

High doses of medroxyprogesterone as the cause of disappearance of adherence of the zona pellucida to an oocyte

Barbara Jodłowska-Jędrych · Marian Jędrych ·
Włodzimierz Matysiak

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Abstract The zona pellucida (ZP) is an external glycoprotein membrane of oocytes of mammals and embryos in the early stage of their development. ZP first appears in growing ovarian follicles as an extracellular substance between the oocyte and granular cells. The zona pellucida markedly affects the development and maturation of the oocyte. The morphology of the ZP-oocyte complex allows a more precise determination of the oocyte maturity. According to numerous experimental studies, ZP is essential for preimplantation embryonic development of humans and other mammals. It prevents dispersion of blastomeres and enhances their mutual interactions. ZP is a dynamic structure responsible for the provision of nutrients to early forms of oocytes in mammals. The aim of the present study was ultrastructural evaluation of the ZP-oocyte contact during inhibited ovulation. Female white rats (Wistar strain) received a suspension of medroxyprogesterone acetate (MPA) in incremental intramuscular bolus doses of 3.7 mg (therapeutic dose), 7.4 mg and 11.1 mg. The animals were decapitated 5 days after the administration of MPA. Ovarian sections were evaluated under a transmission electron microscope (TEM) Zeiss EM 900. Morphometric analysis of ZP was conducted using the cell imaging system by Olympus. In females exposed to

therapeutic doses of MPA, ZP showed the structure of granular-fibrous reticulum of a medium electron density with single cytoplasmic processes originating from the surrounding structures. The oocyte cell membrane generated single, delicate processes directed toward ZP. Microvilli of the oocyte were short and thin. In the group receiving 7.4 mg of MPA, ZP had the structure of a delicate, loose granular-fibrous reticulum, and the oocyte cell membrane generated single microvilli directed toward ZP. In both those groups, the close ZP-oocyte contact was observed. Otherwise, in the group exposed to the highest MPA doses (11.1 mg), thicker and more numerous oocyte microvilli were found, which did not penetrate ZP matrix. They were dense, irregularly separated contour, forming a barrier between ZP and oocyte. The present findings are likely to suggest that MPA has inhibiting effects on the synthesis of binding proteins and causes the loss of the oocyte contact with ZP.

Keywords Zona pellucida · Oocyte · Medroxyprogesterone · Transmission electron microscopy

Abbreviations

ZP Zona pellucida
MPA Medroxyprogesterone acetate
O Oocyte

B. Jodłowska-Jędrych (✉) · W. Matysiak
Department of Histology and Embryology,
Medical University of Lublin,
Radziwiłłowska 11,
20-080 Lublin, Poland
e-mail: b.jedrych@gmail.com

M. Jędrych
Department of Mathematics and Biostatistics,
Medical University of Lublin,
Jaczewskiego 4,
20-067 Lublin, Poland

Introduction

The zona pellucida (ZP) is an external glycoprotein membrane of oocytes of mammals and embryos in the early stage of development (Dunbar et al. 2001; Prasad et

al. 2000; Sinowatz et al. 2001a, b; Zhao and Dean 2002). ZP first appears in growing ovarian follicles as an extracellular substance between the oocyte and granule cells (Rankin et al. 2000).

Ultrastructural studies in mouse oocytes from primary follicles reveal the presence of electron-dense matrix filling the depressions among oocyte microvilli (Zhao and Dean 2002). Such a matrix is considered to be the first element of the developing ZP. The ovarian follicle growth coincides with changes in the structure and thickness of ZP (Qi et al. 2002; Vanroose et al. 2000).

The site of ZP biosynthesis has not been entirely explained. According to some authors, ZP is synthesized by the oocyte (Epifano et al. 1995; Haddad and Nagai 1977; Kimura et al. 1994). Observations of mouse oocytes conducted by Wassarman et al. (2004) suggest a considerable involvement of the oocyte Golgi apparatus in modifying protein particles, which are the precursors of ZP glycoproteins. The highest activity of this cell organelle was observed in gametes located in growing ovarian follicles. A large number of secretory follicles were found in the circumferential part of the cytoplasm. The studies mentioned suggest that the synthesis of ZP takes place inside the oocyte. Moreover, as shown by the findings of other studies in mice, the oocyte is the sole element responsible for the synthesis of this structure (Epifano et al. 1995; Haddad and Nagai 1977; Kimura et al. 1994).

