

**BABES-BOLYAI UNIVERSITY CLUJ-NAPOCA  
FACULTY OF BIOLOGY AND GELOGY**

**OBTAINING AND *IN VITRO* CULTURE OF HUMAN EMBRYOS.  
STRUCTURAL AND ULTRASTRUCTURAL STUDIES.**

**Summary of the Ph.D. Thesis**

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**CLUJ NAPOCA  
2010**

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Key Words: In vitro fertilization, ultrastructure, oocyte, sperm, embryos, immature, mature, postmature

**Introduction:**

Even if it seems surprising, in human reproduction, naturally are more failures than successes due to natural causes and exogenous influences.

In vitro fertilization has a modest success rate at the first glance (25-30%), but it still represents a way of solving infertility for a lot of couples.

For the embryologist the main goal is to obtain high quality embryos, with high implantation potential. Starting from this premise we considered that new investigations regarding sperms, oocytes and embryos ultrastructure, may bring new data about their quality. This new data we can further use in order to improve ovarian stimulation protocols, sperm preparation techniques and embryos culture systems.

We believe that every little step which finally can lead to a new birth is very important for us.

The investigations lasted a long period of time because the material we use it was very hard to obtain, due to the fact that the couples sometimes refused to sign the agreement forms.

The observations I have made taking in account the bibliographic data available and my own data during the last 10 years led us to investigate:

1. the sperms maintained for 24 hours in culture medium kept the characteristic features about mobility and aspect, but if the sperms were put together with fresh oocytes were not able to fertilize the oocytes.

2. at follicular puncture, the oocytes can be : immature, mature and postmature. For fertilization are used mature oocytes, because the immature ones do not fertilize, but the immature oocytes can be matured in vitro if are kept for 24 hours in incubator in culture medium. As a consequence, the immature oocytes matured in vitro can be fertilize.

**Objectives:**

We want to explain why the immature oocytes do not fertilize, why the immature oocytes matured in vitro fertilize. We considered that a comparative ultrastructural study of oocytes (in different stages of maturation) can provide new informations to explain this behaviour. We should mention that in literature are no data available regarding this subject.

**Materials and method:**

The biologic material was obtained after correct information of the patients and after we obtained all the approvals needed in order to start. The male biological material is represented by sperms. The sperm sample is recommended to be obtained with maximum 30 minutes before using it, by masturbation.

Natural, the sperms separate from seminal fluid at female genital tract level. The research data shows that in seminal fluid are factors that inhibit capacitation (Yanagimachi 1994), that prolonged exposure of sperms to seminal fluid inhibit sperms capacity to undergo acrosomal reaction in vitro (Rogers and colab. 1983), and decrease the sperms capacity to fertilize (Kanwar și colab. 1979). In our laboratory, we try to prepare the semen very fast, to avoid all the problems mentioned early, using the "swim-up" technique. In order to obtain a higher fertilization rate, we modified the technique. The number of centrifugations, the culture medium quantity added, the incubation period

were parameters modified. The experience of the embryologist and the semen particularities were the parameters that allowed us to change the parameters mentioned before in order to obtain the higher number of superior quality sperms.

The female biologic material is represented by oocytes, harvested by follicular puncture, under ultrasound guidance.

After the follicular puncture, the oocytes are assessed using optic microscope (40X and 125X magnification) to establish their maturity grade. The most important argument to assess the oocyte maturity is to be able to determine the correct timing for insemination in order to obtain the highest percentage of fertilized oocytes. After Rabe (2000) the oocyte can be:

1. mature oocytes: clear cytoplasm, first polar body visible, the corona radiata cells have a low density, the cumulus cells are even having a typical aspect in optical microscopy.
2. postmature oocytes: the cytoplasm and zona pellucida are slightly visible, cumulus oophorus and corona radiata have high cellular density.
3. immature oocytes: the oocyte is invisible, the cumulus oophorus cells have a high density and the corona radiata cells are very dense.

In our laboratory, we use this model to assess the oocyte degree of maturation.

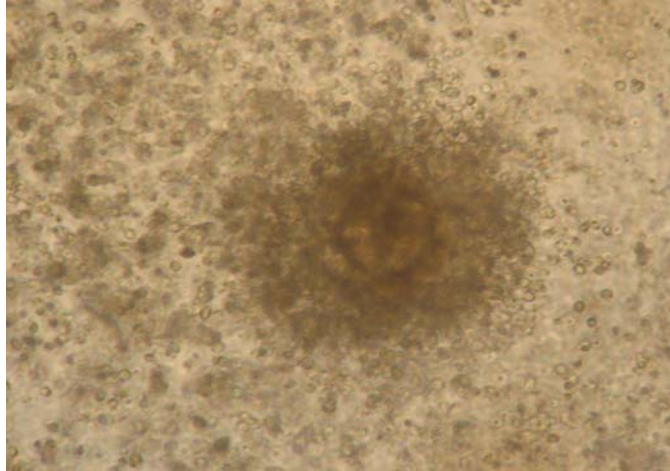


Fig.1 Immature oocyte (original).



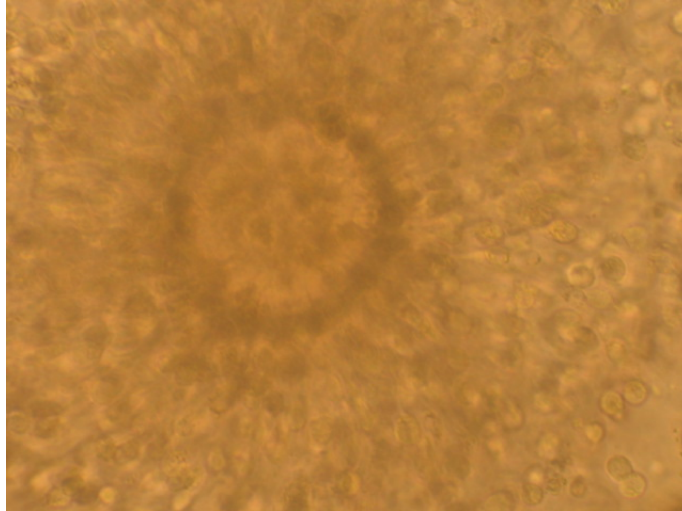


Fig.2 Mature oocyte (original).

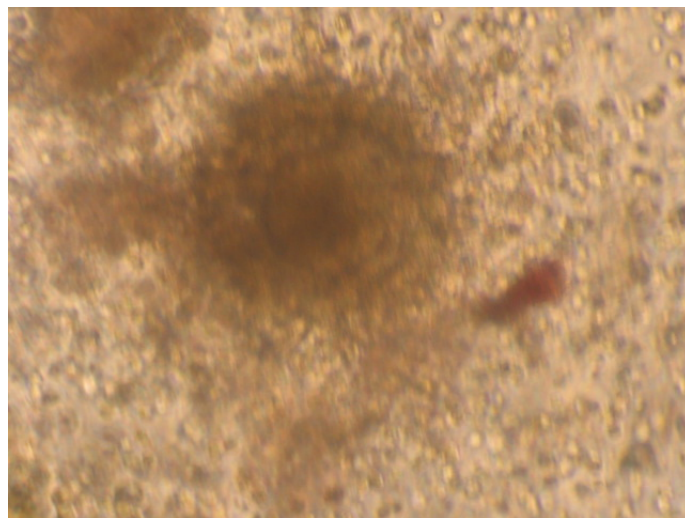


Fig.3 Postmature oocyte (original).

We cultivated in vitro for 24 hours the immature oocytes. We have performed the insemination for mature oocytes after 2 hours post follicular puncture and after 30 minutes for postmature oocytes. We used 100.000 100% well motile sperms for each droplet in order to achieve fertilization. The fertilization control was made after 16 hours post insemination. The fertilized oocyte has 2 pronucleus. The presence of pronucleus is the first visible sign of fertilization, the both pronucleus must be present in the same time and in an aligned position.

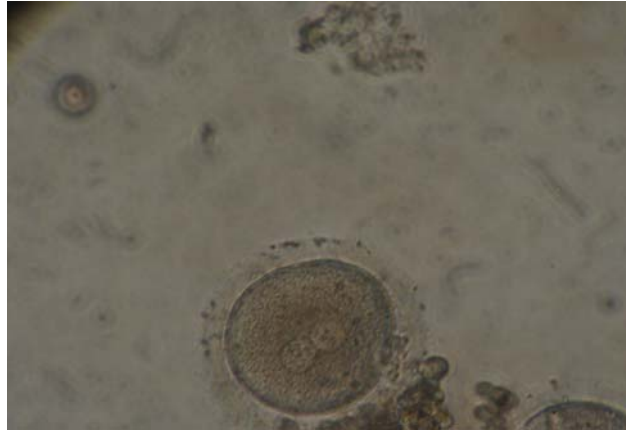


Fig.4 . Human 2 PN stage oocyte (original)

Excellent quality embryos, obtained by in vitro techniques are one of the main goal of in vitro culture methods. In the last years, the human reproductions techniques developed very much, now almost all the pathologies can be solved and correct, minus one: the embryos quality. By oocyte quality we understand their capacity to implant and to produce a viable pregnancy. Apparently one of assisted reproduction techniques limitation is the incapacity to obtain good quality oocyte.

To evaluate human embryos we used Edward's evaluation system used in Bourn Hall Clinic United Kingdom:

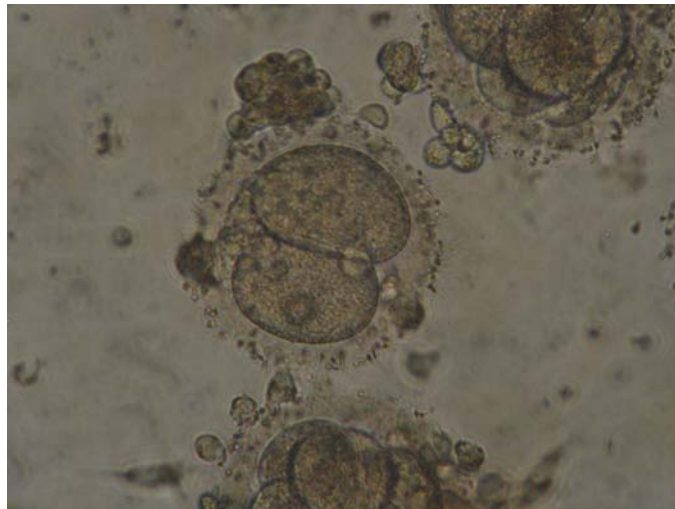


Fig.5. 2 cells stage human embryo (original).



Fig. 6. 3 cells stage human embryo. (original).

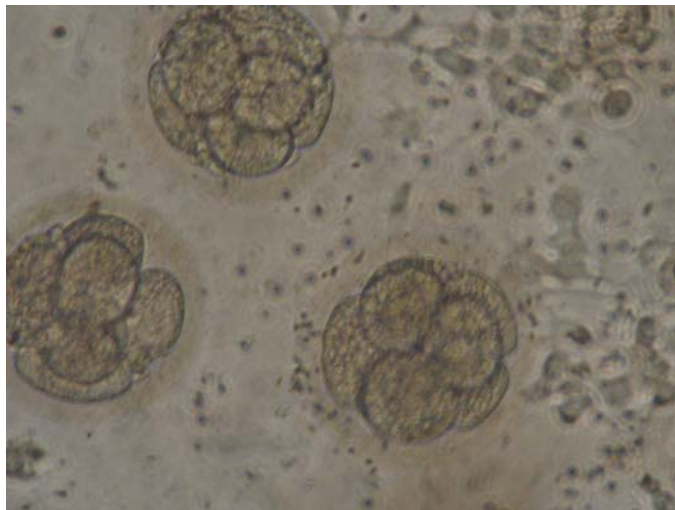


Fig.7. 6 cells stage human embryo (original).

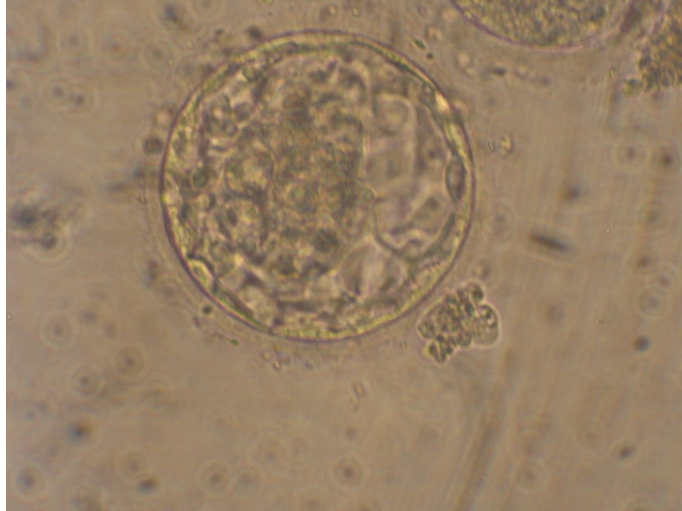


Fig.8. Human blastocyst (original).



Fig.9. Human blastocysts(original).

