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Chapter IV

A Picture of Zona Pellucida as Seen by Way of a Transmission Electron Microscope

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Abstract

Introduction: The outer, glycoprotein membrane surrounding the mammal ovum and embryos is evident as a clear girdle (the zona pellucida – ZP). It first appears in the primary follicles as an extracellular substance between the oocyte and the granulosa cell, soon forming a

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corona radiata. The ultra-structure study of mice oocytes with the primary follicle shows the presence of an electron-rich matrix filling the gaps between the microvilli of the ovum. That matrix, which exists as a protrusion surrounding the oocyte, is considered to be the first element of the growing zona. With growth of the follicle, the structure, thickness and the texture of the zona pellucida changes. The zona pellucida has a fibrous structure, and it is built of the glycoprotein complexes: ZP1, ZP2, ZP3 and ZP4. These glycoproteins determine the specific structure of the external surface of the zona pellucida which is non-homogenous. The visualization of an oocyte surface in a scanning microscope shows that the zona pellucida has a structure that resembles a fibrous net with a variable number of fenestrations.

The materials: We used mature ovaries extracted from a female white rat (aged 2-3 months), which, beforehand, underwent ovulation stimulation by accepted procedures. Two experimental models were chosen: in the first one, an ovulation stimulating substance was used (human chorionic gonadotropin – hCG); in the second one, an ovulation stimulating substance and a substance stimulating the ovaries to produce multiple follicles, was employed (hCG and human menopausal gonadotropin –hMG). The material for the research was preserved in a few stages, and then examined by way of a transmission electron microscope (TEM). The ultra-structural examination was performed on mature ovarian follicles. The research was approved by the Local Ethical Committee.

Results: The ultra-structural images of the zona pellucida were diverse. In the majority of the ovarian follicles that were examined, the zona pellucida was a delicate, granular-fibrous net of low electron density. In that structure the microvilli came from both the ovum and the granulosa cells. Furthemore, the microvilli were numerous, short and had diversified diameter, whereas the granulosa cells, were seen to have irregular, cytoplasmic extensions of various length and thickness. Inside some of the microvilli, the transporting vesicles (which contained a substance of varied electron density) were visible.

Conclusion: The diversity of structural images observed in this study shows that the zona pellucida structure is dynamic. The examinations performed with the use of a transmission electron microscope enabled a more detailed observation of the zona pellucid, as well as the evaluation of its relations with its neighboring structures, namely the ovum and granulosa cells. The presence of diverse ovum and corona radiata microvilli in the zona pellucida, signifies a reciprocal interaction of all of the structures described in this study.

Keywords: Zona pellucida, transmission electron microscope, granulosa cells, oocyte

Introduction

The zona pellucida (ZP) is an outer glycoprotein membrane surrounding the growing and mature oocyte, as well as the embryos at their early stage of development (Magerkurth 1999, Prasad 2000, Sinowatz 2001(1), Zhao 2002). The zona pellucida has a flexible fibrous structure of various thickness of 2-25μm, depending on the species (Qi 2002). It is built of the glycoprotein complexes ZP1, ZP2, ZP3 and ZP4.

The particular proteins are synthesized, secreted and organized in a specified order in the ovum and granulosa cells (Liang 1993, Lee 1993, Dunbar 1994), and the lack of one of these proteins brings about the lack of the zona pellucida, and thus female infertility (Qi 2002). During fertilization, the interaction of spermatozoa with ZP3 activates an acrosome reaction and expose additional molecules which are involve in secondary binding of spermatozoa to ZP2.

The role of ZP1 is to cross-link the ZP2 and ZP3 into one filament (Epifano 1995). The glycoprotein also determines the specific structure of the external surface of the zona pellucida (which is non-homogenous). The visualization of an oocyte surface in a scanning microscope shows that the zona pellucida has a structure that resembles a fibrous net with a variable number of fenestrations (Ström Holst 2000).