However, the majority of authors provide evidence that the zone pellucida is created by both the oocyte and granule cells, with greater impact on the latter (Bogner et al. 2004; Rankin et al. 2000; Sinowatz et al. 2001a, b).

ZP has a fibrous structure. It consists of the complex of four glycoproteins, designated as ZP1, ZP2, ZP3 and ZP4. The active synthesis of ZP proteins decreases at the end of oogenesis, and de novo synthesis is infeasible after implantation (Bleil and Wassarman 1980; Shimizu et al. 1983).

Glycoproteins determine the specific structure of the surface of the external ZP, which is not uniform. Visualization of canine oocytes by scanning electron microscopy has shown that ZP has a structure of fibrous network with the presence of a varying number of fenestrations (Ström Holst et al. 2000). Moreover, according to Ström Holst et al. (2000), there are no morphological differences in the envelope depending on the oocyte age.

Analyzing the surface structure of ZP of human oocytes, Magerkurth et al. (1999) divided ZPs into several types, i.e., type A—the surface of ZP has the form of a network with numerous pores and depressions; type B—a similar structure, yet the depressions are shallower and smaller in diameter compared to class A; type C—a spongy, uneven surface, very few or even no pores: the network structure almost disappears and type D—characterized by an almost

smooth external surface: traces of pores and depressions visible only in some places.

ZP constituting the natural surrounding provides the oocyte with natural microenvironment and has an important influence on its development and maturation. ZP and granule cells forming the corona radiata are responsible for the biological functions of the gamete. The morphology of the ZP-oocyte complex allows a more precise determination of the oocyte maturity.

Many of the published experimental studies confirm the crucial role played by ZP during the preimplantation embryonic development of man and other mammals (Bauskin et al. 1999; Parillo et al. 2005; Wassarman et al. 2004; Zhao and Dean 2002). ZP is an integral part of the embryo in the normal course of an early period of development (Jelinkova et al. 2003). It prevents the dispersion of blastomeres, created in the process of blastulation, and enhances their mutual interactions.

As a dynamic structure, ZP is responsible for the delivery of nutritional components to the early form of a fetal ovum in mammals (Wassarman et al. 2001; Zhao and Dean 2002).

The thickness of ZP varies. In man, it ranges from 13 to 16 μm (Dunbar and Wolgemuth 1984) or from 15 to 18 μm (Rankin et al. 1996). In mice, ZP is 7 μm thick (Florman et al. 1984).

In the studies on extracorporeal fertilization of human oocytes, Bertrand et al. (1995) have demonstrated that the thickness of ZP is different both in fertilized and in non-fertilized oocytes. ZP thickness was significantly smaller in fertilized oocytes in which the mean value was 16.6 μm , whereas in non-fertilized oocytes, it was 18.9 μm . The authors suggest that with ZP thickness of 22 μm or more the chances of fertilization are considerably lower.

The attempts to reduce ZP thickness using laser radiation have shown no influence of such changes on fertilization, blastulation, implantation or early abortions (Petersen et al. 2002). The results were similar to the observations made in groups of animals whose oocytes were not exposed to laser radiation.

The experimental studies in dogs, cats, horses and elephants prove that an increase in the diameter of the ovarian follicle significantly coincides with an increase in the thickness of ZP (Barber et al. 2001).

A decrease in ZP thickness before implantation depends on its mechanical stretching resulting from subsequent stages of embryonic development (Confino et al. 1997). The theory of ZP mechanical stretching assumes its centrifugal “thinning” caused by the growth of the embryo. According to the authors, a change in ZP thickness may be caused by an enzymatic effect of the trophoblast or the specific environment of the uterine tube and uterus. The smallest thickness of ZP occurs shortly before implantation at the site where ZP

will disrupt, i.e., in the area of the blastocyst pole, which is implanted into the mucosa of the uterus.

ZP is an indispensable element of the fertilization process. It performs three fundamental functions: it binds a spermatozoon—thus preventing interspecies fertilization; it induces the acrosome reaction of the spermatozoon; and, finally, immediately after fertilization, it prevents polyspermy (Baker et al. 2000; Bleil and Wassarman 1990; Herrler and Beier 1999; Serrano and Garcia-Suarez 2001; Thaler and Cardullo 2002; Yanagimachi 1994; Zhao and Dean 2002).

It seems that the adherence of ZP to the oocyte is necessary for a proper function of the oocyte. The oocyte sending cytoplasmic processes into ZP ensures transmission of signals to the environment as well as their reception.