## Results regarding the quality of male biologic material-semen quality

During 10 years we performed 5558 sperm counts. One of the analyzed parameters was the sperms number. We considered 5 mio/ml as an important value, Bostofte et all (1982) considered this values as the limit value for IVF.

Diagnosis	azoospermia	Oligospermia			normospermia
		3524 cases= 63,39%			1588 cases=28.59 %
		<5mio/ml	between 5 and 10mio/ml	Between 10 and 25 mio/ml	>25 mio/ml
No. Of cases	<b>446 cases</b>	893 cases	1279 cases	1352 cases	1588 cases
Percentage	<b>8.02%</b>	16.06%	23.01%	24.32%	28.59%.

Table.1 Number of cases/ diagnosis

The percentage of oligospermia cases is 63,39% and normospermia is only met in 28, 59% of cases, this situation being an alarming one.

If we add azoospermia and severe oligospermia we can conclude that the number of cases with serious problems from this point of view is unexpected high.

### Diagnosis/cases

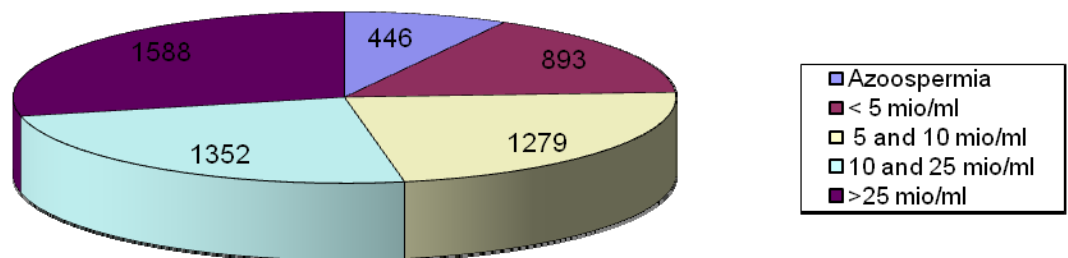


Chart 1. Diagnosis/cases

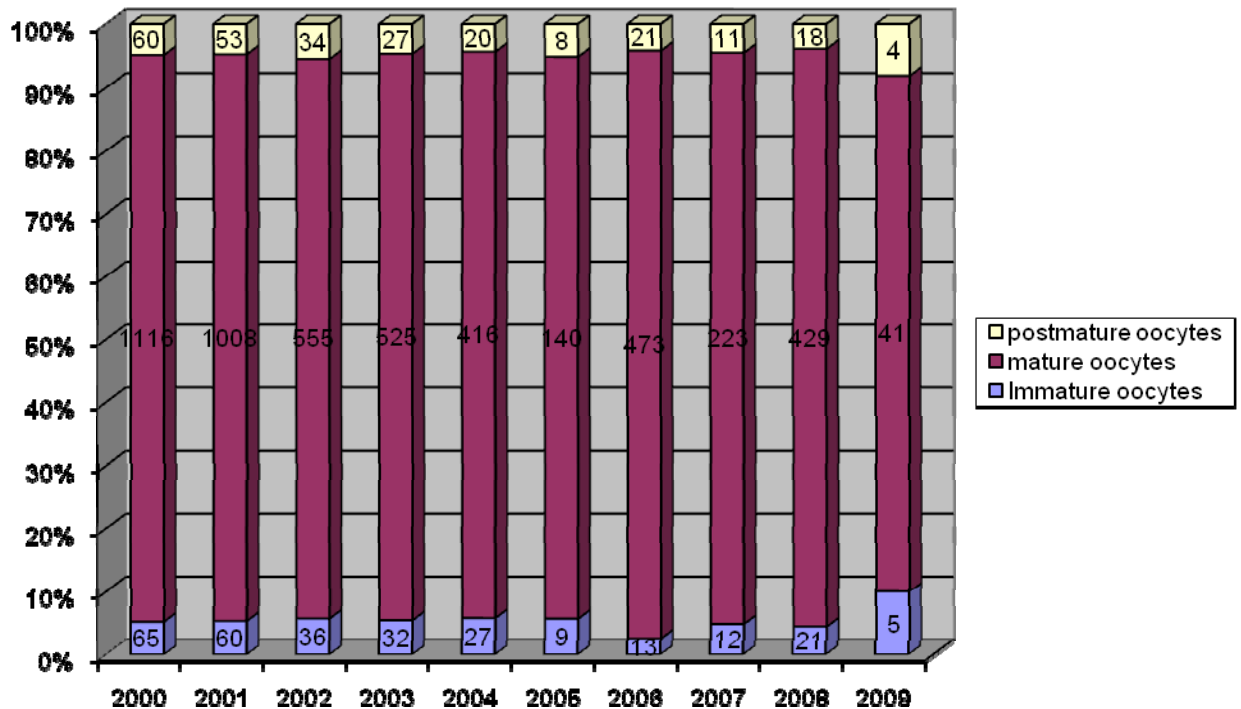
### Results regarding the quality of female biologic material-oocytes quality.

The oocytes were divided in 3 main groups: immature, mature, postmature.

Year	Immature oocytes	Mature oocytes	Postmature oocytes
2000	65 (5,24%)	1116 (90%)	60 (4,76%)
2001	60 (5,35%)	1008 (89,9%)	53 (4,76%)
2002	36 (5,76%)	555 (88,80%)	34 (5,44%)
2003	32 (5,48%)	525 (90%)	27 (4,62%)
2004	27 (5,83%)	416 (89,95%)	20 (4,32%)
2005	9 (5,73%)	140 (89,18%)	8 (5,09%)
2006	13 (2,56%)	473 (93,30%)	21 (4,14%)
2007	12 (4,88%)	223 (90,65%)	11 (4,47%)
2008	21 (4,49%)	429 (91,67%)	18 (3,84%)
2009	5 (10%)	41 (82%)	4 (8%)
Total	280 (5,2%)	4926 (90,1%)	256 (4,7%)

Tablel. 2 Immature, mature and postmature oocytes obtained during time.

### Immature, mature and postmature oocytes obtained



The percentage we had obtained is similar with the date published in literature. (Yong și colab. 1998).

### Development rate of embryos.

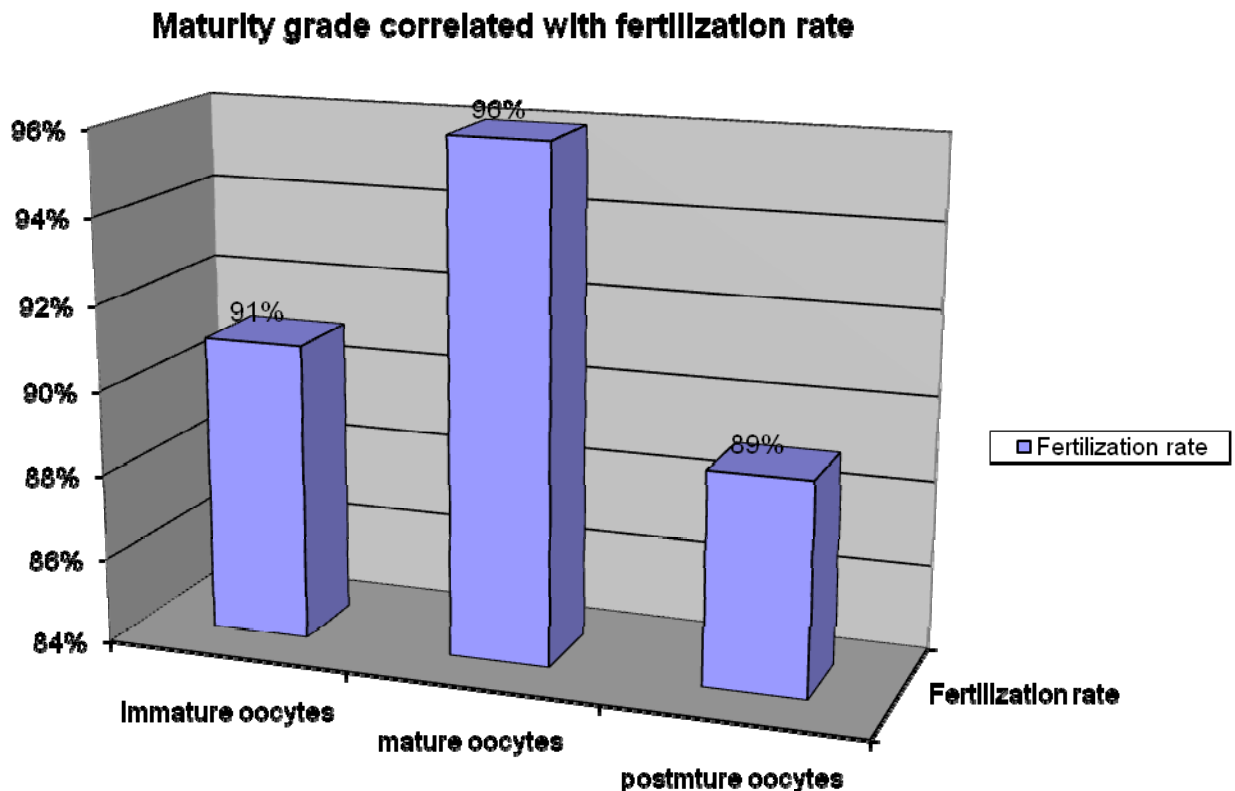
In vitro preimplantational development is under the influence of a great number of factors. The culture medium composition, the quality of male biological material, the laboratory environment, the hygiene conditions and infections prevention and the quality of oocytes are factors that permit us to obtain good quality embryos. The oocyte quality can be influenced in positive and negative way by ovarian stimulation protocol (duration, dosage and medication chosen).

We had obtained 5462 oocytes, 287 were immature, 4919 were mature and 256 postmature.

The oocytes insemination was made at different time intervals, for postmature oocytes the insemination was performed at 30 minutes post follicular puncture and for mature oocytes at 2 hours. The immature oocytes were matured for 24 hours and then inseminated with fresh sperm. The fertilization rate was 91% for the mature oocytes, 96% for the mature oocytes, 89% for the postmature oocytes.

Maturity grade	Immature oocytes	Mature oocytes	Postmature oocytes
Fertilization rate	91%	96%	89%

Table.3 Oocyte maturity grade/fertilization rate.



## **Results regarding ultrastructural studies upon biological material.**

### **Ultrastructural male biological material studies.**

The ultrastructural studies upon seminal material were made using fresh sperm and sperms fixed after different time period:

- fixed immediately after swim-up preparation.
- fixed after different period of time (1,3,6,18 hours) after swim-up preparation in order to see if, in absence of oocytes the capacitated sperms presents ultrastructural modifications. These investigations started from our observation, that the sperms that spent 12 hours in culture medium in absence of oocytes have not the capacity to fertilize, if after this period of time are put together with oocytes. This situation occurred when the oocytes were immature and in order to be fertilized, the oocytes spent 24 hours in culture medium. In this case we had to obtain a fresh semen sample.

On the other hand, the ultrastructural studies performed upon prezygotes and embryos in different developmental stages showed the presence of sperms in zona pellucida. As a results these aspects were also studied.

### **Seminal material studies.**

The images we had obtained on fresh sperms revealed the typical aspect of a human sperm. The head of the sperm has the typical head. The anterior half of the head (sharp shape) is covered by the acrosome which has a lower electronoptical density. The acrosome has an internal membrane, acrosomal matrix and an external membrane.

The neck of the sperm presents the proximal centriole from where axonema begins. The first region of the tail is the intermediate piece, where is located the mitochondrial sheath around axonema. (Fig.10)

On transversal section at the intermediate piece level are visible the sperm plasmalemma, the thin layer of cytoplasm, the sheath mitochondria and the 9 doublets of microtubules. (Fig.14)





Fig.10

Even in normospermic cases, we found a large number and diversity of abnormal, mobile forms, undetectable at optic microscope.(Fig.11, 12, 13.)



