Effect of Anti Zona Antibody on In Vitro Growth and In Vitro Maturation of Intact Follicles

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Tujuan: Mengetahui pengaruh antibodi anti *zona pellucida* terhadap perkembangan (*in vitro growth* = IVG) dan pematangan (*in vitro maturation* = IVM) folikel.

Tempat: Laboratorium biologi dan reproduksi Fakultas Kedokteran Hyogo, Nishinomiya, Jepang.

Rancangan/rumusan data: Studi eksperimen pada hewan coba.

Bahan dan cara kerja: Dilakukan pengambilan 80 folikel intak secara mekanik dari ovarium mencit (C57BL/6 x DBA2-F1 *mice*) usia 16 hari kemudian dilakukan inkubasi pada medium yang mengandung antibodi anti *zona pellucida* selama 8 hari. Antibodi ini diambil dari kelinci yang disuntikkan komponen ZPA dan ZPC mencit. Serum kelinci normal digunakan sebagai kontrol. Folikel dikelompokkan menjadi 4 kelompok dengan masing-masing terdiri dari 20 folikel. Kelompok 1 sebagai kontrol, kelompok 2 diinkubasi dengan serum kelinci normal, kelompok 3 diinkubasi dengan anti ZPA dan kelompok 4 diinkubasi dengan anti ZPC. Setelah 8 hari seluruh folikel dipindahkan ke medium IVM untuk dinilai perubahannya menjadi folikel antral.

Hasil: Secara morfologis tidak dijumpai perbedaan bermakna pada perkembangan folikel antara kelompok kontrol dan perlakuan. Tetapi antibodi anti *zona pellucida* mempengaruhi perkembangan folikel antral pada kelompok perlakuan. Secara statistik dijumpai perbedaan bermakna dalam jumlah folikel antral antar kelompok 3 dan 4 dengan kelompok 1 dan 2. Pada kelompok 3 (anti ZPA) 9 dari 20 folikel (45%) berkembang menjadi folikel antral sedangkan pada kelompok 4 (anti ZPC) 11 dari 20 folikel (55%) berkembang menjadi folikel antral, dibandingkan dengan kelompok 1 dan 2, masing-masing 100% dan 85% folikel pre antral berkembang menjadi folikel antral. Kemudian seluruh folikel antral ditransfer ke medium IVM dan diinkubasi selama 16-17 jam. Pada kelompok 1, 100% folikel mengalami mucifikasi, sedangkan pada kelompok 2, 3 dan 4 masing-masing sebesar 75%, 55% dan 15% folikel mengalami mucifikasi. Setelah dilakukan denudasi *germinal vesicles* (GV) dijumpai sebanyak 5% pada kelompok 1,5% pada kelompok 2, dan 10% pada kelompok 4. Sedangkan pada kelompok 3 tidak dijumpai GV. Metafase 1 dijumpai sebanyak 40% pada kelompok 1,35% pada kelompok 2,50% pada kelompok 3 and 50% pada kelompok 4. Sedangkan metafase 2 dijumpai sebanyak 55% pada kelompok 1,60% pada kelompok 2, dan 40% pada kelompok 3. Tidak dijumpai metafase 2 pada kelompok 4. Beberapa oosit yang berdegenerasi dijumpai pada kelompok 2 (5%), kelompok 3 (5%) dan kelompok 4 (30%). Terdapat perbedaan bermakna dalam hal pematangan folikel (IVM) antara kelompok kontrol dan perlakuan.

Kesimpulan: Antibodi anti *zona pellucida* mempengaruhi proses perkembangan dan pematangan folikel in vitro. Efeknya pada fertilisasi masih harus diteliti lebih lanjut.

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Kata kunci: folikel intak, perkembangan folikel (IVG), pematangan folikel (IVM), antibodi anti *zona pellucida*, folikel antral, mucifikasi, *germinal vesicles*, metafase-1, metafase-2

Objective: *To determine effect of anti zona antibody on in vitro growth (IVG) and in vitro maturation (IVM) of intact follicles.*

Setting: *Laboratory of Developmental Biology and Reproduction Hyogo College of Medicine, Nishinomiya, Japan.*

Design/data identification: *Experimental animal study.*

Material and methods: *Intact follicles were collected from 16-days old mice (C57BL/6 x DBA2F1 mice) by mechanical treatment and incubated at antiserum for eight days. Antiserum was prepared from rabbits immunized synthetic peptides of mouse ZPA and ZPC. Normal rabbit serum was used as control. Intact follicles were divided into 4 groups, group 1 as a control (no rabbit serum), group 2 with normal rabbit serum (control), group 3 with anti-ZPA antibody and group 4 with anti-ZPC antibody. After IVG the intact follicles were transferred to IVM medium without antisera to assess antral formation and mucified of follicles.*