Stanger et al. (2001) questioned the role of ZP as an indispensable early embryonic element. In the studies on in vitro fertilization in man, it was observed that the oocytes from induced cycles had thin or no ZP, which demonstrated that the environment provided was insufficient for the development of blastomeres and the embryo. Finally, the percentage of successful implantations was as low as 0.7.

Similar opinions were presented by Zhao and Dean (2002). In their experimental studies in mice, the authors found that the development of oocytes, with a structurally defective or missing ZP progressed through subsequent stages of folliculogenesis and then isolated *only few/single* oocytes, could be fertilized in vitro. The defect in ZP matrix significantly affected the decrease in the number of mature follicles and reduced the follicle lifetime period.

For therapeutic purposes aimed at stimulation of ovulation, some other methods are also used, which pharmacologically inhibit maturation of ovaries and thus inhibit ovulation. In order to inhibit ovulation, synthetic derivatives of progesterone are used. To achieve a prolonged action, medroxyprogesterone acetate (MPA) is applied. MPA has strong progestagenic and antigonadotrophic effects. The chemical compound is a derivative of 17 α -hydroxyprogesterone and shows high affinity for progesterone receptors. The prolonged action of the steroid is a result of slow absorption from the site of injection. After intramuscular administration, it inhibits the secretion of pituitary gonadotrophins, which in turn makes the maturation of ovarian follicles impossible in women at reproductive age. It inhibits luteinizing hormone (LH) release, occurring in the middle of the cycle, thus halting ovulation. MPA slightly decreases follicle-stimulating hormone (FSH) concentrations and, consequently, does not inhibit the growth of ovarian follicles. MPA does not show estrogenic activity, and its androgenic action is almost lacking. Twenty-four hours after intramuscular administration of a single dose of

MPA, ovulation is inhibited; the maximum concentration is achieved after approx. 4 to 20 days.

The morphology of the ZP-oocyte complex allows a more accurate determination of the oocyte maturity. Studying the fine structure of this complex in the state of inhibited ovulation after administration of MPA should provide an additional morphological criterion, which may increase our current knowledge on implantation effectiveness of the oocyte. The aim of the study was ultrastructural evaluation of the ZP contact with the oocyte after the administration of MPA.

Material and methods

Twenty mature female white rats of Wistar strain (age range 2.5–3.0 months, body weight 250–300 g) were studied. Animals were housed in metal cages with a light regime of 14 h light/8 h dark and room temperature of 21 to 23°C. Rats were fed on a standard diet and had free access to water. Effects of stress were reduced to a minimum. Animals were randomly divided into three experimental groups (D1–D3) and one control group (C).

The study design was approved by the Ethical Committee of the Medical University of Lublin.

The day of the estrus cycle was determined by vaginal swabs (9 a.m., each morning) according to the Freeman method (Freeman 1994).

MPA was administered in three incremental doses. Animals received the suspension of MPA (Pharmacia NV/SA, Belgium).

The animals were divided into three experimental groups, with 5 rats each, as follows:

- D1—a single dose of 3.7 mg (therapeutic dose) per 300 g body weight
- D2—a single dose of 7.4 mg
- D3—a single dose of 11.1 mg

Experimental animals were decapitated 5 days after administration of MPA. Control animals received an intramuscular injection of 0.1 ml of saline (0.9% sodium chloride) per 100 gram body weight and were decapitated 14 h after saline injections.

Ultrastructural examinations

The samples for TEM examinations was prepared as follows: first, they were fixed in 4% glutaraldehyde for 4 h and rinsed in cacodylate buffer for 12 h; and then fixed in 2% osmium tetroxide (OsO₄) for 1 h and rinsed in distilled water for 2 min. Next, the specimens were dehydrated in a series of alcohols with increasing concentrations: 30%, 50%, 70%, 90%, and 100%. The tissue

fragments were placed in a solution of propylene oxide and embedded in epoxy resin Epon 812 (MNA, DDSA, DNP-30) according the following schedule:

- propylene oxide + resin (2:1) for 1 h
- propylene oxide + resin (1:1) for 1 h
- propylene oxide + resin (1:2) for 1 h

The resin and tissue fragments were placed in gelatinous capsules and polymerized at the temperature of 60°C for 24 h. The blocks were sliced using the RMC MT-7 ultramicrotome cutter (USA). Semithin slices (1 µm) were stained with methylene blue; preliminary selection of ovarian follicles was performed using light microscopy.