Fig.11



Fig. 12

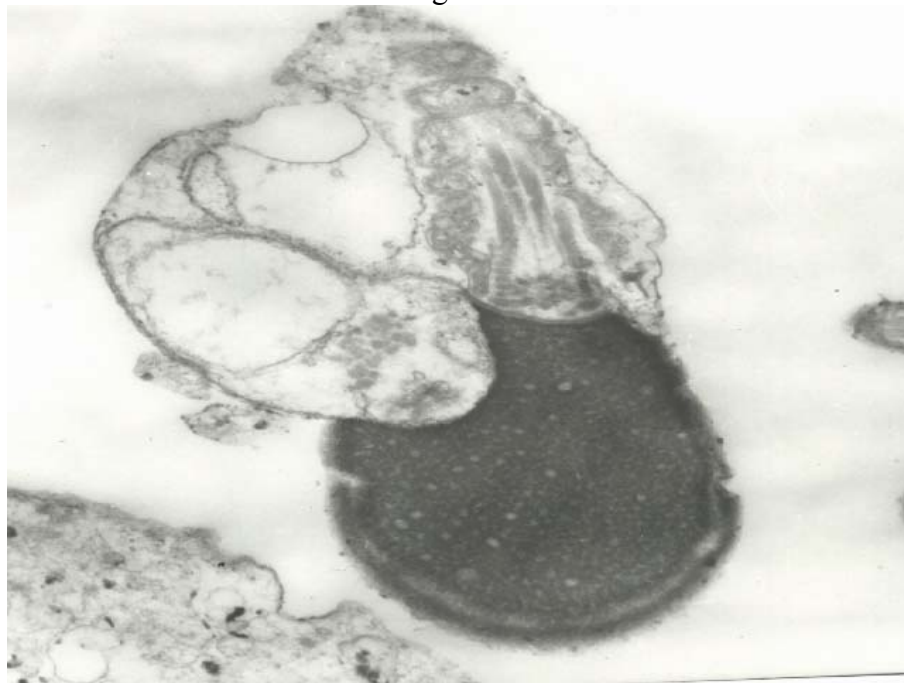


Fig.13

The observations made on sperms fixed after 3 hours of incubation in culture medium shows that the sperms have a normal ultrastructural aspect.(Fig.14)

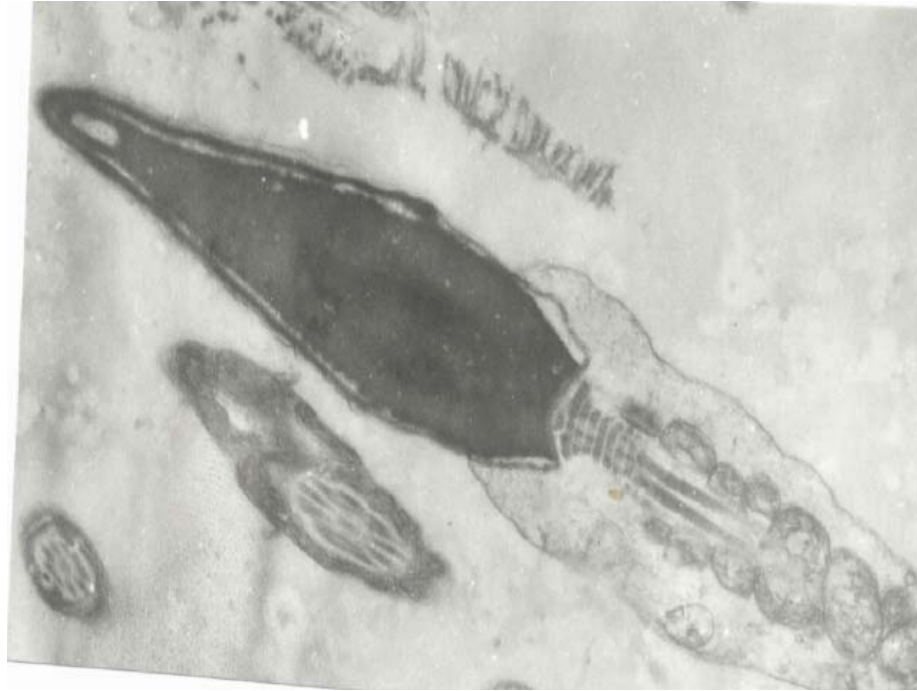


Fig.14.

After 6 hours of incubation the culture medium becomes more acid (this modification can be seen due to the medium colour modification), probably due to hyaluronidase, which is released from acrosome. The ultrastructural aspect of the these sperms is typical.(Fig.15.)

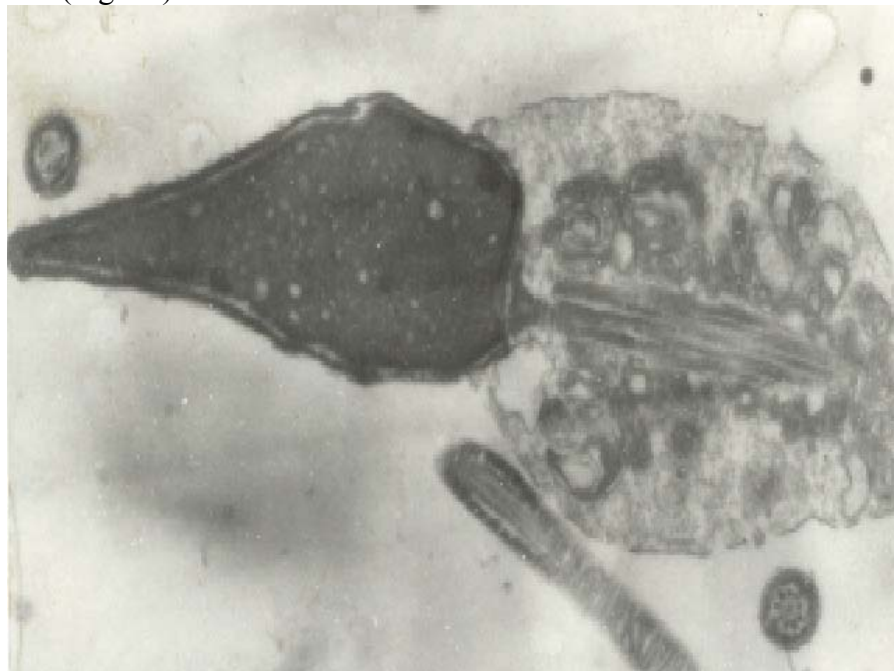


Fig.15

Significant ultrastructural modifications were noticed after 18 hours of incubation. A large number of sperms (30-40%) shows acrosome dilatations, the acrosomal matrix has a electronoptic aspect less dense and even vesicles are present at this level(Fig.16).

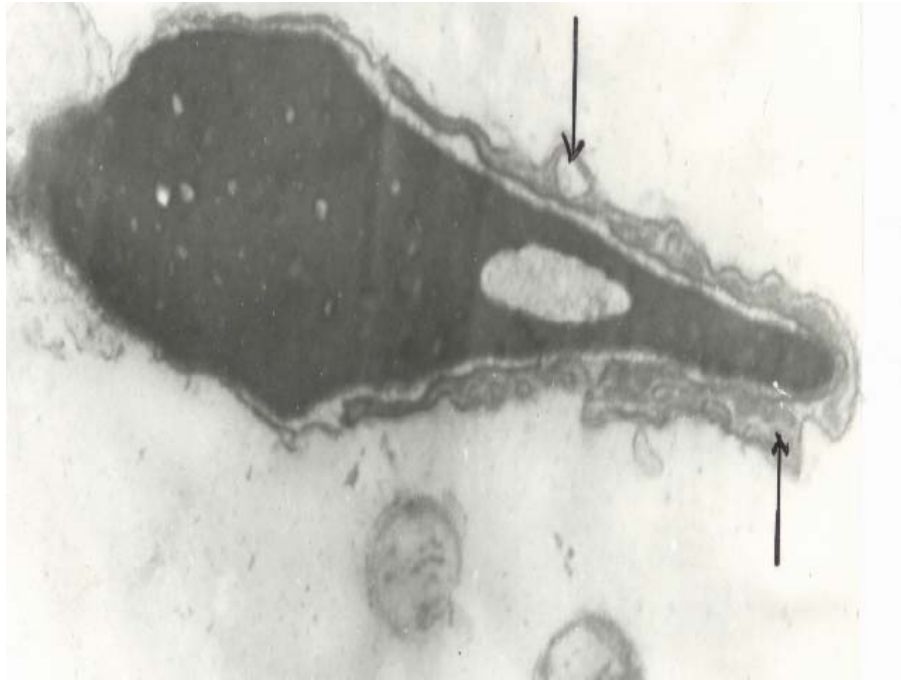


Fig. 16

In few cases, the head of the sperm has a lighter aspect, probably due to cromatine decondensation, which can be interpreted that the sperm is dead.(Fig.17).

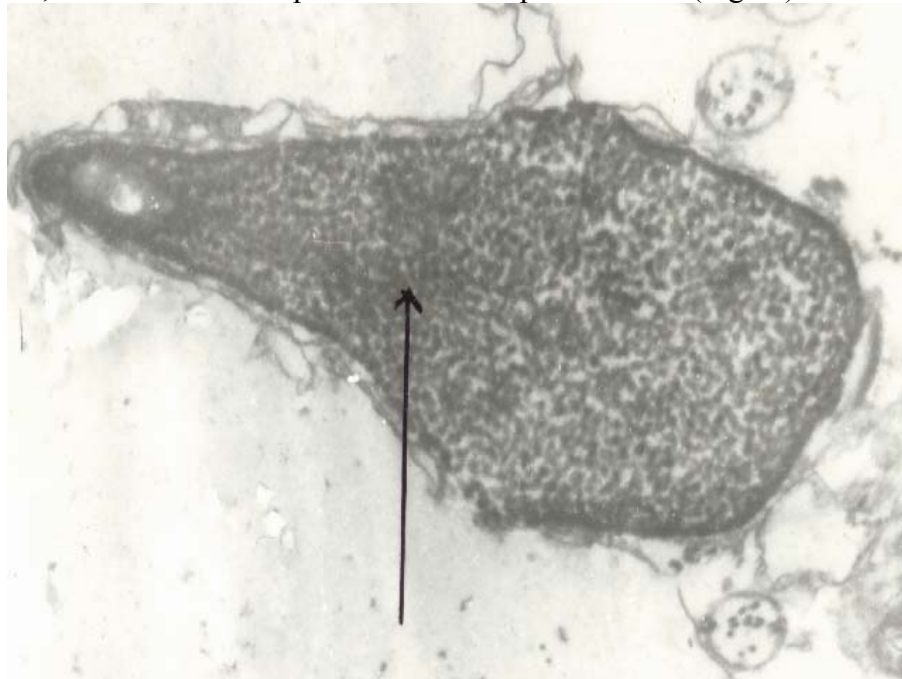


Fig. 17

The aspect of the acrosome at sperms incubated for 18 hours is the reason why these sperms can not fertilize, even if they are mobile and the optical aspect is normal. Probably, during the 18 hours spent in the culture medium, the proteic molecules located on the sperm head, molecules that should recognize specific parts from the ZP3 glycoprotein from zona pellucida are modified and the sperms are unable to link to the zona pellucida.

In this context, the affirmation found in literature that the sperms keep their fertilization potential for 24 hours should be considered with a certain reserve. Obviously we made this remark studying the sperms *in vitro*, and it may be possible that *in vivo* the conditions are different, so the modifications seen in our study are not present *in vivo*.

**Studies of male biological material on immature unfertilized oocytes.**

At 18 hours post insemination, when we performed the fertilization control, in the zona pellucida of immature unfertilized oocytes we found sperms attached at this level.

The images we had obtained in electronic microscopy showed:

- the majority of sperms found in the zona pellucida are closer to the external part of the zona.(Fig.18, 19)