Moreover, as the authors specify, there are no significant morphological differences around the zona depending on the oocyte's age. The changes of ZP are significant after fertilization. In the fertilized oocyte, the surface of the zona pellucida is more compact and smooth, with the pores being of a lesser diameter (Magerkurth 1999, Vanroose 2000). What is more, the oviduct cells' secretion penetrates into the pores reducing their size (Vanroose 2000).

The zona pellucida first appears in the primary follicles as an extracellular substance between the oocyte and the granulosa cells, hence forming a corona radiata (Rankin 2000). The ultra-structure study of mice oocytes with the primary follicle shows the presence of an electron-rich matrix filling the gaps between the microvilli of the ovum (Zhao 2002).

That matrix, which exists as a protrusion surrounding the oocyte, is considered to be the first element of the growing zona. With the growth of the follicle, the structure's thickness, as well as the texture of the zona pellucida, change (Qi 2002, Vanroose 2000). However, the location of the biosynthesis of the zona pellucida has not been fully explained, but one group of scientists puts forth that it is produced by the oocyte (Haddad 1977, Kimura 1994, Bartel 2007, Epifano 1995).

The observations of mice ovum performed by Wasserman and his associates show, that the oocyte Golgi apparatus play a significant part in modifications of the protein particles which are the precursors of the zona's glycoprotein (Wassarman 2004). The authors have observed the biggest activity of these cellular organelles is seen in the gametes which exist in the growing ovarian follicles. In the peripheral part of the ovum's cytoplasm, they have also observed a large amount of secretory vesicles. The above study suggests that the synthesis of the zona pellucida takes part inside the oocyte. Moreover the inner surface of the ZP is more closely packed than the outer surface (Qi 2002). These different studies on the mice' zona pellucida suggest that only the oocyte is responsible for ZP synthesis (Eberspaecher 2001, Haddad 1977, Kimura 1994, Epifano 1995, Qi 2002). However, the majority of scientists have come to the opinion that the zona pellucida is created with the participation of both the ovum and the granulosa cells, with a prevalence of the second one (Bogner 2004, Rankin 2000, Sinowatz 2001(1, 2)). Hence, an overview of the available literature shows that in human, ape, rabbit, dog, pig and cow ovaries, the zona pellucida is formed with the participation of these two structures (Cariño 2002, Grootenhuis 1996, Martinez 1996, Dunbar 1994, Sinowatz 1995, Kölle 1998).

The zona pellucida constitutes a significant element in fertilization and in early embryogenesis. In these processes, it has three basic functions: it is responsible for sperm binding – preventing inter-species fertilization; it induces the acrosome reaction of the sperm, and after the fertilization, it prevents polyspermy (Baker 2000, Bleil 1990, Bronson 1970, Modliński 1979, Serrano 2001, Soupart 1975, Thaler 2002, Zhao 2002). Experimental procedures confirm the zona pellucida's crucial role in the pre-implantation development of the human embryo and those of other mammals (Epifano 1994, Wassarman 2004, Zhao 2002). Research has shown that the ZP is an integral part of the embryo in its initial stage of development. Furthemore, it prevents scattering and reinforces the integration of blastomeres formed during the cleavage process. Moreover, the zona pellucida nourishes and protects the developing ovum and the embryo (in its early stage) against some viruses (Zhao 2002, Wassarman 2001, Vanroose 2000). It seems that the adherence of the ZP to the oocyte is also a vital condition for the ovum to function. In this respect, the oocyte and granulosa cells enable the sending of mutual signals by sending cytoplasmic extentions into the ZP.

The purpose of this study was the ultra-structural evaluation of the zona pelllucida and its contacts with neighboring structures in the experimental ovulation stimulation of an animal model.

For medical purposes, pharmacological procedures are used to stimulate ovulation. In order to stimulate ovarian follicles, and, consequently, to obtain oocytes in supported fertilization techniques, usually, natural, exogenous gonadotropins are used.