Results: *There were no significant differences in morphological appearance of grown intact follicles at the microscopic level between control and antibody-treated group. However antral formation from preantral follicles during follicle culture was interfered with anti-ZPA and anti-ZPC antibody. There were significant differences ($p<0.05$) in antral formation of group 3 and 4 compare to control (group 1 and 2). In anti-ZPA group there were 9 of 20 (45%) preantral follicles become antral follicles and in anti-ZPC group there were 11 of 20 (55%) preantral follicles become antral follicles comparing with group 1, 100% of preantral become antral follicles and group 2, 85% of preantral become antral follicles. After transferred to IVM medium, 20 of 20 (100%) follicles in group 1, 15 of 20 (75%) follicles in group 2, 11 of 20 follicles (55%) in group 3 and 3 of 20 (15%) follicles in group 4 are mucified. After mechanically denuded, germinal vesicles (GV) are found 5% in group 1, 5% in group 2, no GV in group 3 and 10% in group 4. Metaphase-1 are found 40% in group 1.35% in group 2. 50% in group 3 and 50% in group 4. Metaphase-2 are found 55% in group 1.60% in group 2,40% in group 3. There is no metaphase-2 in group 4. Some oocyte degeneration are found in group 2 (5%), group 3 (5%) and group 4 (30%). There were significant differences in maturation of oocytes between control and antizona antibody group.*

Conclusion: *Antizona antibody influenced both of IVG and IVM of intact follicles. The result of fertilization should be elucidated further.*

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Keywords: *intact follicles, IVG, IVM, anti zona antibody, antral formation, mucified, germinal vesicles, metaphase-1, metaphase-2*

INTRODUCTION

The zona pellucida (ZP) is a matrix composed of glycoproteins which surrounds all mammalian oocytes and mediates several important roles such as binding sperm in a species-specific manner, inducing the acrosome reaction, preventing polyspermy and protecting the embryo prior to implantation. The ZP contains receptors that mediate initial interactions between sperm and egg as a prelude to fertilization.[1-10]

The ZP is composed of three proteins in both mice and humans: ZP1, ZP2 and ZP3 which are synthesized and secreted during oocyte growth and follicular development. Cloning of the ZP genes from a number of species has resulted in the introduction of an alternative nomenclature of ZPA (ZP2), ZPB (ZP1), ZPC (ZP3) based on gene size.[1,3,4,9]

Mouse ZP is an extensive three-dimensional array of long interconnected filaments that consist of structural repeat. The filaments are constructed by alternating molecules of ZP2 and ZP3, randomly cross-linked by ZP1 through intermolecular disulfide bonds. It forms a spherical shell of remarkably uniform thickness which is penetration of this shell by spermatozoa plays a crucial role in mammalian fertilization. Any inability of spermatozoa to penetrate the zona pellucida inevitably leads to infertility.[1,3,4,8,9]

Bidirectional communications which traverse the zona pellucida between oocyte and granulose cells through microvilli and gap junctions are essential for oocyte and follicular development. Since the zona pellucida is formed at an early stage of oocyte growth, circulating antibodies against zona pellucida may impair ovarian function. Antibodies against the zona pellucida may interfere follicular development and oocyte maturation.[11]

In premature ovarian failure (POF) subjects, a variety of auto-antibodies and possible target antigens have been reported so far, including antibodies binding to the zona pellucida. Grootenhuis et al (unpublished observations), recently found three of 34 POF patients positive for IgG antibodies toward human recombinant ZP3. Autoantibodies to ZP have been also described as a cause of infertility in women. In women with unexplained infertility, these antibodies were seen in 5.6% of the cases, whereas in the normal controls positivity was seen in only 1.7%. ZP antibodies were thought to interfere with the sperm-oocyte interaction, thus inducing infertility.[12,13]

This study will describes the influence of the anti-zona antibodies on follicle growth and maturation in animal experimental.

MATERIALS AND METHODS

Specific-pathogen-free female mice F1 (C57BL/6 x DBA/2) - Japan SLC Inc. Shizuoka Japan were used as animal experiments. Mouse ovaries were taken from 16 days old mice and immersed in collection medium. Antiserum was prepared from rabbits immunized synthetic peptides of mouse ZPA and ZPC conjugated diphteria toxoid.