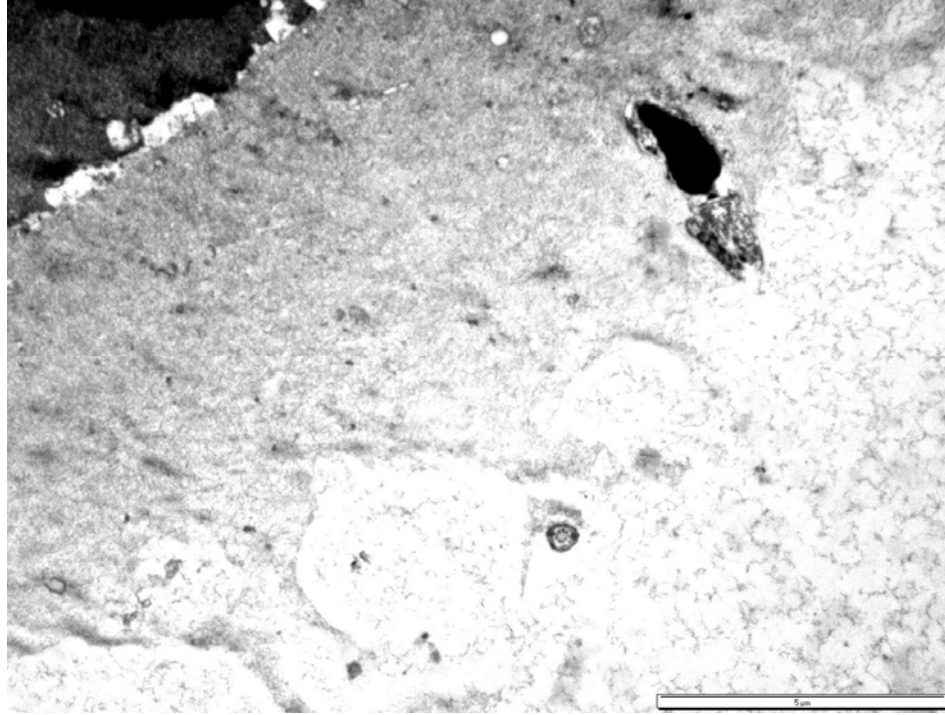


Fig. 18



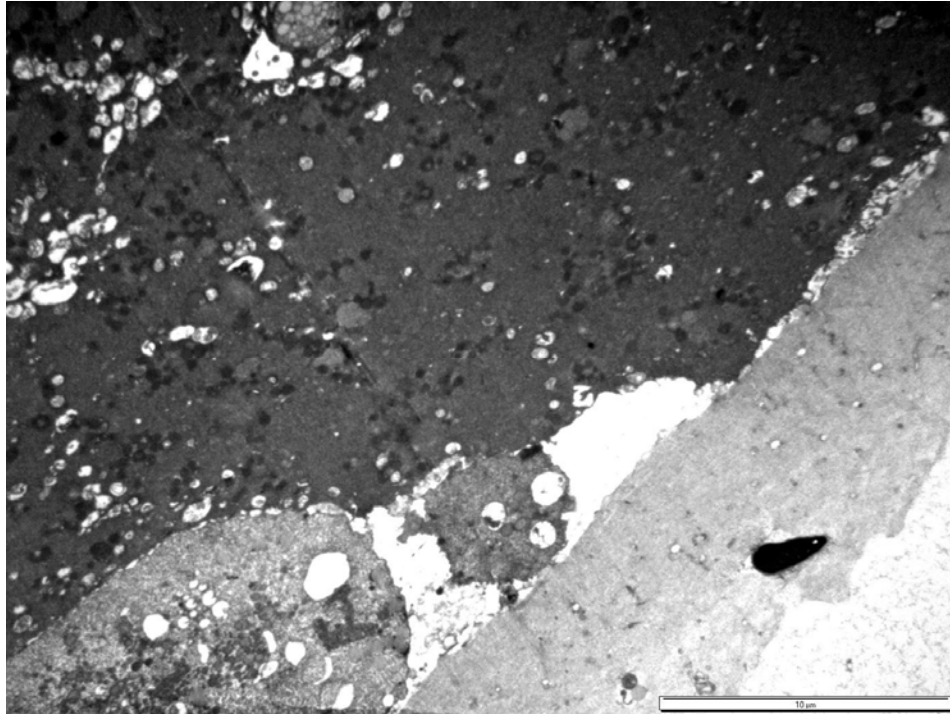


Fig. 19

The outer acrosomal membrane seems to be fenestrated, which shows a potential start of the acrosomal reaction. Zona pellucida shows a light rarefaction of its macromolecular structure suggesting a certain enzymatic activity (Fig.20). The head presents a typical aspect with no signs of chromatin decondensation.

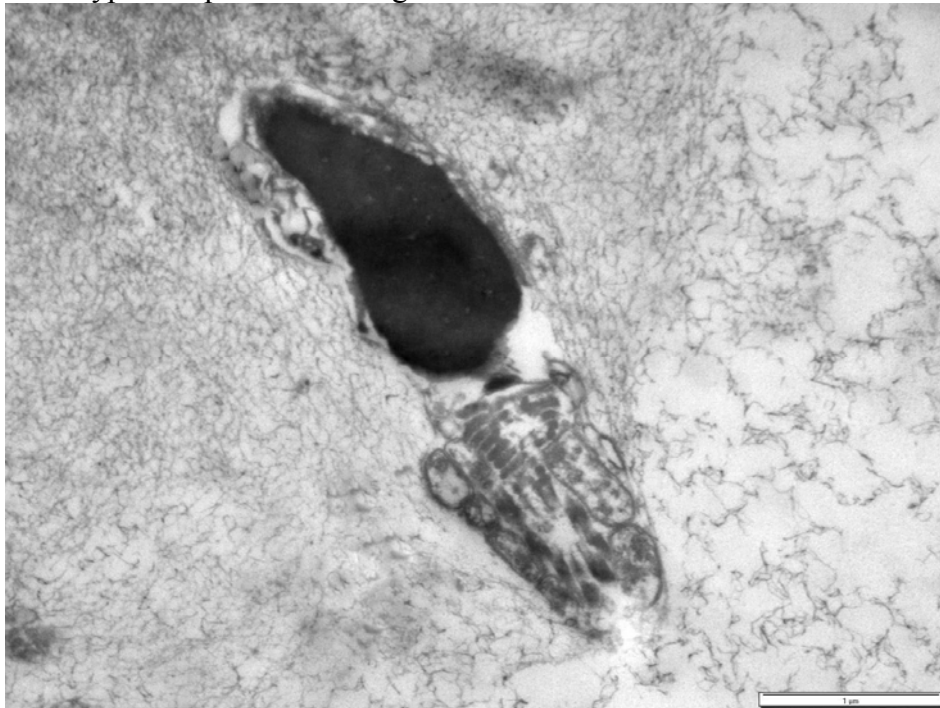


Fig. 20

The prezigot stage, the 3,5,8 cells stage shows sperms caught in the zona pellucida.(Fig.21, 22).

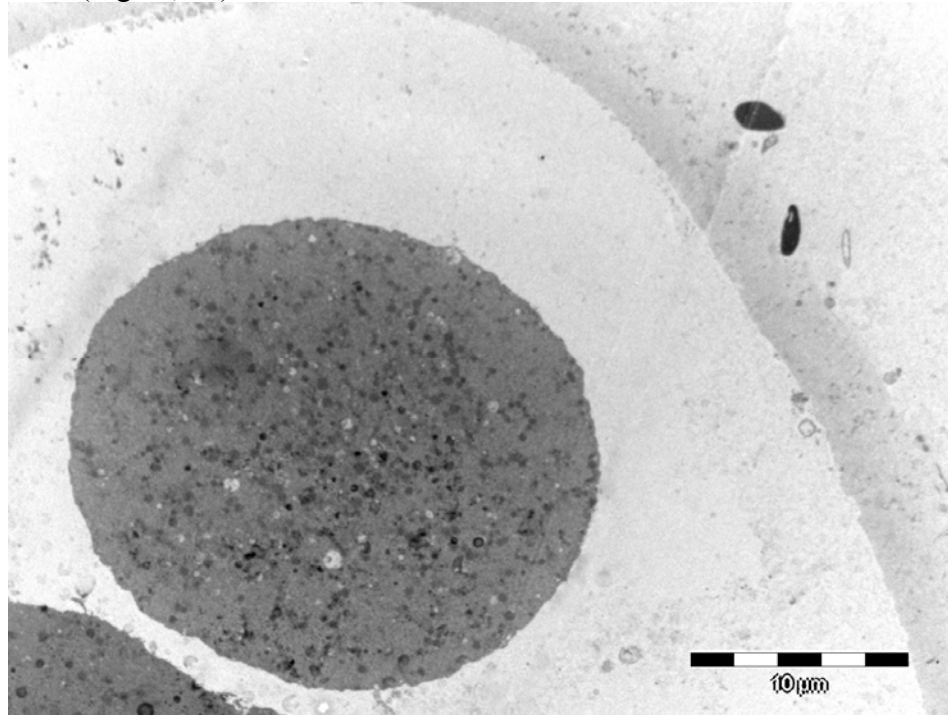


Fig. 21

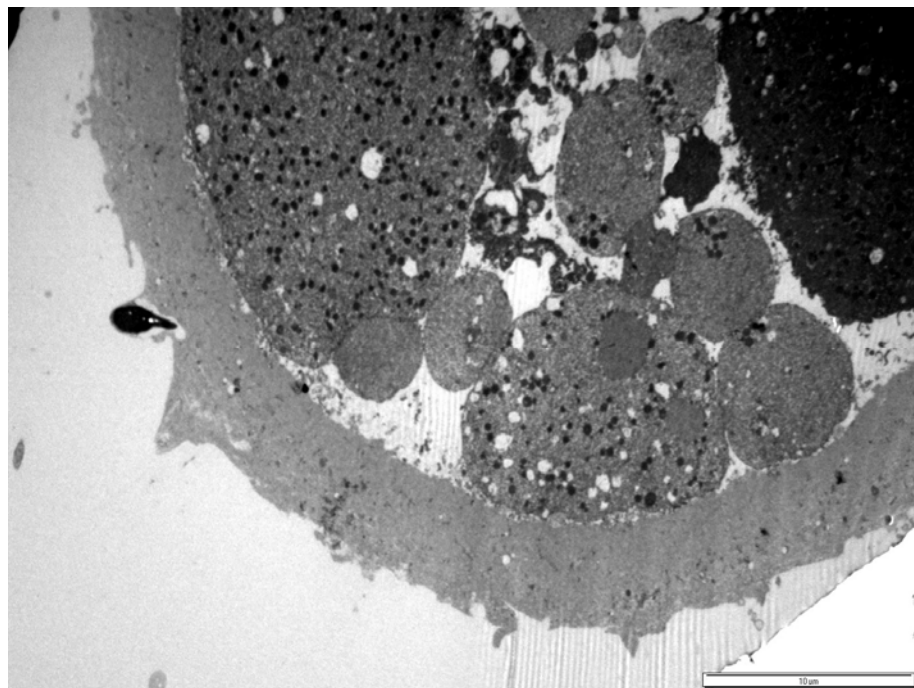


Fig. 22

Even in the blastocyst stage sperms can be seen in zona pellucida. In Fig. 23 a trophoblastic cell can be seen and also a sperm's head.



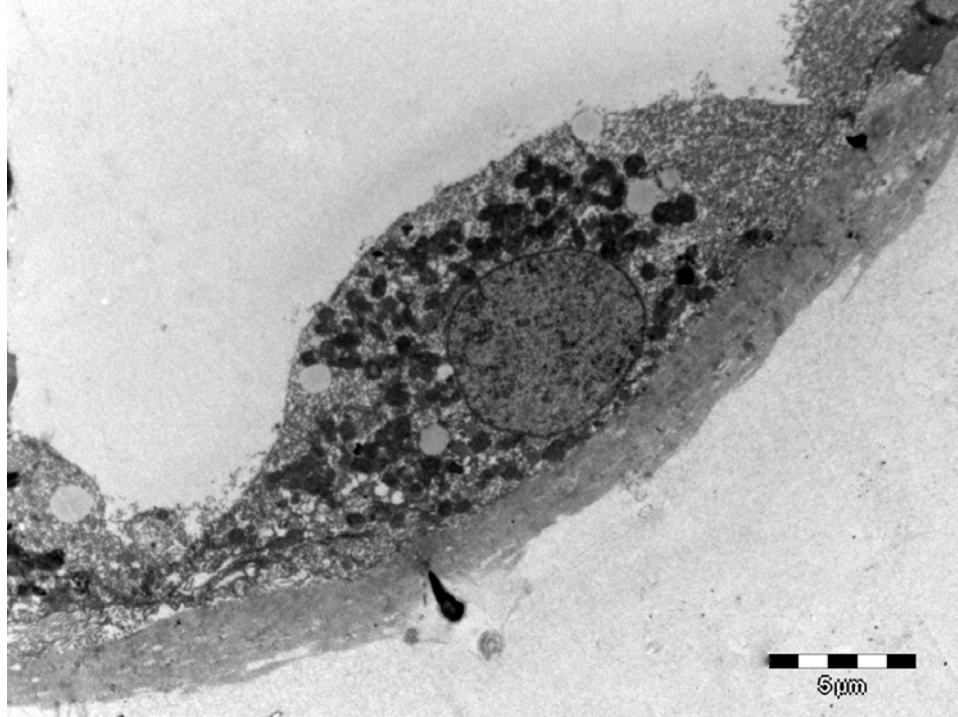


Fig. 23

### Conclusions upon sperms:

- sequential ultrastructure of sperms that spent different time period (3,6,18 hours) in culture medium in absence of oocytes has never been described before in literature.
- our study shows that the ultrastructure of sperms after one and six hours of incubation is normal, the sperms being able to fertilize.
- after 10-12 hours alteration processes occur, showing that the sperms are unable to fertilize.
- the ultrastructural sperm modifications we had described after 18 hours of incubations are:

- acrosome dilatation
- less dense electronoptic aspect of acrosomial matrix
- presence of vacuoles in acrosomial matrix
- fenestration of external acrosomial membrane
- less coloured aspect of the nucleus, which shows degenerescence

The acrosome is the most affected structure, presenting a beginning of acrosomial reaction. These modifications explain why the sperms are not able to fertilize if they spend 18 hours in culture medium. The affirmation found in literature, that the sperms keep their fertilization potential for days is not supported by our results.

The aspect of sperms caught in zona pellucida at different embryonic stages present a remarkable chromatinic stability. The sperms show the same electronoptic aspect even after 4-5 days spent in zona pellucida which proves the zona pellucida's conservation capacities.

### **Studies upon ultrastructure of oocytes, in different maturity stages.**

One of the criteria used in laboratories for oocyte quality is the degree of oocyte maturation. Using the data from literature and our own observations made during the years, the oocytes can be divided in three categories: immature, mature and postmature. For this clasification we took in consideration the optical aspect of the complex oocyte-cummulus oophorus/corona radiata. The optimal stage for fertilization is mature oocyte. **Deep** investigations allows a pertinent carachterization of all three types of oocytes and the possible differences between them at ultrastructural level.

The ultrastructural details found can allow us to establish a link between ultrastructural and optical aspect of the oocytes.

We used for our study the biological material (sperms, oocytes and embryos) only after we received the information and the accept forms signed by the owners.

The analisys of hundred of imges obtained shows aspects described previously in literature, but also a lot of aspects are new, undescribed in literature.

#### **Mature oocyte**

Using optical microscopy we are able to identify mature oocyte by the aspect of cummulus and especially corona radiata. The cummulus cells are eqally distributed. The cells of corona radiata are radiar positioned around the oocyte. (Fig. 24, 25).