Materials and Methods

The materials for the study were mature ovaries extracted from a female white rat (aged 2-3 months), which, beforehand, underwent ovulation stimulation by accepted procedures. Two experimental models were chosen: in the first one an ovulation stimulating substance was used (human chorionic gonadotropin – hCG) in a dose 150 IU (Choragon 1500 manufactured by Ferring Pharmaceuticals, Germany); in the second one, an ovulation stimulating substance and a substance stimulating the ovaries to produce multiple follicles (hCG and human menopausal gonadotropin –hMG) in a dose 7,5 IU FSH and 7,5 IU LH (Menopur manufactured by Ferring Pharmaceuticals, Germany), was employed. The substances were inoculated intramuscularly. The research was approved by the Local Ethical Committee. For a comparative analysis of the structures of the follicular ovarian follicle, these were extracted from a control group (5 rats) that received saline-0.9% NaCl, in the form of a one time intramuscular injection in a volume of 0,1 ml. The animals were killed 14 hours following the administration of the saline. The timing of this was made on the basis of the data resulting from an earlier, similar experimental studies on female Wistar rats performed, among other experiments by Dalita Ben-Yosef and Alexander Durlinger (Ben-Yosef 1996, Durlinger 2000).

The material for the research was preserved and then examined by using a transmission electron microscope (TEM). In their preparation, the fragments of studied tissue were placed for 4 hours in the fixative I – 4% glutaraldehyde, and subsequently they were placed for 12 hours in a buffering agent – cacodylic acid. The fixative II was 2% osmium tetroxide – OsO_4, in which the tissue was left for 1 hour, and then it was rinsed in distilled water for 2 minutes. In the next phase of the research, the material was dehydrated in a number of alcohols of growing concentration – starting with 30% and concluding with 100%.

Subsequently, the tissue fragments were placed in propylene oxide, and then they were submerged in an epoxy “resin” Epon 812 (MNA, DDSA, DNP-30) in a routine procedure.

The resin with the examined tissue fragments were placed in gelatin capsules and then underwent a 24 hour polymerization at 60 °C. The blocks were then cut with an ultramicrotome MT-7, a product of RMC (US). The semi-thin cuts of 1 μm thickness were stained with methylene blue, and subsequently, an initial selection of ovarian follicles was performed in an optical microscope. The ultra-thin cuts of 80 nm were stained with Reynolds mixture (lead nitrate + trisodium citrate). The ultra-structural evaluation and photographic documentation were done with a transmission electron microscope: Zeiss type EM 900. The ultra-structural examination was performed on mature ovarian follicles.

Results

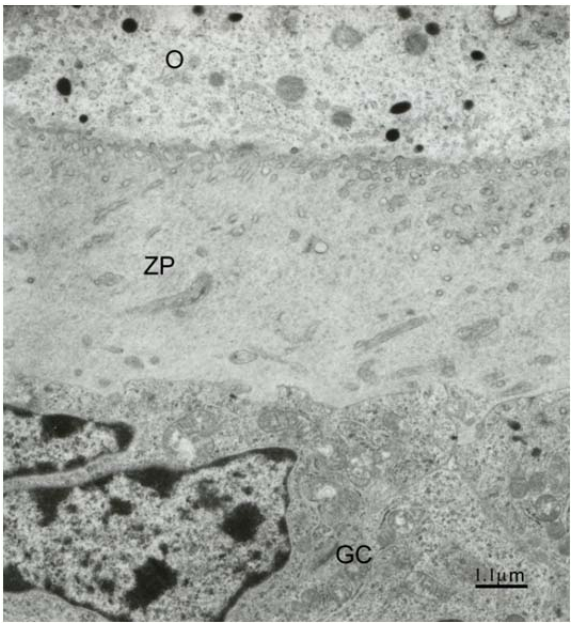
The Group After Treatment with Human Chorionic Gonadotropin (hCG)

The ultra-structural images of the zona pellucida after hCG stimulation were diverse. In the majority of the examined ovarian follicles, the zona pellucida was a delicate, granular-fibrous net of low electron density. In that structure, the microvilli came from both the ovum and the granulosa cells. The microvilli were numerous, short and had a diversified diameter (Figures 1, 2).

