### Journal für

# Reproduktionsmedizin und Endokrinologie

- Journal of Reproductive Medicine and Endocrinology -

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J. Reproduktionsmed. Endokrinol 2006; 3 (2), 94-97

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Indexed in EMBASE/Excerpta Medica/Scopus

Krause & Pachernegg GmbH, Verlag für Medizin und Wirtschaft, A-3003 Gablitz

## Premature Ovarian Failure Syndrome May Be Induced by Autoimmune Reactions to Zona Pellucida Proteins

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Autoimmunity is thought to be involved in pathogenesis of the premature ovarian failure (POF) causing infertility. The zona pellucida (ZP), an extracellular matrix surrounding the oocyte, is considered to be pathogenic among autoantigens, because many contraceptive vaccine research has shown that anti-ZP antibodies impair ovarian function in animal experiments. In this article we describe the importance of ZP in oocyte growth and the possible effects of anti-ZP antibodies on ovarian failure. Clinical experiments were conducted to detect anti-ZP antibodies in POF patients by dot immunoassay and immunofluorescent staining method. Also, as an animal model experiment, we examined whether or not peptide ZP antigens of same-species amino acid sequences could produce autoantibodies reactive to ZP. The results showed that antibodies were produced by immunisation with the antigens and histological examination revealed that the number of growing follicles was considerably reduced. These results conclusively indicated that self-ZP protein is a possible pathogenic antigen for autoimmunity causing POF. J Reproduktionsmed Endokrinol 2006; 3 (2): 94–7.

Key words: anti-zona antibody, premature ovarian failure, zona pellucida, infertility

**P** remature ovarian failure (POF) refers to a range of symptoms from amenorrhea to cessation of ovarian function before age 40 associated with elevated FSH levels, which occurs in approximately 1 % of women. As for pathogenic mechanisms of POF, genetic causes, autoimmune causes, iatrogenic factors and environmental factors are indicated, but in many cases no cause is identified and they are classified as "idiopathic" POF [1]. Among these pathogenic mechanisms, up to 30 % of cases are considered to be due to autoimmune causes [2].

Autoimmune ovarian failure is clinically divided into two categories (Tab. 1). One is associated with somatic autoimmune disorders including endocrine and non-endocrine autoimmune disease. The other is isolated autoimmune ovarian failure. In this group, various degrees of ovarian dysfunction are observed without other autoimmune disorders [3–5]. Early studies showed various

Table 1. Two categories of autoimmune ovarian failure

1. Associated with other autoimmune disorders	
<ul> <li>Endocrine <ul> <li>Adrenal/thyroid/IDDM/MG</li> </ul> </li> <li>Non-endocrine <ul> <li>Chronic candidiasis/ITP/SLE/RA</li> </ul> </li> </ul>	
2. Isolated autoimmune ovarian failure	
<ul> <li>Autoimmune oophoritis</li> <li>Autoantibodies to <ul> <li>steroid-producing cells and enzymes</li> <li>gonadotropin (FHS/LH) receptors</li> <li>zona pellucida</li> </ul> </li> </ul>	
IDDM = Insulin-dependent diabetes mellitus; MG = Myasthenia gravi	

IDDM = Insulin-dependent diabetes mellitus; MG = Myasthenia gravis ITP = Idiopathic thrombocytopenic purpura; SLE = Systemic lupus erythematosis; RA = Rheumatoid arthritis

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incidences of ovarian autoantibodies in POF patients using whole ovarian homogenates. Variations in detection of ovarian autoantibodies are likely to be due to study design such as adopted methods, antigen preparation and criteria for assessment in different laboratories [6]. Several ovarian components such as steroid producing cells and enzymes, gonadotropin receptors and zona pellucida (ZP) are postulated as target antigens [7–9] but the actual pathogenic antigens still remain unclear. Recently, several growth factors and cell surface molecules have been identified as critical substances for oocyte growth [10]. These components are also candidates for a target antigen causing POF. In this article, we focus on the relationship between ovarian failure and anti-ZP antibodies.

#### Structure and Function of Zona Pellucida

The ZP is an extracellular matrix that surrounds growing oocytes, ovulated eggs and pre-implantation embryos, and is known to be involved in several important events during fertilisation (Fig. 1A). It has been shown that ZP mediates relatively species-specific sperm-egg recognition and induction of acrosome-reaction events that are critical to penetration of the ZP. Following fertilisation, ZP prevents the penetration of additional sperm and protects the pre-implantation embryo during passage down the oviduct [11, 12].

On the other hand, bidirectional communication between the oocyte and surrounding granulosa cells is essential for follicle development. It is realised via microvilli, which penetrate the ZP and form gap junctions with the oocyte's membrane [13]. Electron microscopic observation of the ZP is shown in Figure 1B. Oocyte and surrounding cumulus granulosa cells communicate with each other through microvilli traversing the ZP. This bidirectional communication is probably important for oocyte growth and follicular development. Structural and functional abnormalities of the ZP thus cause impairment of ovarian function. In addition, some ZP components were found in the intercellular spaces of granulosa cells [14, 15]. This suggests that ZP antibodies interfere with granulosa cell function, although it is unknown whether the components are produced by granulosa cells themselves or penetrated into the spaces from the oocyte.