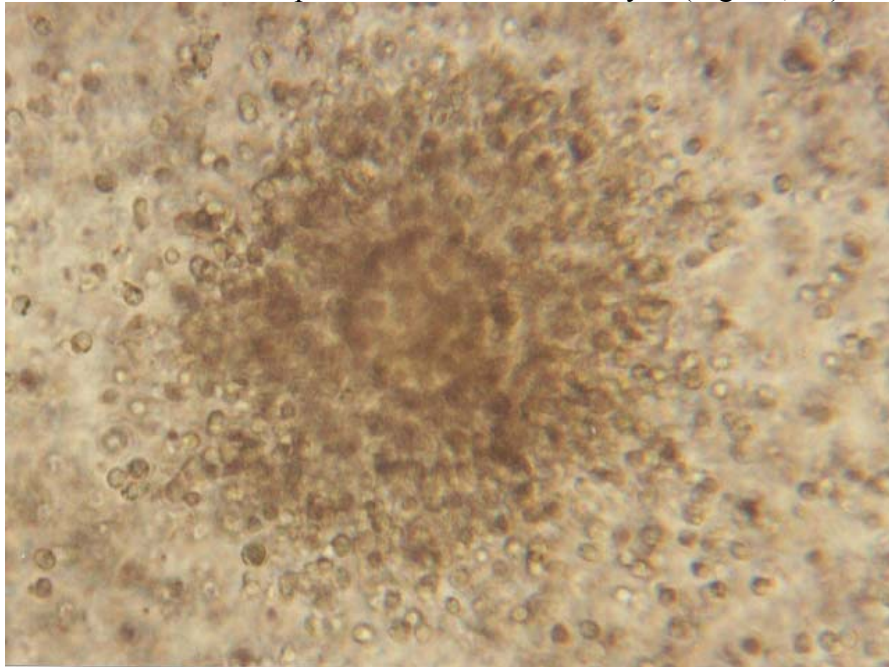


Fig. 24

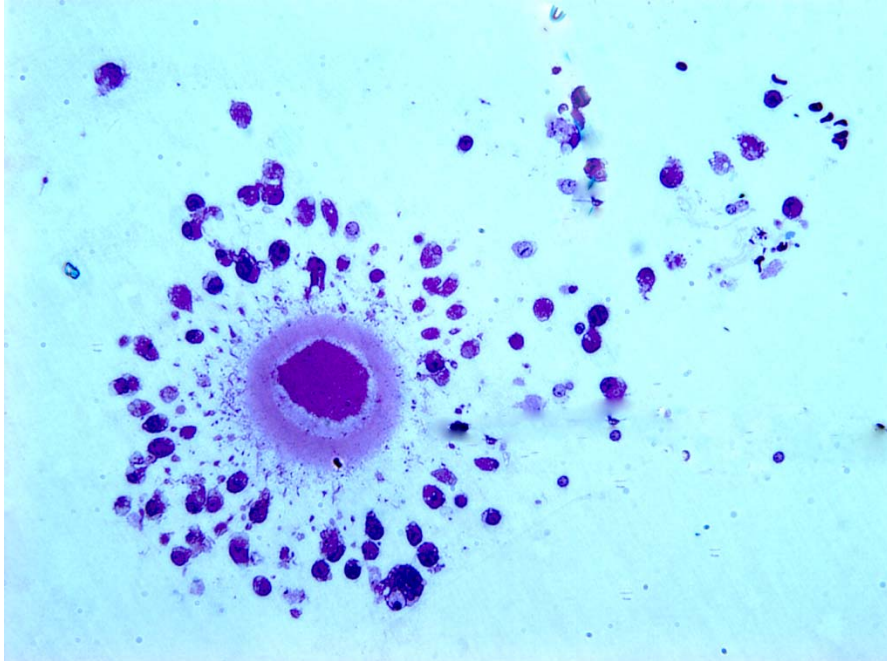


Fig. 25

### Ultrastructure of mature oocyte

#### Corona radiata

The ultrastructure of corona radiata's cells is different from the ultrastructure of the oocyte, having a great number of vesicles (containing a lipidic material-probably estrogenic hormones). Golgi system, large number of mitochondria, RER, vesicular REN, lipidic vesicles are present. The nucleus is round or irregular and has a reticulated nucleolus. (Fig. 26, 27.)

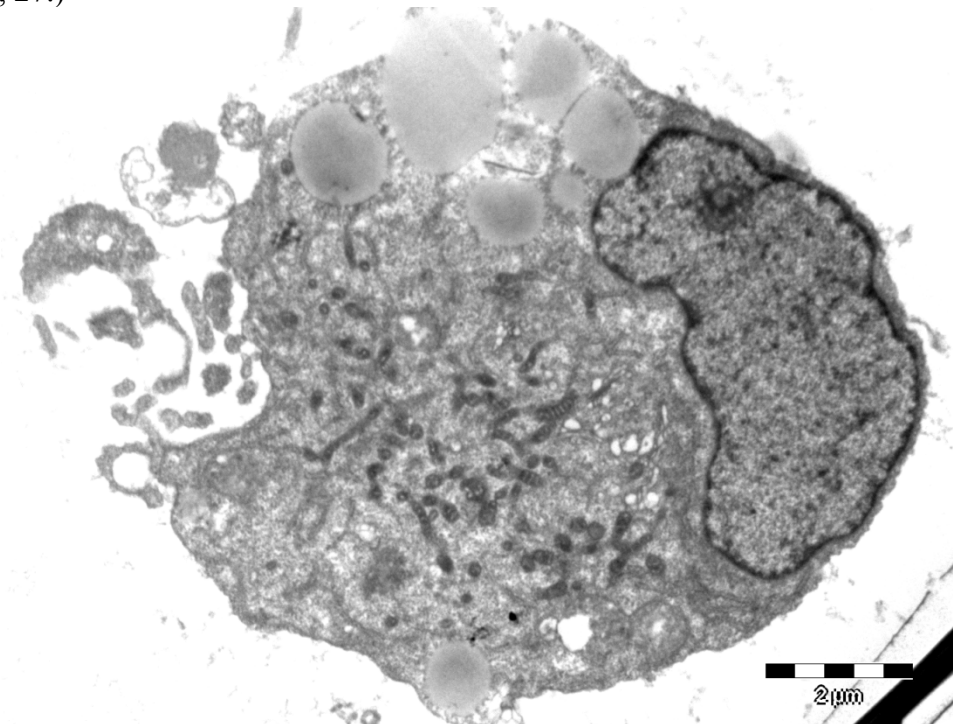


Fig. 26

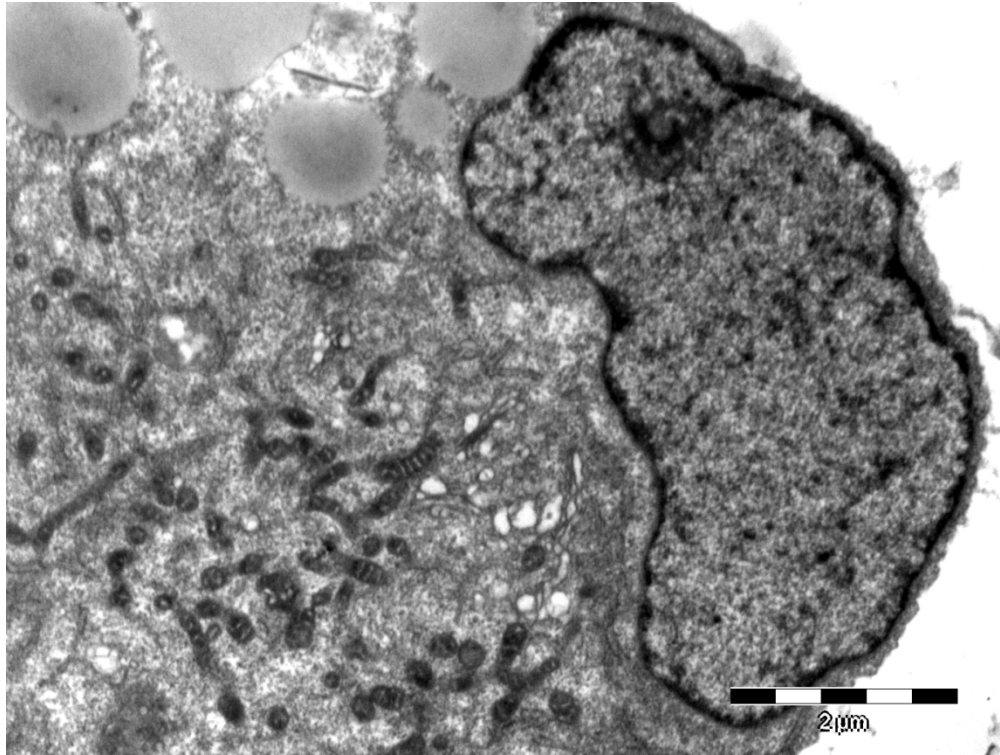


Fig. 27

Zona pellucida is seen in optical microscopy as a gelly shell around the oocyte. The SEM images present the zona pellucida as a **tesatura** of filamentous glycoproteic macromolecules (10-15 μm thick). Zona pellucida has a greater density on the internal surface and lower on the external surface (in electronic microscopy). The outer layer is rare and shows a lot of irregularities compared with the uniform inner layer. (Fig. 28, 29).



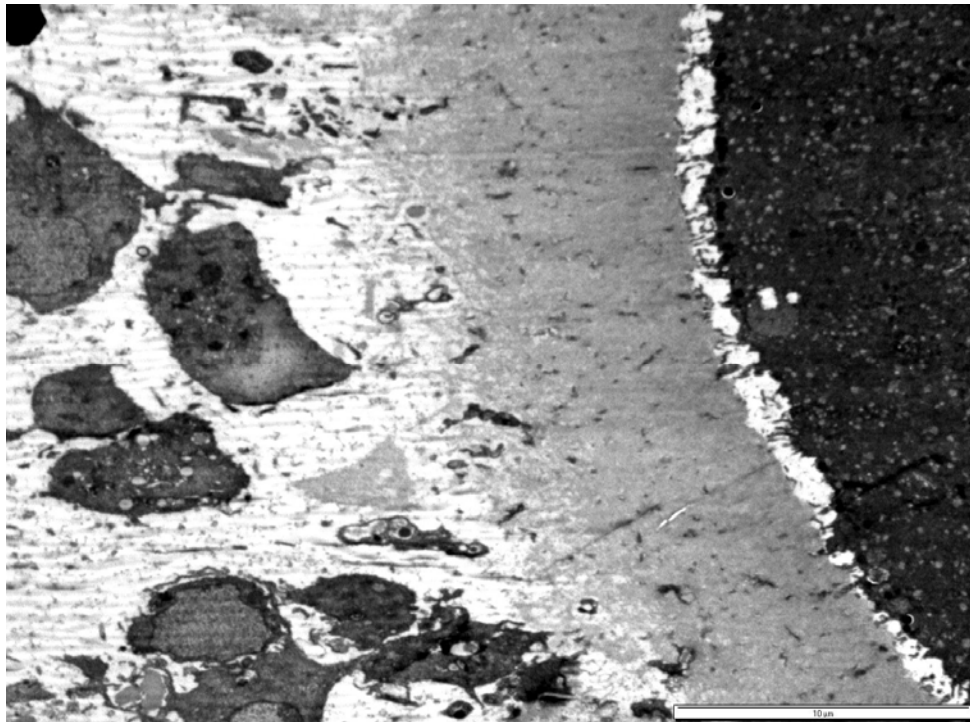


Fig. 28

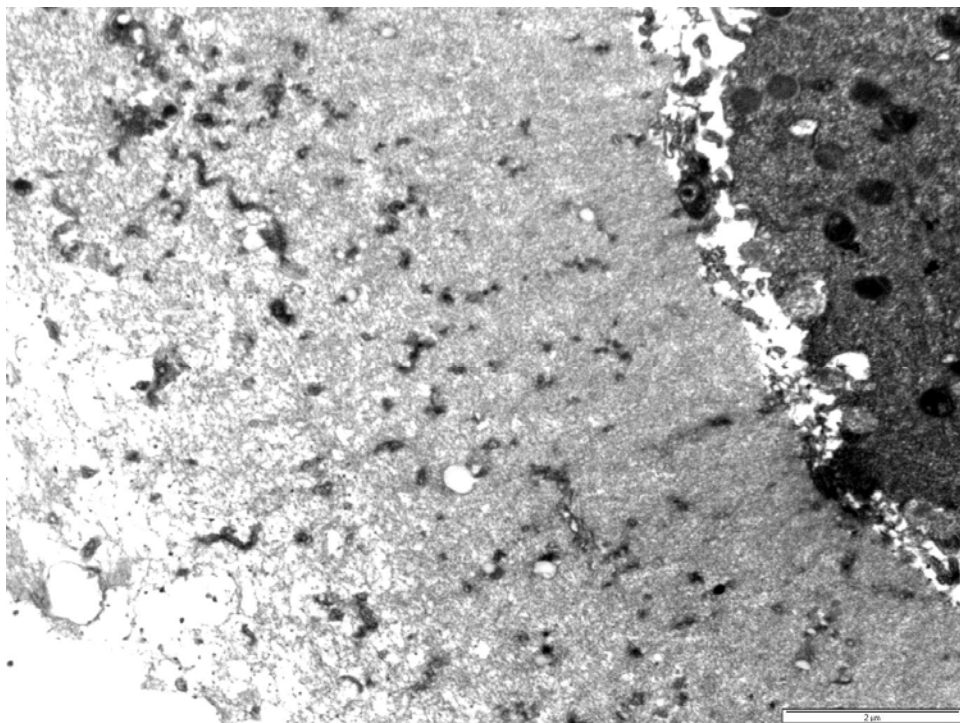


Fig. 29

Oocyte cytoplasm contain:  
 -mitochondria in large number, round shaped with high electronoptical density

Only REN is present. RER and the ribosomes are not present. The REN vesicles contain an amorph material which has the same density and aspect as the material from zona pellucida.