Moreover, it was evident that the granulosa cells were sending irregular, cytoplasmic extensions of various lengths and thicknesses into the ZP (Figure 3). What is more, inside some of the microvilli, the transporting vesicles (which contained a substance of varied electron density) were visible.

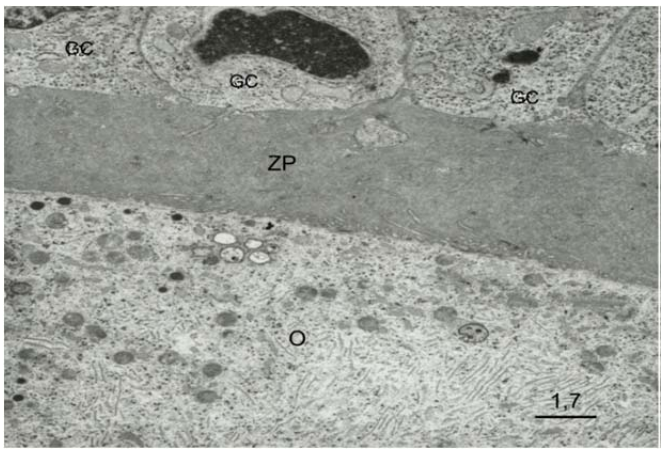
The Group after Treatment with Both Human Chorionic Gonadotropin (hCG) and Human Menopausal Gonadotropin (hMG)

In comparison with the previous group, the zona pellucida after hCG and hMG stimulation was more densely packed. In addition, singular ovarian vesicles were observed. In these, the ovum was seen to have sent between 11 and 19 microvilli towards the zona pellucida, where the microvilli had ¼th of the zona pellucida's thickness, and where few granular cell microvilli were present (Figures 4, 5).



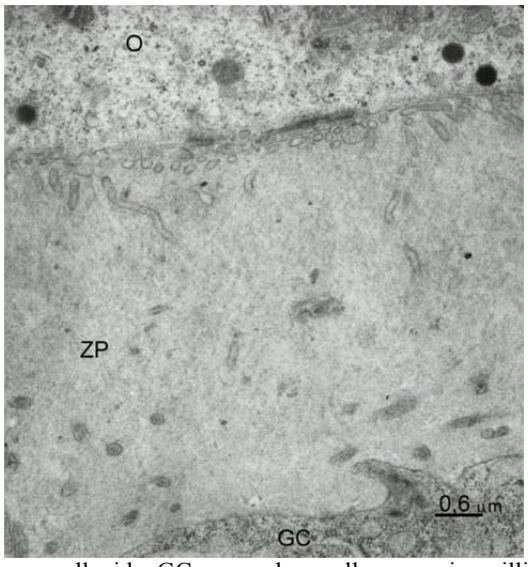
O – oocyte, ZP – zona pellucida, GC – granulose cell.

Figure 1. A microscopic image of the zona pellucida. The zona pellucida is of granular-fibrous structure with visible microvilli of diversified diameter (group hCG).



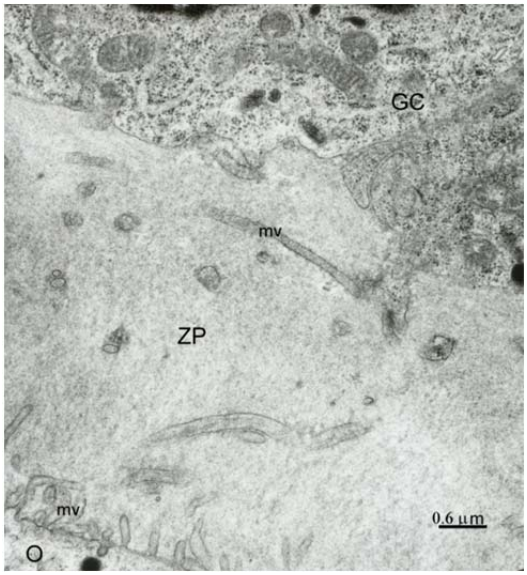
O – oocyte, ZP – zona pellucida, GC – granulose cell.