In this experiments, intact follicles were collected from 16 days old mice by mechanical dissection with 27-G needles. Collected follicles were cultured on a plate equipped with a Transwell-COL membrane insert (3.0-μm pore size, 12-mm diameter; Corning Coaster Corp, Cambridge, MA) and incubated at 10% antiserum for six days. Normal rabbit serum was used for a control. The in vitro growth (IVG) medium was 18 ml a-minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS), 200 μl glutamine (SIGMA), 200 μl insuline-transferrin-selenium (ITS-SIGMA), 100 μl penicillin-streptomycin solution (invitrogen Tokyo - Japan) and 27 μl follitropin (Serono - Geneva Swiss). About 80 intact follicles were incubated in 4 ml of IVG medium under 5% CO_2, 5% O_2 and 90% N_2 at 37°C for 6 days. Half of the medium was changed every other day.

After in vitro growth culture, the intact follicles were transferred to the in vitro maturation (IVM) medium without antisera to assess antral follicle maturation. The IVM medium composed of IVG medium adding by 80 μl EGF and 10 μl hCG. Sixteen hours afterward, we assesed the mucified follicles with expanded granulosa cells. By gentle pipetting oocytes could be seen with the first polar body were assessed as metaphase II stage, and those with neither a germinal vesicle nor a polar body were assessed as metaphase I stage.

RESULTS

Eighty intact preantral follicles stage were collected from fresh mouse ovaries and subjected to IVG culture. At day 6^{th}, 57 follicles had grown become antral follicles. In group 1 (control) 100% antral formation was occured, in group 2 (NRS - control group) 85% preantral become antral follicles. The percentage was significantly lower in antizona antibody group, group 3 (anti ZPA) and group 4 (anti ZPC) were only 45% and 55% preantral follicles grown become antral follicles as shown in Table 1.

Table 1. In vitro growth of preantral follicles

Antisera	Used follicles	Antral formation	%
Control	20	20	100[a]
NRS	20	17	85[b]
Anti ZPA	20	9	45[c]
Anti ZPC	20	11	55[d]

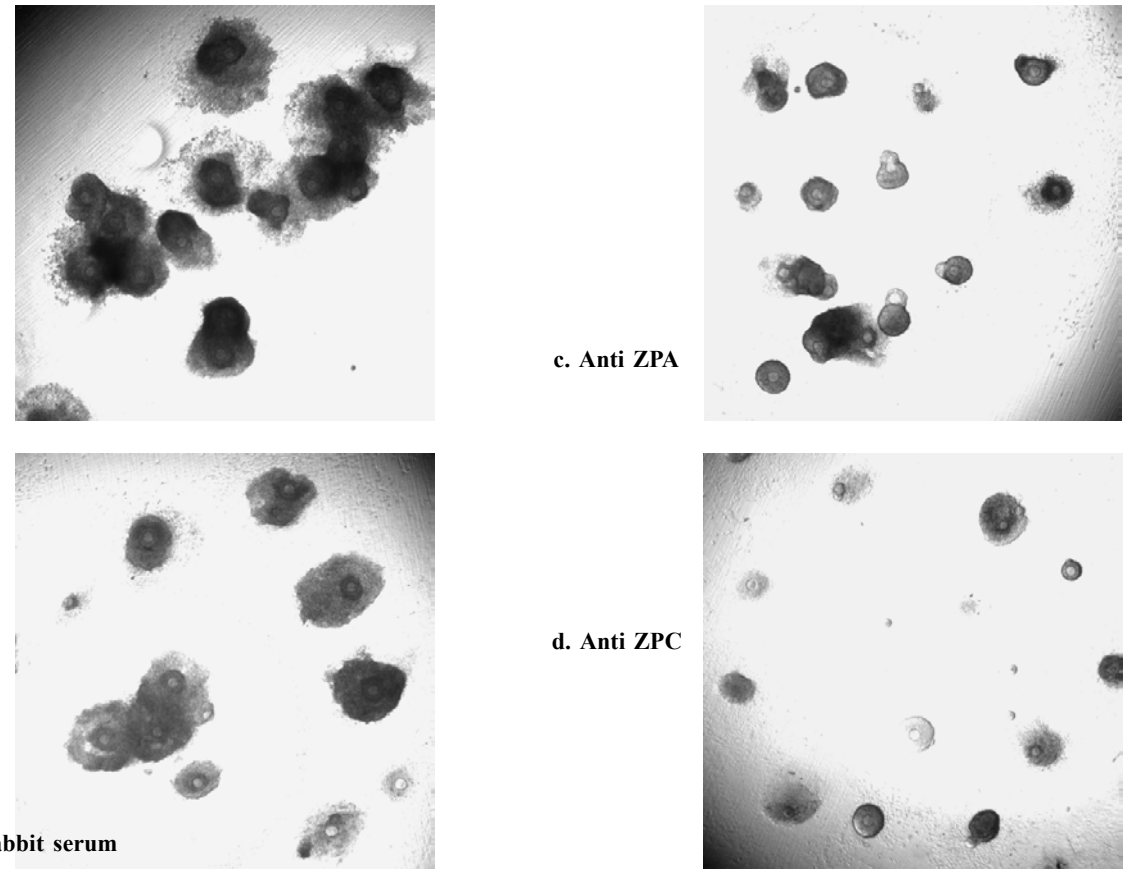
Note: [ab] significantly different with [cd] (P<0.05, X^2 test)