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**Figure 1.** Role of the zona pellucida (ZP) in fertilisation and oocyte maturation. **A:** Schematic drawing of sperm-ZP interaction (modified from [11]). The capacitated sperm attaches and then tightly binds to the ZP (**a**, **b**). These interactions are mediated by ZP3 which serves as a primary receptor for sperm. The acrosome reaction in the bound sperm is induced which is essential for sperm penetration of ZP (**c**). ZP2 maintains acrosome-reacted sperm in the ZP during penetration (**d**). Following fusion of egg and sperm, cortical granules disintegrate to induce the zona reaction that prevents polyspermy (**e**, **f**). Proteolytic cleavage of ZP2 and ZP3 is involved in the zona reaction. After fertilisation ZP still remains to protect the fertilised eggs. **B:** Electron microscopic picture of a mouse oocyte section, demonstrating the zona pellucida (ZP), framed by a granulosa cell (GC) and the oocyte (OV). Protruding micro-villi of the granulosa cell are visible within the ZP.

#### **Genetically Knockout Mice**

The ZP is composed of three glycoproteins in various animal species named ZP1, ZP2, and ZP3. Recently, ZP1, ZP2, and ZP3 knockout mice were produced and new findings were reported [16–18] that ZP1 knockout mice formed morphologically-abnormal ZP and had reduced fecundity [16]. ZP2 knockout mice formed very thin layer of ZP on the oolemma which was barely visible under an optical microscope and the female mice were infertile. [17] ZP3 knockout mice completely lacked the ZP structure and were also infertile [18]. These results indicate that ZP3 is essential for ZP formation and the presence of the other components, ZP1 and ZP2, is insufficient for mouse follicle development and fertility.

It is interesting that each knockout mouse showed different degrees of deficiency in zona formation. This suggests that ZP1, ZP2, and ZP3 proteins may contribute differently in zona formation and oocyte growth. Further studies revealed that the infertility in ZP2 and ZP3 knockout mice was due to disruption of follicular formation. These results clearly demonstrated that ZP2 and ZP3 are involved in oocyte growth and follicular development and also indicated that genetic deficiency of ZP2 and ZP3 may cause ovarian failure in infertile patients.

#### Contraceptive Vaccine Development Using ZP Antigens

Since it was reported that antibodies against ZP blocked fertilisation [19, 20], a lot of research has been done to develop a contraceptive vaccine. However, it was very difficult to avoid ovarian failure associated with abnormal estrus cycles and hormone levels even when recombinant proteins were used for immunisation [21–23]. Strong ovarian disruption was often induced by immuni-

sation with ZP antigen from different species including monkeys and dogs. The defective ovarian histology resembled that from human ovarian failure [24–26].

#### **Incidence of Anti-ZP Antibodies**

Shivers and Dunbar [27] first reported the presence of anti-ZP antibodies in infertile women by indirect immunofluorescent staining using pig ZP. Since then, a large number of studies have been reported associating anti-ZP antibodies and ovarian failure. 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 [28, 29]. The anti-ZP antibody was also found in low responders to gonadotropin stimulation during *in vitro* fertilisation and embryo-transfer (IVF-ET) treatment [30]. However, controversial results have been also reported. Such conflicting results are attributed to the methodological differences.

On the basis of these observations, we designed experiments to detect anti-zona auto-antibodies by dot immunoassay using human zona pellucida. The human ZP was isolated by pipetting from oocytes that had failed fertilisation in a clinical program of IVF-ET under informed consent. The preparation was solubilised at 70 °C for 30 minutes in phosphate buffer saline (PBS). 1 µl containing 0.01 µg of protein was dotted onto a nitrocellulose membrane. After blocking with bovine serum albumin containing PBS, the membrane was incubated with the patients' sera and subsequently treated with an anti-human IgG antibody conjugated with horseradish peroxidase. Color development was performed by chloronaphthol and H<sub>2</sub>O<sub>2</sub>. Densitometric values showing more than twice over standard deviation (SD) values of the control group (healthy males) were assessed as positive. 50 %



**Figure 2.** Detection of anti-ZP antibodies in POF patients by dot immunoassay. The human zona pellucida was isolated from unfertilised oocytes in a clinical program of IVF-ET under informed consent. Solubilised protein (0.01 µg) was dotted onto a nitrocellulose membrane. The membrane was incubated with the patients' diluted sera and subsequently treated with an anti-human IgG antibody conjugated with horseradish peroxidase. Color development was performed by chloronaphthol and H<sub>2</sub>O<sub>2</sub>. Densitometric values showing more than twice over the SD values of the control group (healthy males) were assessed as positive. 50 % (5/10) of POF patients showed positive reaction for anti-zona antibodies, while 11.8 % (2/17) were positive in the other infertile women.