Ultrathin slices (80 nm) were stained with Reynolds liquid (lead nitrate + sodium citrate; Reynolds 1963). Ultrastructural evaluation and photographic documentation were performed using TEM Zeiss EM 900. Evaluation involved only mature cavitory ovarian follicles.

Morphometry

The thickness of ZP was measured in methylene blue-stained semithin sections (1 µm) at 200× using the cell imaging system by Olympus version A, eight measurements of ten semithin sections of each ZP.

Statistics

In each group, the maximum, the arithmetic mean ± standard deviation (SD) and standard error (SE) were calculated. To test for significant intergroup differences ($p < 0.05$), the Kruskal-Wallis one-way analysis of variance by ranks and multivariate analysis were applied (Brandt 1998). The calculations were made using STATISTICA 8.0 software.

Results

Electron microscope

ZP of animals in group D1 revealed a granular-fibrous network with a medium electron density. Single cytoplasmic processes coming from the surrounding structures were found within ZP. Oocyte membranes produced single, delicate processes directed toward ZP (Fig. 1). The oocyte microvilli were short and thin. Ultrastructural analysis disclosed a close contact between the analyzed structures. In group D2, the ZP structure (Fig. 2) was similar to that in group D1, yet cytoplasmic processes inside the ZP network were more numerous compared to group D1. Oocyte microvilli were short and thin, but cytoplasmic processes of granule cells were considerably thicker.



Fig. 1 Fine structure of contact between the zona pellucida (ZP) and oocyte (O). Group D1 (3.7 mg MPA). Note the granule cells (gc) of the corona radiata adjoining ZP. N Nucleus of gc, is intercellular space

In group D3, oocyte microvilli were more numerous, thicker and did not penetrate the matrix of ZP (Fig. 3). Only single-oocyte cytoplasmic processes extended toward ZP. Fine structure was observed in the majority of ovarian follicles in this group. ZP had the structure of a delicate, loose granular-fibrous network with lower electron density compared to the previous groups.

Measurements of ZP thickness showed significant differences ($F=291.219$, $p < 0.001$) between the experimental groups, with the lowest thickness of 2.28 µm in group D1 and the largest thickness of 10.767 µm in group D3 (Table 1). The lowest mean thickness of ZP was found in group D2;

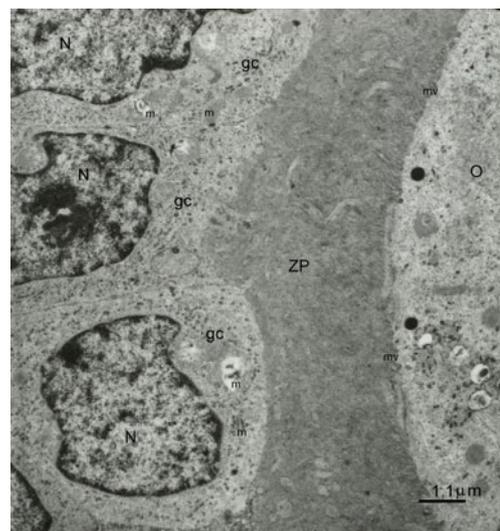


Fig. 2 Fine structure of contact between the zona pellucida (ZP) and oocyte (O). Group D2 (7.4 mg MPA). Note the short, thin microvilli (mv) of the oocyte (O)

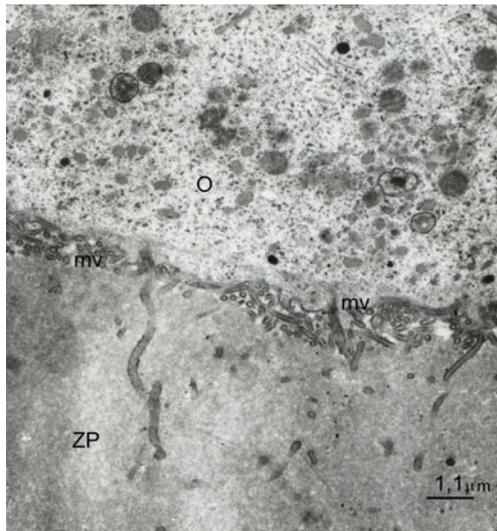


Fig. 3 Fine structure of contact between the zona pellucida (ZP) and oocyte (O). Group D3 (11.2 mg MPA). Abundant microvilli (mv) of the oocyte (O) facing the zona pellucida (ZP), yet not penetrating ZP3

the highest (8.369 μm) was found in group D3 (11.2 mg MPA), i.e., in the group of rats receiving MPA in the highest dose. The greatest variation in ZP thickness was observed in group D1 (3.7 mg MPA, the therapeutic dose; Table 1).