Antral formation of preantral follicles in control and anti-zona antibody group could be seen in figure 1. After detached from culture membrane, antral follicles were transferred to IVM medium and incubated for 16 hours. Finally 49 follicles survived and mucified at day 7th. Meiotic progression was assessed after removal of mucified granulosa cells. Germinal vesicles (GV) are found 5% in group 1, 5% in group 2, no GV in group 3 and 10% in group 4. Metaphase-1 are found 40% in group 1, 35% in group 2, 50% in group 3 and 50% in group 4. Mature oocyte or metaphase-2 are found 55% in group 1, 60% in group 2, and 40% in group 3. There is no metaphase-2 in group 4. Some oocyte dege-neration are found in group 2 (5%), group 3 (5%) and group 4 (30%). There were significant differences in maturation of oocytes between control and anti-zona antibody group as shown in Table 2.

Table 2. In vitro maturation of antral follicles

Antisera	Used follicles	Mucified	GV	M-I	M-II	Degeneration
Control	20	20 (100%)	1 (5%)	8 (40%)	11 (55%)[a]	–
NRS	20	15 (75%)	1 (5%)	6 (30%)	12 (60%)[b]	1 (5%)
Anti ZPA	20	11 (55%)	–	10 (50%)	8 (40%)[c]	1 (5%)
Anti ZPC	20	3 (15%)	2 (10%)	10 (50%)	– d	6 (30%)

Note: [ab] significantly different with [cd] (p<0.05, X^2 test)



a. Control

c. Anti ZPA

b. Normal rabbit serum

d. Anti ZPC

Figure 1. Antral formation of preantral follicles in control dan anti-zona antibodies group

DISCUSSION

There were no significant differences in morphological appearance of grown intact follicles at the optical microscopic level between the control and antibody-treated groups (as shown in Figure 1) although antral follicles in anti-zona antibodies groups seems to be smaller. However antral formation from preantral follicles during follicle culture was interfered with anti-ZPA and anti-ZPC antibody which is fewer in treatment groups (Table 1), and after in vitro maturation the mucification and oocyte maturation rates in the antibody-treated group were also significantly lower than those of the control group (Table 2). This results are very similar with Koyama and collegues study in 2005.[11]

Green et al by their study explained that mouse ZP are constructed by alternating molecules of ZP2 (ZPA) and ZP3 (ZPC), randomly cross-linked by ZP1 (ZPB) through intermolecular disulfide bonds.[3,4] This means that ZP2 (ZPA) and ZP3 (ZPC) are essential for ZP formation. Other study about genetically knockout mice revealed that ZP2 (ZPA) knockout mice formed very thin layer of ZP and the female mice were infertile which is ZP3 (ZPC) knockout mice completely lacked the ZP structure and were also infertile. These suggest that ZP2 (ZPA) and ZP3 (ZPC) are involved in oocyte growth and follicular development.[10]

Our study indicated that anti-ZPA antibody and anti-ZPC antibody inhibited both of follicular growth and oocyte maturation. In vivo experiment of immunized hamster by recombinant protein of hamZP2 resulted in the production of self-reactive antibodies that were bound to their own zona pellucida. Significant decreases in the number of follicles at every stage of development including primordial follicles were observed by histological examination. Anti-zona antibody probably induces ovarian dysfunction by impairing communication between oocyte and granulosa cells, there by leading to infertility or POF.[11] Recent study by Koyama et al showed that 50% of POF patients have positive reaction for anti-zona antibodies.[10]

Shivers and Dunbar study showed the presence of anti-ZP antibodies in infertile women by direct immunofluorescent staining using pig ZP.[14] Some researchers found a significantly higher incidence of anti-ZP antibodies in unexplained infertile women than other cases. They also showed the presence of such antibodies in anovulatory infertile women. The anti-ZP antibody was also found in low responders to gonadotropin during in vitro fertilization (IVF).[15,16]

It is possible that if anti-zona antibodies are present in the follicular fluid because it inhibited both of follicular growth and maturation. However their effect on fertilization should be elucidated further.

CONCLUSIONS

The zona pellucida is important for follicular growth and oocyte maturation. Anti-zona antibodies caused ovarian dysfunction through the impairment of follicular growth and oocyte maturation in the animal experiments. This present results suggest that we must consider the adverse effect of autoimmunity to zona pellucida on ovarian function.

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