The Golgi complex is located in the central area of the oocyte, and is present in small number.

The mature oocytes does not have nucleus, and in the cytoplasm close to the first polar body, the division **fuse** is present.

At the periphery of the cytoplasm are present the cortical vesicles, which are characteristic to the mature oocyte. These vesicles contain proteins and enzymes which play a crucial role in blocking polyspermy. (Fig. 30, 31).

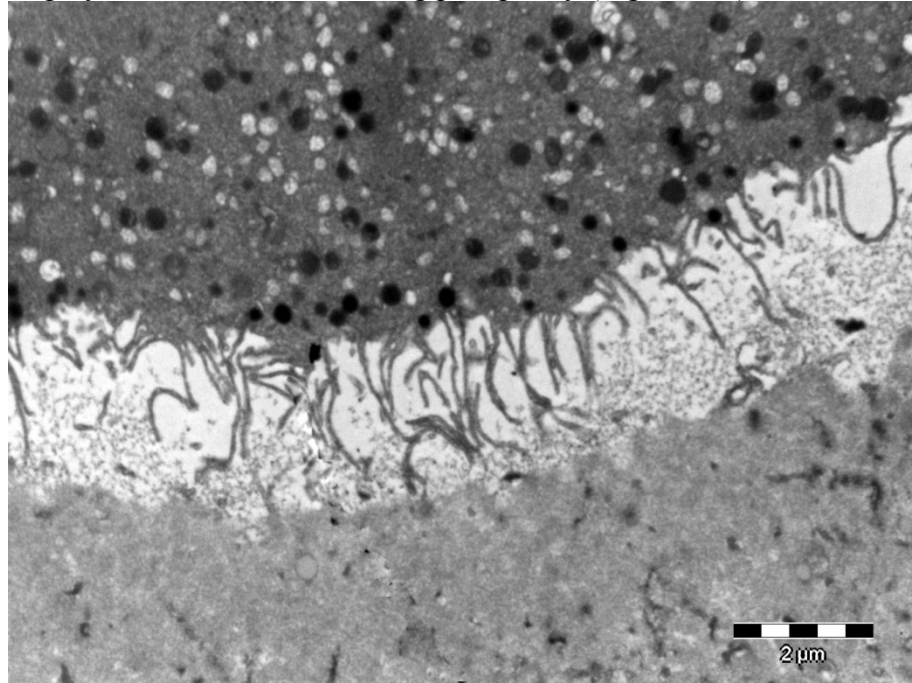


Fig.30

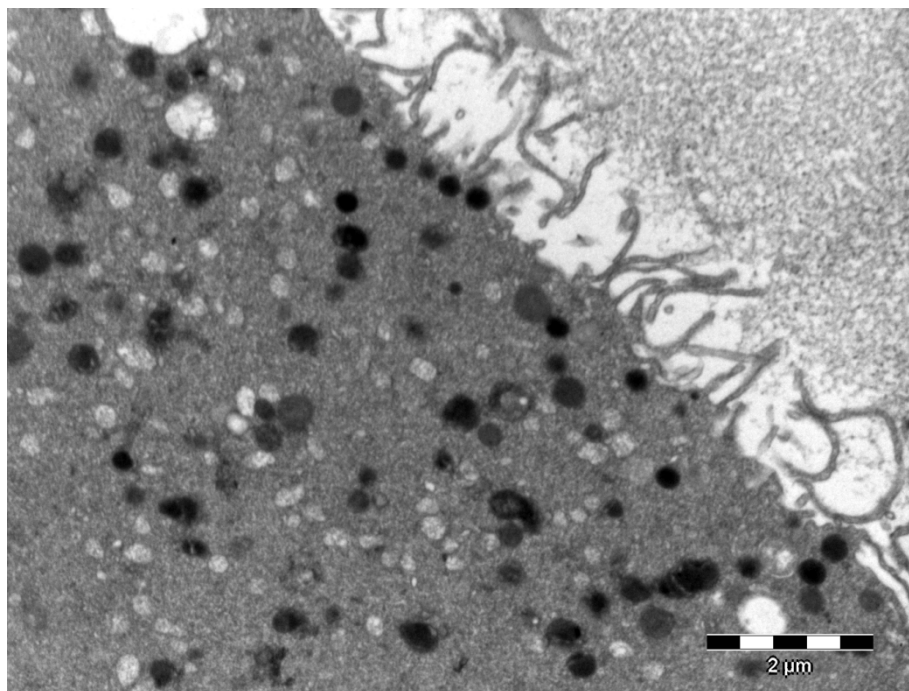


Fig. 31

### **Immature oocyte**

Using optical microscopy the immature oocyte can be identify due to cummulus and corona radiata aspect. The cummulus has an irregular aspect. The corona radiata cells are packed tight around the oocyte, making the identification of immature oocyte easy. (Fig.32)

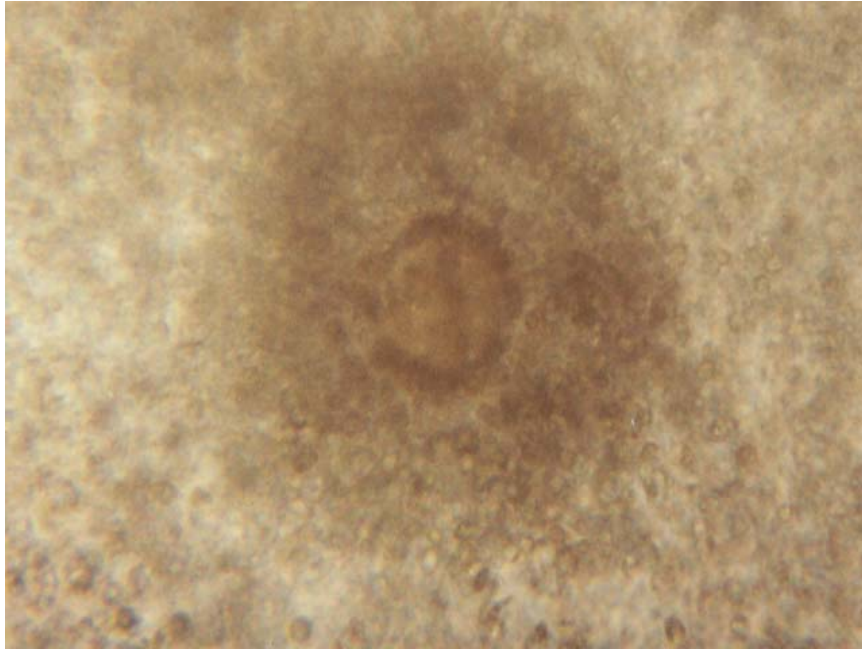


Fig.32.

### **Ultrastructure of immature oocyte**

The ultrastructural aspect of immature oocytes fixed immediately after follicular puncture presents aspects unquoted in literature.

Corona radiata. The corona radiata cells are tightly packed (piriform form) which can be easily seen in electronic microscopy. (Fig.33).



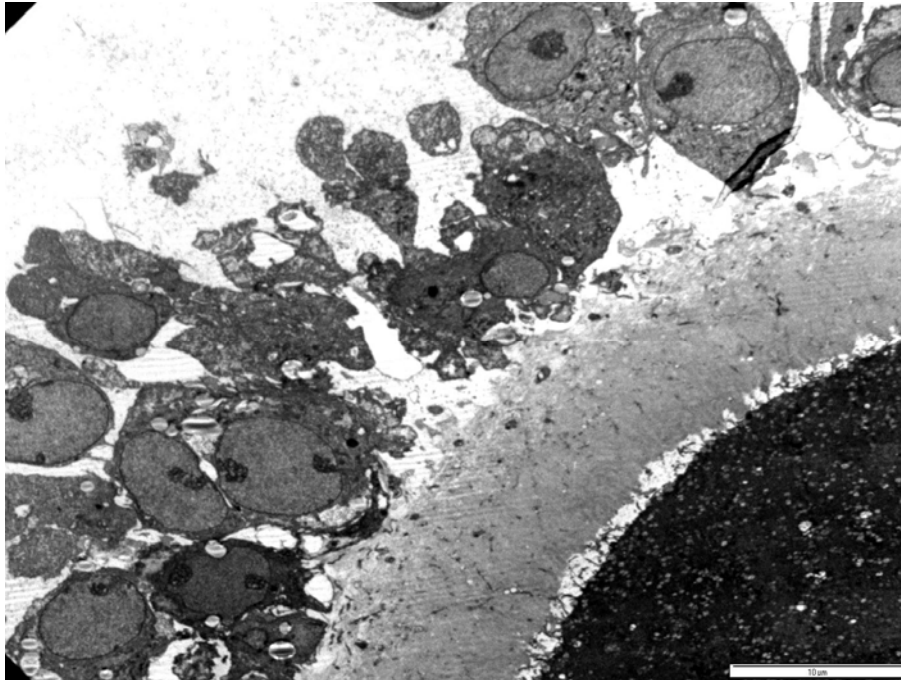


Fig. 33

The corona radiata cells have 2 reticulat nucleoli which prouves the existance of an intense proteic synthesis process.(Fig. 34,35).

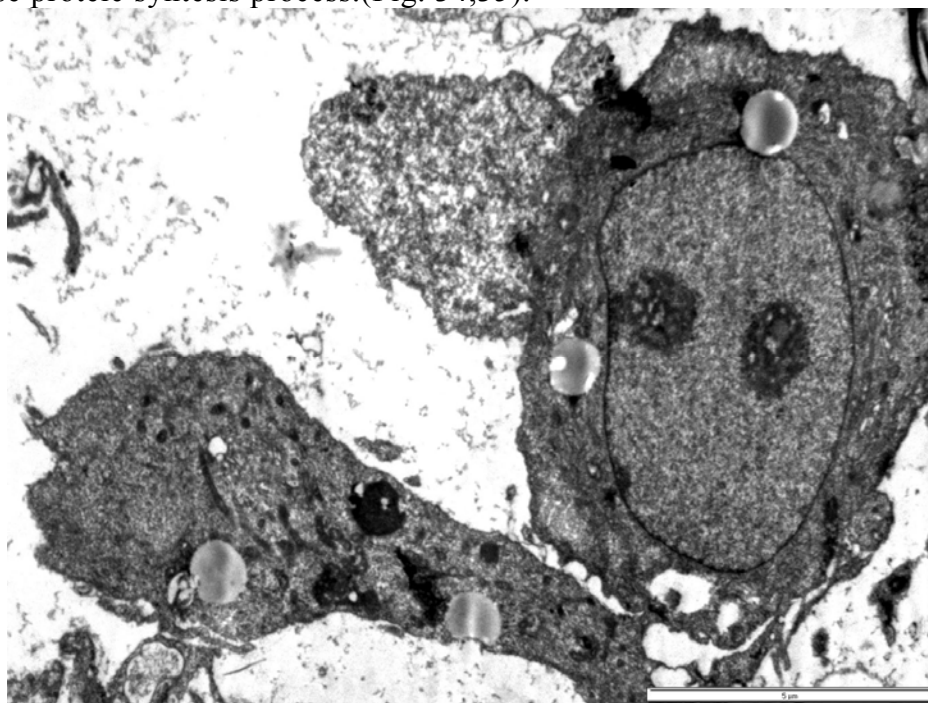


Fig. 34.

