theca cells.

Authors' contributions

LI participated in the design of the study, conducted the research, and drafted the manuscript. LR conducted the research, collected the data, and provided research materials. S provided research ideas and critically reviewed the manuscript. N critically reviewed the manuscript. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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References

1. Sabatini R, Caggiono R, Rabe T. Adverse effects of hormonal contraception. **J Reprod Med Endokrin 2011**; 8: 130–156.
2. Bauskin AR. Characterization of human zona pellucida glycoproteins. **Hum Reprod 1999**; 5: 534–540.
3. Gupta SK, Bansal P, Ganguly A, et al. Human zona pellucida glycoproteins: functional relevance during fertilization. **Int J Chemtech Res 2015**; 83: 50–55.
4. Lefievre L. Four zona pellucida glycoproteins are expressed in human. **Hum Reprod 2004**; 19: 1580–1586.
5. Drell DW, Dunbar BS. Antibodies to rabbit and pig zonae pellucidae distinguish species-specific and shared antigenic determinants. **Biol Reprod 1984**; 30: 445–457.
6. Araujo VR, Gastal MO, Figueiredo JR, Gastal EL. In vitro culture bovine preantral follicles: a review. **Int J Chemtech Res 2014**; 12: 1–78.
7. Sumitro SB, Aulanni'am A. Zona pellucida 3 (ZP3) has proper biochemical properties to be considered as candidate antigen for immunocontraceptive vaccine. **Reprod Tech 2001**; 1: 51–53.
8. Rankin TL, Tong ZB, Castle PE, et al. Human ZP3 restores fertility in Zp3 null mice without affecting order-specific sperm binding. **Development 1998**; 125: 2415–2424.
9. Naz R. Vaccine for human contraception targeting sperm Izumo protein and YLP12 dodecamer peptide. **Int J Chemtech Res 2016**; 23: 857–868.
10. Gupta SK, Bhandari B. Acrosome reaction: relevance of zona pelucida glycoprotein. **Asian J Androl 2011**; 13: 97–105.
11. Borillo J. Antibodies to two ZP3 B cell epitopes affect zona pellucida assembly. **J Reprod Immunol 2008**; 78: 149–157.
12. Mubarakati NJ, Aulanni'am A, Sumitro SB, Ciptadi G. In silico study of conserved region from recombinant human ZP3 (rhZP3) produced in E.coli BL21 competent cells for developing woman immunocontraception. **Int J Chemtech Res 2015**; 8: 243–248.
13. Pantiwati Y. Passive immunization of anti bZP3 (Zona pellucida 3) in wistar rat (*Rattus novergicus*) and mouse (*Mus muscullus*). **Media Peternakan Fakultas Peternakan Institut Pertanian Bogor 2012**; 1: 163–169.
14. Arket CL, Gittens JE, O'Brien MJ, et al. Intercellular communication via connexin gap junctions is required for ovarian folliculogenesis in the mouse. **Dev Biol 2001**; 233: 258–270.
15. Gittens JE, Barr KJ, Vanderhyden BC, Kidder GM. Interplay between paracrine signaling and gap junctional communication in ovarian follicles. **Cell Sci 2005**; 118: 113–122.

16. Sanchez F, Smitz J. Molecular control of oogenesis. **Biochim Biophys Acta 1822**; 2012: 1896–1912.
17. Spicer LJ. Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: influence of follicle size on responses to GDF9. **Biol Reprod 2007**; 78: 243–253.
18. Jameson JL, de Groot J. *Endocrinology: adult and pediatric*. 7th ed. Philadelphia: Elsevier Inc; 2010.
19. Kierszenbaum AL, Tres LL. *Histology and cell biology: an introduction to pathology*. Canada: Elsevier; 2012.
20. Strauss JF, Williams CJ. *Yen & jaffe's reproductive endocrinology*. China: Elsevier; 2014.
21. Magoffin DA. Ovarian theca cell. **Int J Biochem Cell B 2005**; 37: 1344–1349.
22. Erickson GF, Shimasaki S. The role of the oocyte in folliculogenesis. **Trends Endocrinol Metab 2000**; 11: 193–198.
23. McPherson RA, Massey HD. *Henry's clinical diagnostic and management laboratory methods*. China: Elsevier; 2011.
24. Oron G, Fisch B, Ao A, et al. Expression of growth differentiation Factor 9 and its 1 reseptor in human ovaries reproductive. **Int J Chemtech Res 2016**; 21: 109–117.
25. Anifandis G, Messini C, Dafopoulos K, et al. Molecular and cellular mechanisms of sperm-oocyte interactions opinions relative to in vitro fertilization (IVF). **Int J Mol Sci 2014**; 15: 12972–12997.
26. Abbas AK, Lichtman AHH, Pillai S. *Basic immunology: functions and disorders of the immune system*. Canada: Elsevier Inc; 2014.
27. Chapeau C. Alkaline phosphatase activity in the theca of ovarian follicles of the hen throughout follicular development. **Poultry Sci 1996**; 75: 1536–1545.
28. Batista EO, Vieira LM, Sa Filho MF, et al. Ovarian follicular growth suppression by long-term treatment with a GnRH agonist and impact on small follicle number, oocyte yield, and in vitro embryo production in Zeu beef cows. **Theriogenology 2016**; 85: 1680–1687.
29. Bagavant H, Thillai-Koothan P, Sharma MG, et al. Antifertility of porcine zona pellucida-3 immunization using permissible adjuvant in female bonnet monkey (Macaca radiate): reversibility, effect on follicular development and hormonal profile. **J Reprod Fertil 1994**; 102: 17–25.
30. 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.
31. Lloyd ML. Immunoglobulin to zona pellucida 3 mediates ovarian damage and infertility after contraceptive vaccination in mice. **J Autoimmun 2010**; 35: 77–85.
32. Vitt UA. In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. **Endocrinologie 2000**; 141: 3814–3820.
33. Mazerbourg S. Growth differentiation factor-9 signaling is mediated by the type I reseptor activin reseptor like kinase 5. **Mol Endocrinol 2004**; 18: 653–665.
34. Dong J. Growth differentiation factor-9 is required during early ovarian folliculogenesis. **Nature 1996**; 383: 531–535.
35. Carabatsos MJ. Characterization of oocyte and follicle development in Growth Differentiation Factor-9-Deficient mice. **Dev Biol 1998**; 204: 373–384.
36. Nilsson EE, Skinner MK. Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. **Biol Reprod 2002**; 67: 1018–1024.
37. Kierszenbaum AL, Tres LL. *Histology and cell biology: an introduction to pathology*. 3rd ed. Canada: Elsevier; 2012.
38. Alves AM, Chaves RN, Rocha RM, Lima LF, Andrade PM, Lopes CA, et al. Dynamic medium containing growth factor differentiation factor-9 and FSH maintains survival and promotes in vitro growth of caprine preantral follicles after long-term in vitro culture. **Reprod Fertil Dev 2013**; 25(6): 9555–9565.

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