Figure 2. The zona pellucida with visible microvilli of diversified diameters and lengths (group hCG).



O – oocyte, ZP – zona pellucida, GC – granulose cell, mv – microvilli.

Figure 3. The zona pellucida – long, granular cell microvilli of diverse diameters in the middle section; short, frequently from the oocyte (group hCG).



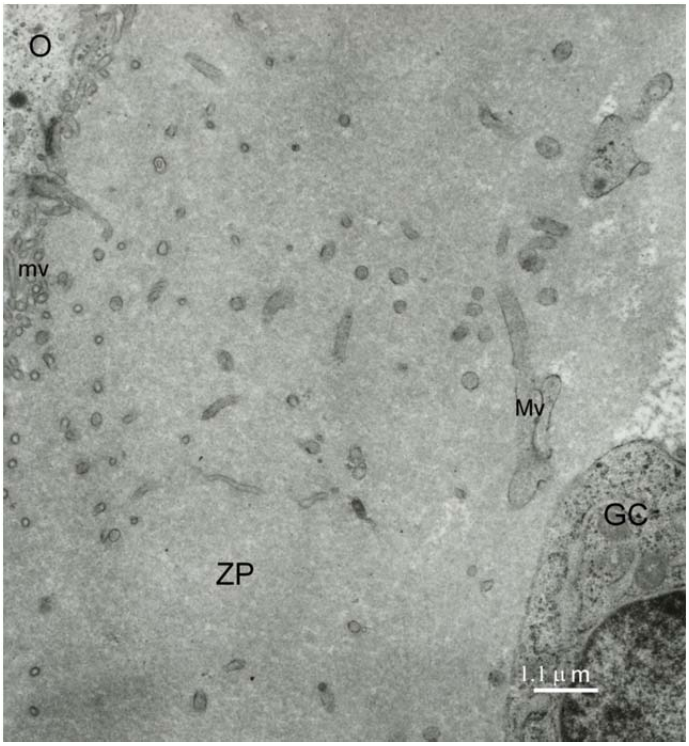
O – oocyte, ZP – zona pellucida, GC – granulose cell, mv – microvilli.

Figure 4. The zona pellucida with oocyte and granulosa cells microvilli of diverse diameters (hCG and hMG group).

The ultra-structural research also allowed for the observation of ovarian vesicles, where the zona pellucida was visible in a granular-fibrous structure; it had a higher electron density than mentioned above (Figure 6).

What is more, the fibers constituting the zona pellucida had various electron densities. Moreover, numerous cytoplasmic extensions penetrated the zona pellucida on the side of the ovum. These had a larger diameter and were longer than the aforementioned ones. In the part where the microvilli were situated, we could also observe vacuoles containing substances of various electron densities. Furthemore, the granulosa cells were seen to have also sent microvilli towards the zona pellucida. These were irregular in shape and of different diameter and length.

The ultra-structural analysis of the research material also allowed for the observation of ovarian vesicles in which the zona pellucida had a micro-fibrous structure.



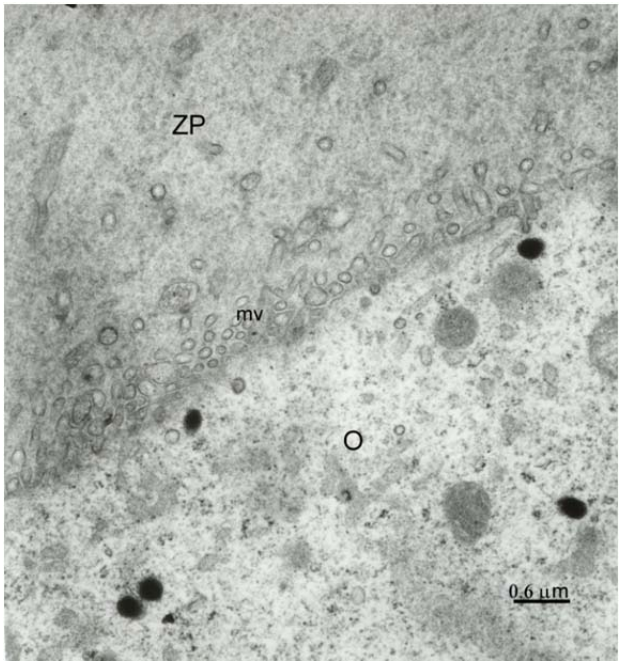
O – oocyte, ZP – zona pellucida, mv – microvilli.