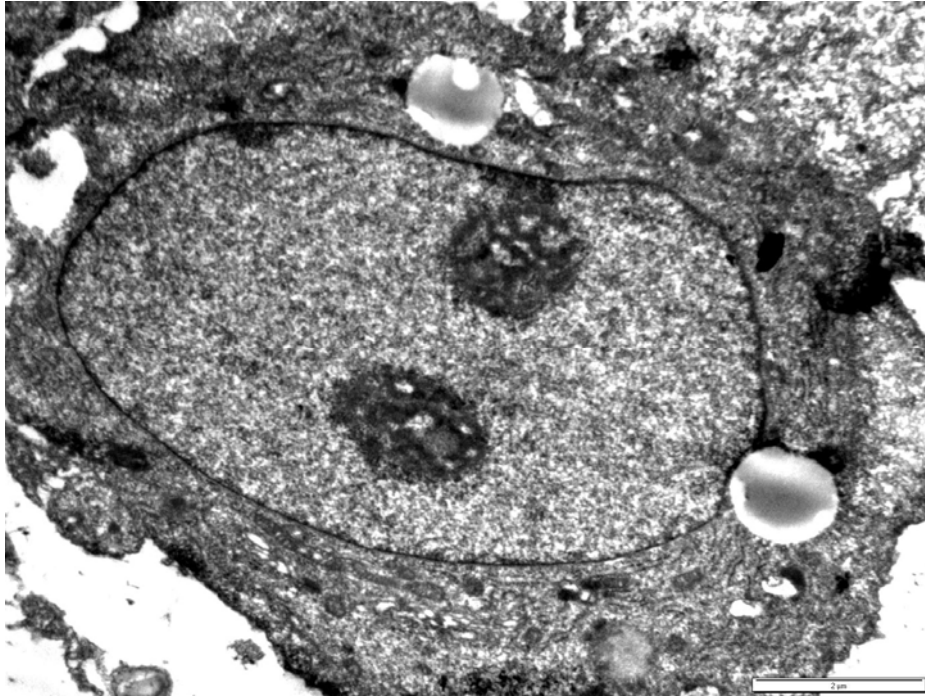


Fig. 35.

Zona pellucida.

Zona pellucida has in general the aspect described earlier, with the outer layer rare and the inner layer denser, with dense electronoptic granules.

The characteristic figure of immature oocytes zona pellucida is that the zona has not completed the structure. From the oocytes start „bridges”, which have the same aspect and density as the material from which is made the zona pellucida (Fig. 36, 37).

The perivitellin space has a large number of microvilli which start from the oocyte. (Fig. 36)

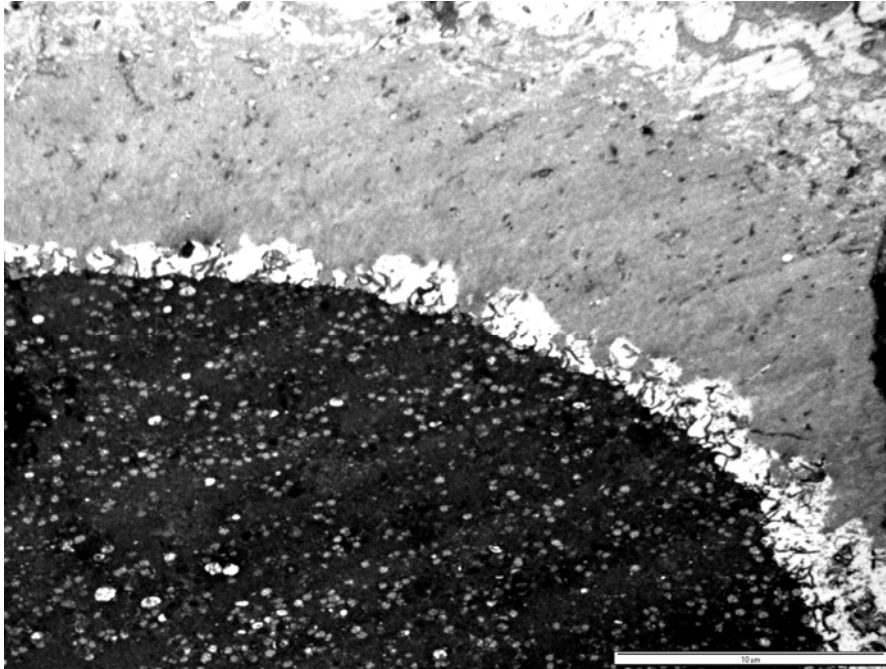


Fig. 36.

#### Cytoplasm.

The cytoplasm of immature oocyte has the same elements described earlier, but are present some differences:

- REN vesicles are in larger number.

- are present a large number of secretion vesicles which contain a material with the same density and aspect as the material from zona pellucida. At high magnification can be seen these vesicles during the exocytosis process in the perivitellin space. During this process the material synthesised in cytoplasm is released in the perivitellin space and will become part of inner layer of zona pellucida. This process has not been quoted in literature.

In peripheral cytoplasm of immature oocyte we observed only a reduced number of cortical vesicles, this can be considered another aspect characteristic to immature oocyte.

(Fig. 37)

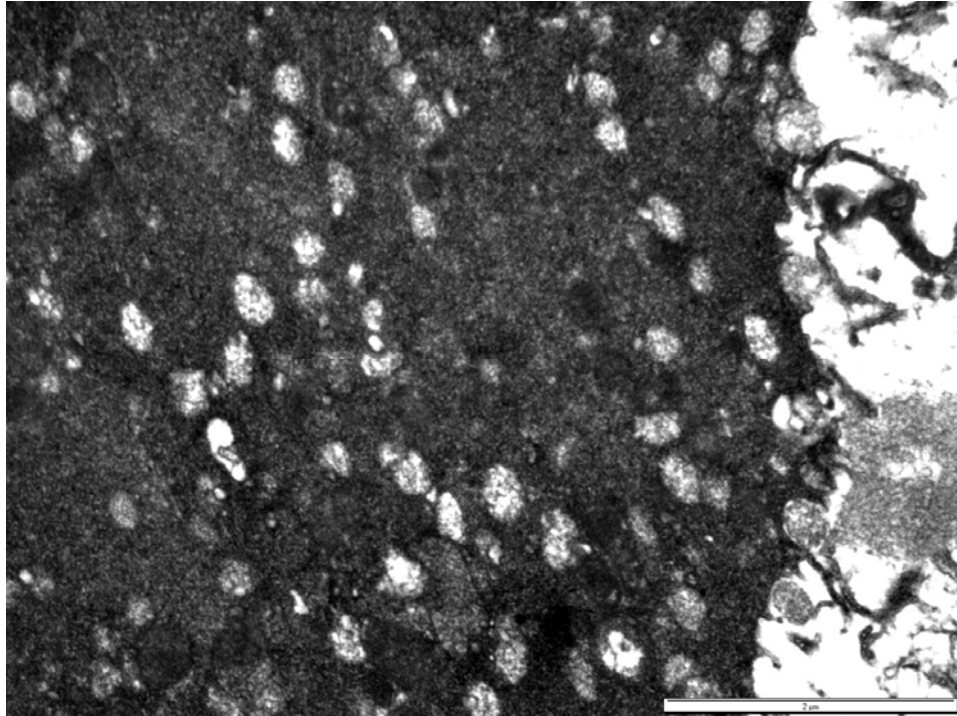


Fig. 37.

**Postmature oocyte.**

The postmature oocyte can be recognized in optical microscopy due to cummulus and corona radiata aspect. The cummulus is reduced in quantity, and the corona radiata cells are very tight packed around the oocyte. (Fig.38)

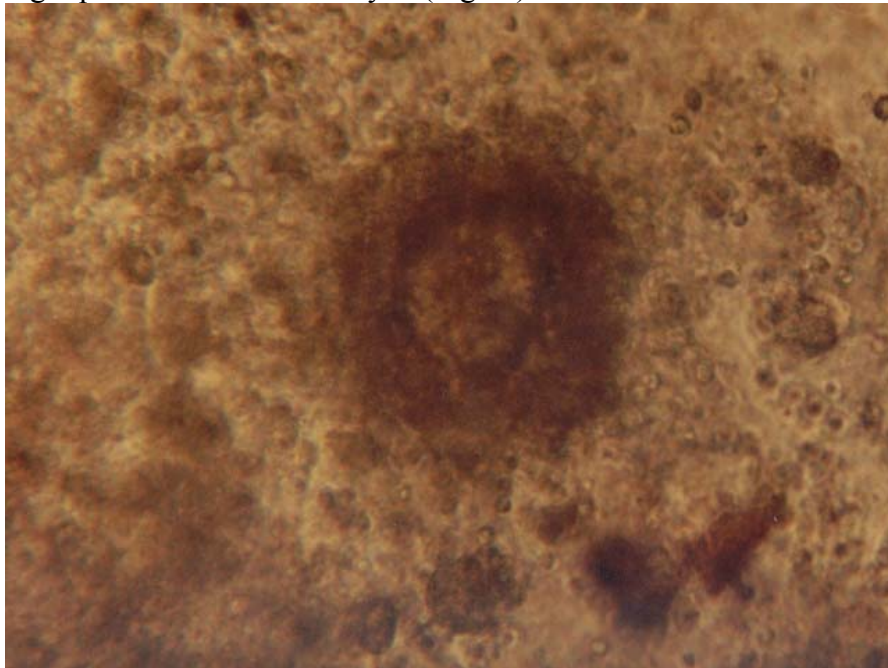


Fig. 38

### Ultrastructure of postmature oocyte.

The corona radiata cells are tightly attached to zona pellucida and present sign of degenerescence-residual bodyes, vacuolic dilatations (Fig. 39), cells fragments (between the cells) (Fig. 40).

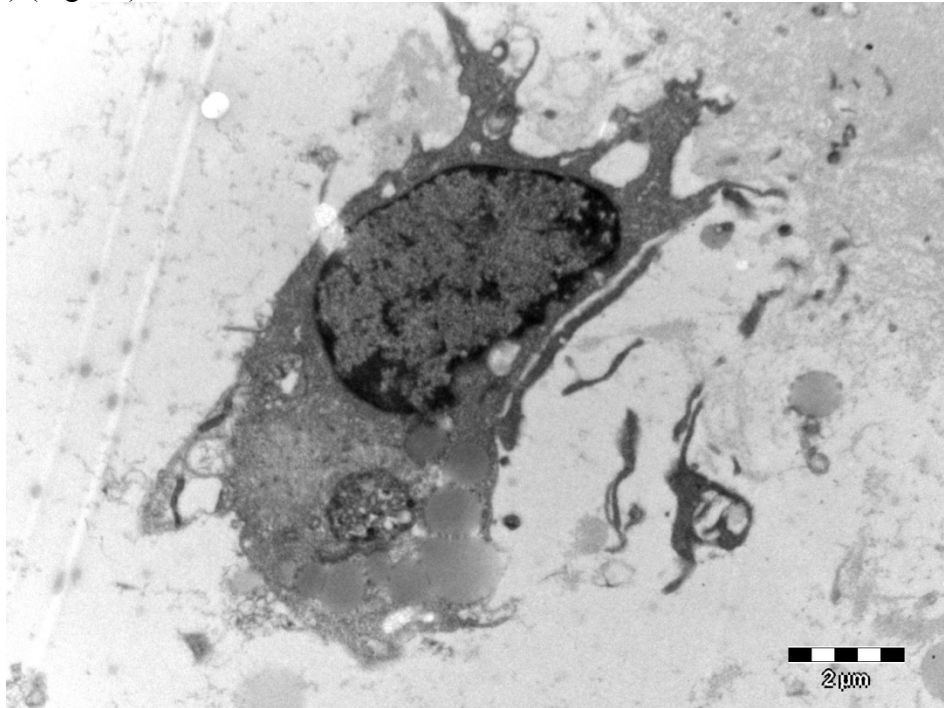


Fig. 39

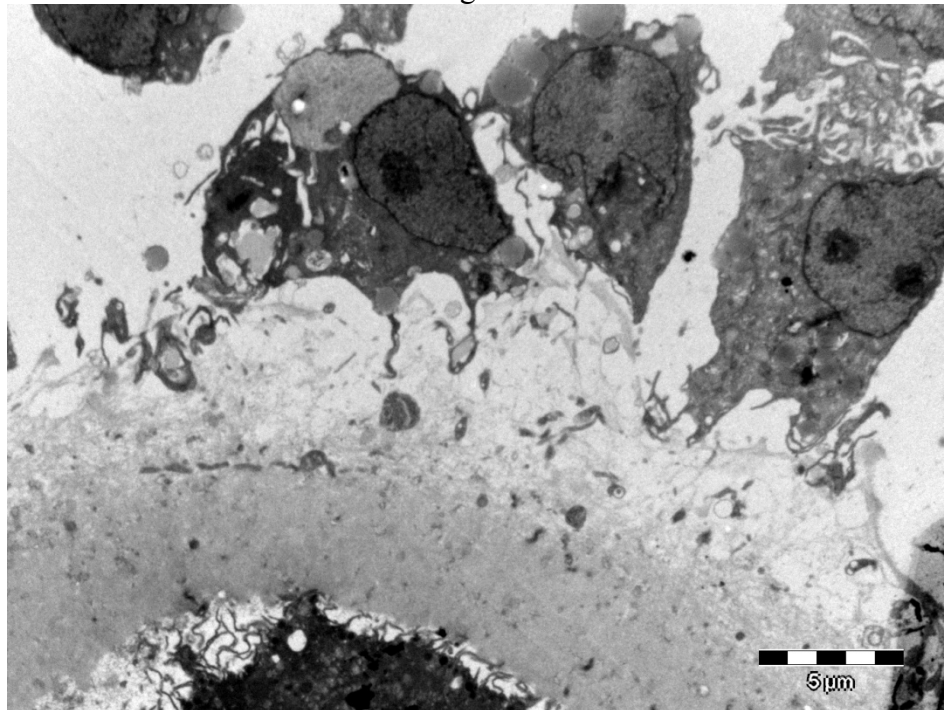


Fig. 40

- In cytoplasm we can see clear signs of celular degenerescence. In the central area autofagic vesicles are present. These vesciles were formed by REN vesicles



dilatation. In periferic area of cytoplasma a large number of mithocondria are present. (Fig. 41) and cortical vesicles (Fig.42).