Comparison of ZP thickness between the experimental groups revealed that ZP thickness was significantly lower in group D1 and group D2 compared to group D3, which did not show differences in comparison with group C (Table 2). ZP thickness was found to be significantly smaller in the ovarian follicles of female rats in groups D1 and D2. ZP thickness in group D3, however, did not reveal significant differences compared to controls, group C.

Discussion

In recent years, an increasing number of studies have been conducted on morphology of the ovarian follicles, largely expanding the available knowledge about the dynamics of the development of oocytes and adjacent structures.

The oocyte, ZP and granule cells of the corona radiata are considered to be the measurement tools for reproduction capacity of mammals. Changes in the composition of the

Table 2 Comparison of zona pellucida thickness in experimental and control groups

	<i>p</i> Values		
D1		0.000	0.000
D2	0.000		0.000
D3	0.000	0.000	0.310
C	0.000	0.000	0.310

Approximate values. A *p* value of 0.000 means that the significance level $p < 0.0005$

structures in question are likely to cause failures in fertilization or embryo development as well as lack of egg implantation.

The results of the present study indicate that both stimulation and inhibition of ovulation induce changes in the examined structures, ZP and corona radiata cells. ZP is a structure sensitive to the effect of substances that inhibit ovulation.

While evaluating the site of ZP and oocyte contact, no microvilli or only single microvilli directed toward ZP were observed. Their number increased together with an increase in MPA doses, yet these were only single cytoplasmic processes. The oocyte membrane generated single microvilli in the direction of ZP. In group D3 receiving the highest dose of MPA, there were sites where the oocyte microvilli did not penetrate the ZP matrix, forming a barrier between ZP and oocyte.

The available literature does not provide much information about the effect of ovulation inhibitors on the ultrastructure of the ZP-oocyte contact. Different observations were reported by Durlinger et al. (2000), who studied the effect of the GnRH antagonist on rat ovaries. The administered substance had inhibiting effects on ovulation, and the microscopic images of ovaries were dominated by granule cells with pycnotic nuclei. Moreover, the changes were present in the oocyte, cumulus oophorus and follicle antrum, yet no changes were observed in ZP. All the changes were progressive and intensified with the duration of observation of the ovarian follicles. Therefore, all the above observations strongly suggest that MPA is responsible for such an ultrastructural image of ZP. MPA makes the maturation of ovarian follicles impossible, but it inhibits their growth only to a certain extent. The ultrastructural

Table 1 Zona pellucida thickness in experimental and control groups (in μm)

Group	Mean (M)	Number	Standard deviation (SD)	Standard error (SE)	Minimum	Maximum
D1	4.448	60	1.341	0.173	3.000	7.500
D2	3.068	55	0.456	0.062	2.280	4.250
D3	8.369	25	1.032	0.206	6.965	10.767
C	8.155	40	0.642	0.101	7.150	10.500
Variance analysis			$F=291.219; p < 0.001$			

studies revealed the granular-fibrous structure of ZP. The ZP matrix showed medium electron density; it is only in group D3 that the density was lower than that in the other groups. The molecular composition of ZP is characteristic of mammals, humans and rats included. Wassarman et al. (2004) studying ZP in mice showed that the ZP external layer had higher electron density compared to the internal layer adjacent to the oocyte. In our material, such a polar differentiation of electron density in ZP was not observed.

The results of ZP thickness measurements varied greatly. The greatest variation was observed in group D1. In group D2, the smallest thickness of 2.280 μm was found compared to the remaining experimental groups. ZP thickness values in group D3 were significantly higher than those in the control group.

Many factors are considered to affect the structure and thickness of ZP. If the granule cells are involved in the synthesis of ZP glycoproteins, then what mechanism is responsible for such a large differentiation of ZP thickness in experimental groups? It should be stressed that the smallest ultrastructural changes were observed in the groups exposed to MPA. The administered MPA may have inhibited the transcription processes in the granule cells.

Biological effects of MPA are caused by MPA itself, its metabolites and FSH. The findings imply that MPA has an inhibiting effect on the synthesis of binding proteins, thus leading to the loss of the oocyte-ZP contact. In addition, injection of 11.2 mg MPA does not impair the synthesis of ZP glycoproteins, as the ZP thickness was similar to that in control animals.

Conflict of interest The authors declare that they have no conflict of interest.

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