Figure 5. Microvilli in the zona pellucida as seen as on the oocyte side (hCG and hMG group).

On the side of the ovum, it had multiple, short microvilli, whereas on the opposite side, where it came in contact with the vesicular liquid, it had irregular edges. On the other hand, in the spots where the zona pellucida had come in contact with the cells of the corona radiata, its structure was penetrated by long cytoplasmic extensions of the granulosa cells. These were of different length and thickness. What is more, there are differences in the length and number of the microvilli between the analyzed groups. However, in the structure of the zona pellucida we did not observed significant differences between the control and the ovulation stimulation group (Figure 7).

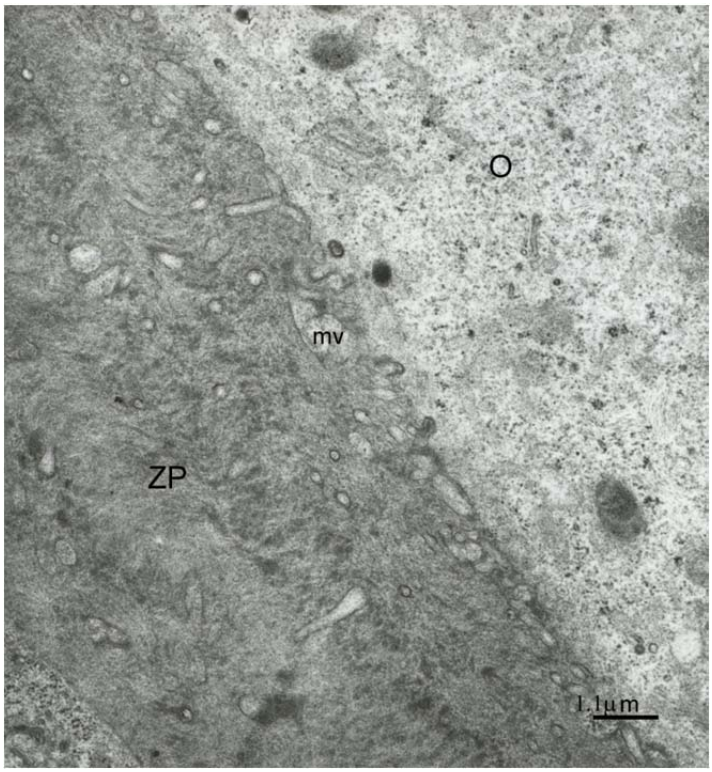
Discussion

The zona pellucida (ZP) plays a substantial role in mammalian reproduction. It is produced during the growth of the ovarian follicle and it provides for protection of the egg.



O – oocyte, ZP – zona pellucida, mv – microvilli.

Figure 6. The zona pellucida displaying high electron density. The ZP fibers are of various electron densities (hCG and hMG group).



O – oocyte, ZP – zona pellucida, GC – granulose cell.

Figure 7. The zona pellucida with the oocyte microvilli of diverse diameters and lengths, few microvilli are visible from the GC (control group).

Thus, it is essential for the proper conduct of fertilization and embryo development in the first few days of life (Ganguly 2010). Observation of the ZP using electron microscopy has allowed for a better understanding of its structure and functions, and knowledge of the construction of the ZP and its physiological transformation has become the basis for the research on its role in disorders of fertility.