Figure 3. Immunofluorescent staining of human oocytes with sera from POF and the other infertile patients. Isolated human oocytes prepared as described in Fig. 2 were treated with patients' diluted sera for one hour at room temperature. After washing with PBS, the treated oocytes were further incubated in FITC-conjugated anti-human IgG for 50 minutes. Observations were carried out under a fluorescent microscope. Each number corresponds to the serum samples in Fig. 2. The immuno-fluorescent intensities did not correlate to the values in the dot immuno-assay. (1)–(5) are oocytes stained with POF patients' sera, (6) and (7) are oocytes stained with other infertile patients' sera. (8) is an oocyte stained with a healthy male's serum showing a negative reaction in dot immunoassay.

(5/10) of POF patients showed positive reaction for antizona antibodies, while 11.8 % (2 of 17) were positive in the other infertile women (Fig. 2). The incidence was significantly higher in POF than in the other infertile women. Figure 3 shows immunofluorescent staining of human oocytes with POF patients' sera. They reacted to human ZP to various degrees. Some sera showed strong reactions, but the intensities did not correlate with the values in the dot immunoassay.

#### ZP Antibody Production by Immunisation with Homologous-sequence Antigens

We conducted a homologous immunisation experiment using hamsters (*Mesocricetus auratus*) to examine whether



**Figure 4.** Increase of antibody titers in ELISA against the synthetic peptide used for immunisation. An 18-mer synthetic peptide of dog ZP2 (88–106 amino acids) was prepared and conjugated with a carrier protein, diphtheria toxoid for immunisation. The immunogen (500 µg) was injected with complete Freund's adjuvant into three 3-month-old dogs at one-month intervals. Antibody titers were determined by ELISA against the cognate peptide antigen.



**Figure 5.** Histological examination of ovarian sections from dog immunised with ZP2 peptide. As compared to the control (**A**), the ovarian section from the immunised dog showed remarkable impairment (**B**). No growing follicle was detected and a fibrous change was observed in the interstitial tissue.

or not ZP2 antigens induce antibody production in same species and if the antibodies produced disrupt normal ovarian function [31]. Hamster cDNA for ZP2 was cloned in our laboratory (NCBI accession #AY876920) and a recombinant protein of hamZP2 (583 amino acids) was produced for immunisation. Ovaries from immunised hamsters were removed for histological examination at 10 weeks after the initial immunisation followed by several booster injections.

Antibodies reactive to homologous recombinant antigens were produced in hamZP2-immunised hamsters. These antibodies were found to be present in the form of complexes with the ovarian zona pellucida in immunised animals. Significant decreases in the number of follicles at every stage of development including primordial follicles were observed by histological examination. Immunisation of hamsters with the homologous recombinant protein resulted in the production of self-reactive antibodies that were bound to their own zona pellucida. The antigen-antibody complex probably induced ovarian dysfunction by impairing communication between oocyte and granulosa cells, thereby leading to POF.

Similar results were obtained in dogs. An 18-mer synthetic peptide of dog ZP2 (88–106 amino acids) was prepared for immunisation in three female dogs. The blood antibody titers by ELISA reached their maximum from the initial immunisation and remained at this level until the end of the experiment (Fig. 4). These animals did not show any estrus cycle, although the control animals of the same age did.

Figure 5 shows a typical ovarian section from an immunised dog. As compared to the control, the ovarian sections from immunised dogs showed remarkable impairment. No growing follicles were detected. A fibrous change was observed in the interstitial tissue, but inflammation reaction such as lymphocyte infiltration was not observed. This suggested that anti-ZP antibody interfered with oocyte-granulosa cell interactions via binding to the ZP. Actually, the potential function of gap junctions traversing the ZP is important for oocyte maturation [13]. In addition, granulosa cells are possibly impaired by antibodies bound to the cell surface, because some ZP components are detected in the intercellular spaces of the granulosa cells [14, 15].

#### Conclusion

POF is a disorder induced by multiple ectopic factors. Since the ZP was found around the oocyte at early stages of folliculogenesis, it has also been considered as a causal factor of POF. This study showed that anti-ZP antibody was detectable with high incidence (50 %) in POF patients when using human ZP as an antigen. The animal experiment revealed that homologous ZP antigen could produce self-reactive ZP antibodies and causes ovarian disorder similar to human POF. We therefore have to take into consideration the fact that anti-ZP autoantibody causes adverse effects on ovarian function as well as fertilisation.

#### Acknowledgment

This work was supported by a Grant-in-Aid of Scientific Research (No. 14370536) from MEXT (Ministry of Education, Science, Culture, Sports and Technology), 2002– 2004 and by the "High-Tech Research Center" Project for Private Universities: matching fund subsidy from MEXT, 2004–2008. The authors appreciate the useful discussions with their colleagues and the technical assistance of the laboratory staff.

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