It is known that the thickness of the ZP has a significant effect on fertilization, as well as on the implantation of the embryo in the uterus, and, subsequentally, normal development embryo (Bertrand 1995, Sholoh 2004, Loret de Mola 1997, Wassarman 2001). Attempts to explain the factors which influence the change in the thickness of the ZP have shown that higher levels of estradiol bring about changes in the thickness of the ZP, as does administration of exogenous gonadotropins (Loret De Mola 1997).

However, in the research of the variation in ZP thickness as it corelates to a woman's age, there were obtained divergent results. Peter et all have demonstrated that thickness of the ZP increases in women after 35 years of age (Petersen 2001). Conversely, Loret de Mola reveal that the ZP of the embryo is thinner in women over age 35 (Loret de Mola 1997). Also smoking is a significant factor in lowering fertility, by way of affecting the thickening of the ZP (Sholoh 2004). Yet, the mechanism of change in thickness of the ZP has not been elucidated. However, Loret de Mola has shown that hCG induces changes in the proteins/carbohydrates composition of the zona pellucida, which he opines probably leads to changes in the thickness of the ZP (Loret De Mola 1997).

In our work, we studied the construction of the zona pellucida of female rats using electron microscopy, doing so after stimulation of ovulation by way of administration of hCG and hMG with hCG. The resulting image was compared with the construction of the ZP in femal rats not treated with hormone stimulation. Human chorionic gonadotropin (hCG) and human menopausal gonadotropin (hMG) are hormones used to induce ovulation. As a result of the application of gonadotropin, the maturation of the oocytes is accelerated and the synthesis of progesterone and estradiol is stimulated (Borges 2009). It has been shown that hCG is beneficial to the build of the ZP, and this fact facilitates fertilization. A higher level of LH or hCG injection triggers a series of interaction between granulosa cells and oocyte (Fritzsche 2006). Furthemore, pregnancy rates increases significantly after stimulation with hMG, therefore, patients not responsive to hCG administration are recommended to be treated with artemisinin-two gonadotropins.

In our study, we observed an increased number of microvilli on the surface of the oocyte in the group receiving the hCG with hMG combination, however, they were shorter in length in comparison with the group treated with hCG alone, and with the control group. It was also revealed that granulosa cells were distributed microvilli within the ZP, and they were of irregular shape, of different diameter and length. What is more, the ZP after hCG and hMG stimulation had a higher electron density than in the hCG treated group alone. In addition, vacuoles containing substances that are within the microvilli are more frequently seen in the group of that was stimulated by the combination of these two hormones. Our research also shows that the use of ovulation stimulation affects the construction of the zona pellucida and the activity of cells involved in its manufacture. The diversity of structural images observed in this study provides evidence that the zona pellucida structure is dynamic.

Moreover, the examinations performed with the use of a transmission electron microscope enabled a more detailed observation of the zona pellucida, as well as the evaluation of its relations with its neighboring structures. The presence of diverse ovum and corona radiata microvilli in the zona pellucida, signifies that there is a reciprocal interaction between all of the structures described in this study.

The importance of similar research is undeniable. An examination performed by way of the assessment of hormone levels or an evaluation of the patient's lifetime history with respect to shape and structure of their ovulatory follicles are some of the indicators suggesting the individual's ability to have children, but in some cases this is not enough. Therefore, in order to assess the treatment used to assist reproduction, more detailed research should be carried out. What is more, morphological analysis of the produced oocytes and the zygotes formed after inseminatio (in-vitro as well), should allow for better determination of the predictive indicators with which to assess the survival of embryos. Examining the properties of the zona pellucida and its birefringence, which play particular roles in the function of the ZP, show that high zona pellucida birefringence is associated with better clinical pregnancy outcome (Borges 2009, Luo 2013). As a study using electron microscopy is non-invasive, it will aid in ascertaining the correct selection of embryos for implantation, which in turn ensures the success of pregnancy (Garside 1997).

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