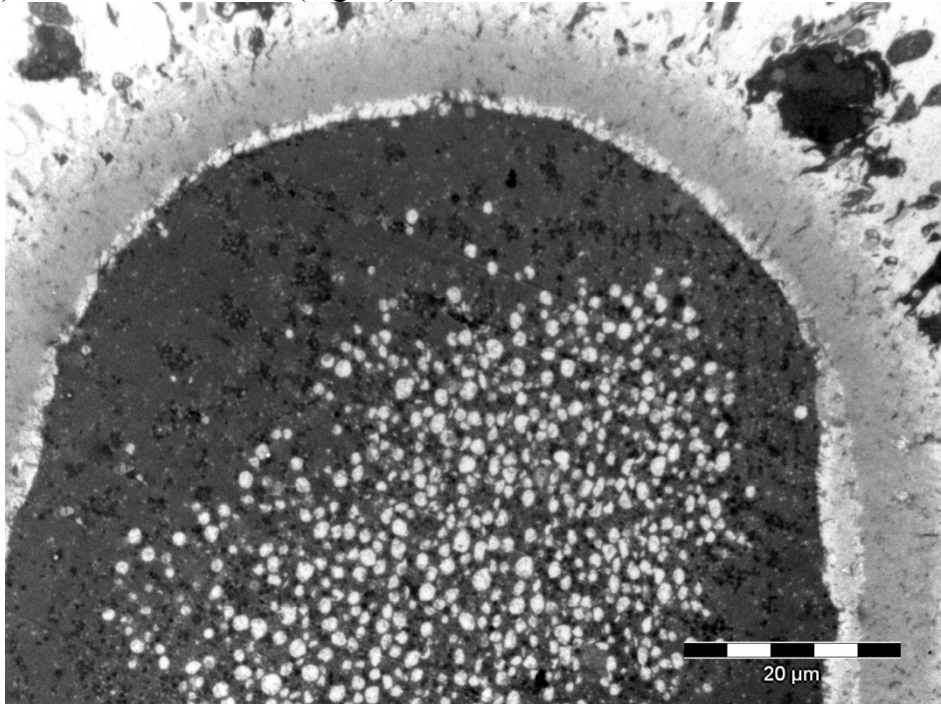


Fig. 41.

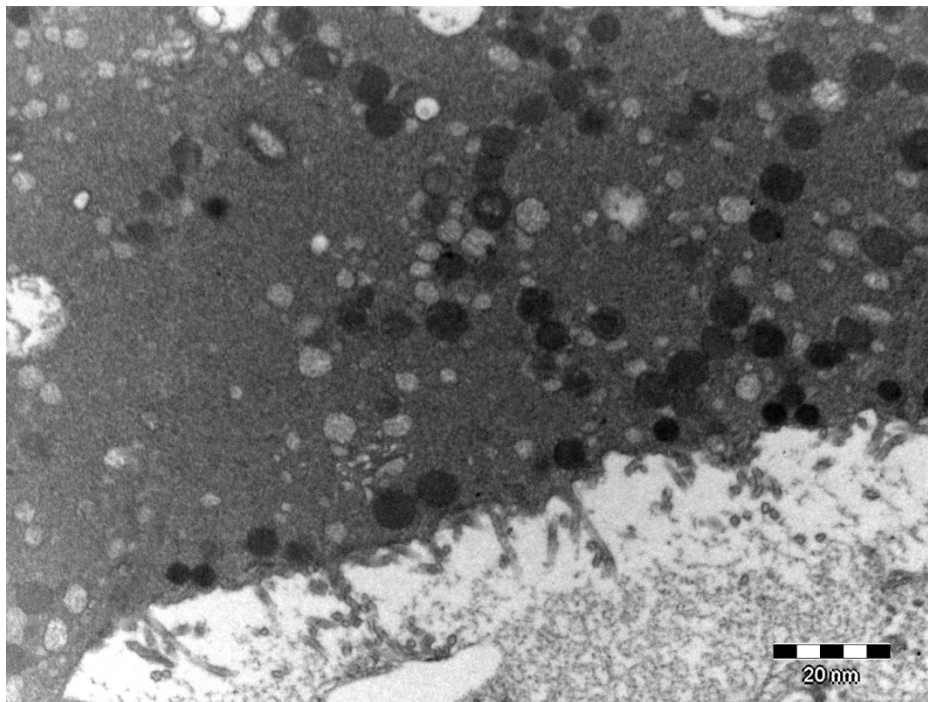


Fig. 42.

### **Unfertilized mature oocytes.**

The mature oocytes found 16 hours postinsemination unfertilized shows some interesting aspects:

-some of the unfertilized mature oocytes presented aspects found in postmature oocytes: sperms attached to zona pellucida, microvilli present in perivitelin space. In cytoplasm we can detect mitochondria, REN vesicles. These data allow us to conclude that in the moment of follicular punction these oocytes allready presented ultrastructural modifications and so, the fertilization was not realized.(Fig. 43)

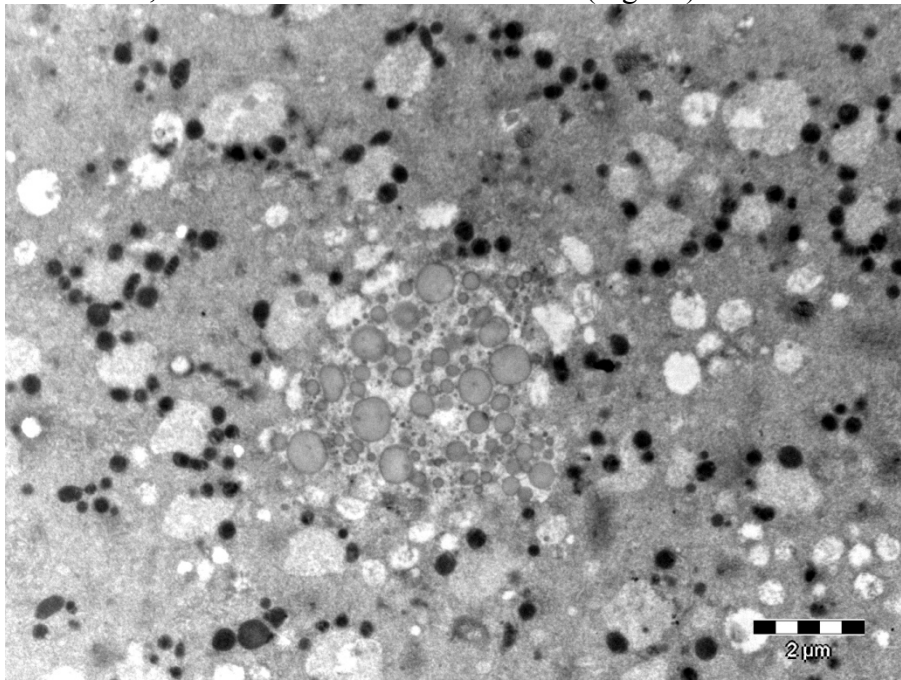


Fig. 43.

Another oocytes presented aspects found in mature oocyte: a large number of mitochondria found in cytoplasm, REN vesicles, microvilli in perivitelin space. In the outer part of zona pellucida sperms are attached.(Fig. 44).An important ultrastructural difference found is that the number of cortical vesicles is lower, compared with mature oocyte. (Fig. 45)

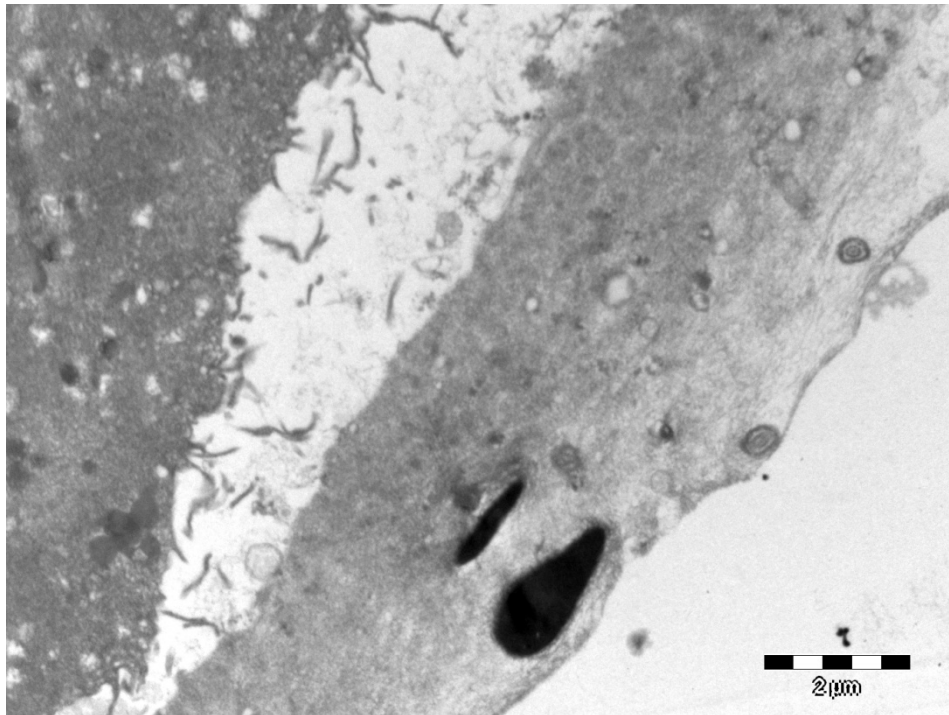


Fig. 44

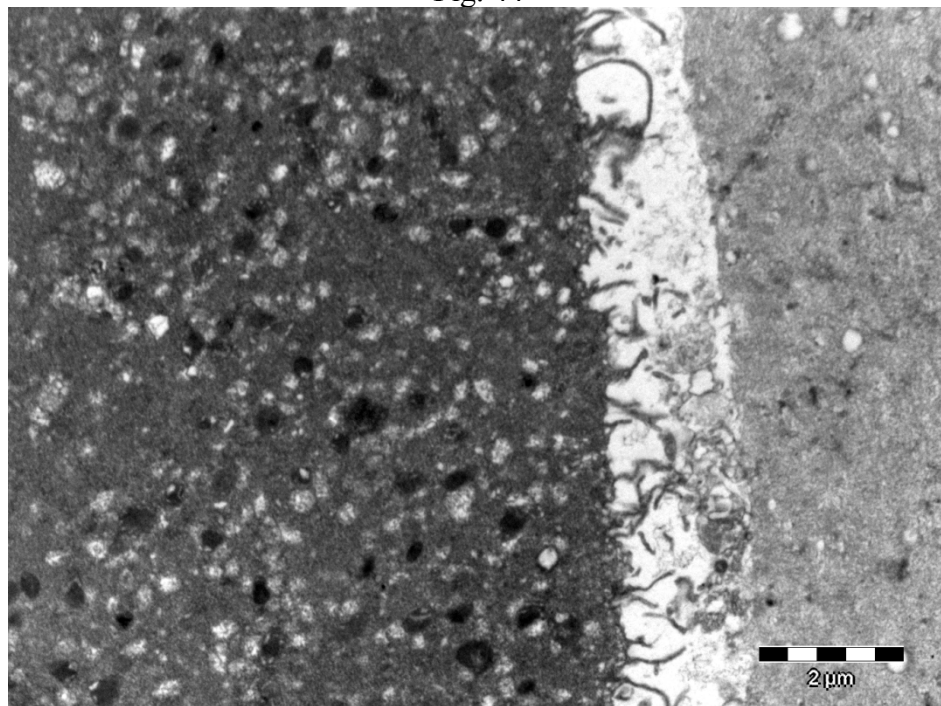


Fig. 45.

#### **In vitro maturation of immature oocytes.**

To increase the success rate during IVF procedures, one target is to obtain a large number of good quality embryos. At one follicular puncture the oocytes obtained are not in the same maturation stage. The immature oocytes do not fertilize. To overcome this problem, the teams tried different cultivation techniques to mature in vitro the immature



oocytes. This kind of techniques are mandatory when, at the follicular puncture are present only immature oocytes.

The cultivation technique implies the identification of maturation degree of oocytes. This can be made using an optical microscope. The immature oocytes found were put in culture medium (Ham's F10 or IVM-Medicult). To induce maturation the immature oocytes were kept in the incubator at 37°C, 5% CO<sub>2</sub>, for 24 hours.

After 24 hours of incubation we found that 90% of the oocytes matured. We inseminated these oocytes with fresh prepared sperm. After 18 hours postinsemination the fertilization control was made and 95-100% of matured oocytes were fertilized. From these oocytes we had obtained good quality embryos, which in some cases implanted and healthy children were born. These results made us to analyze the ultrastructure of in vitro matured oocytes and in vivo matured oocytes and we found that they are similar. We can conclude that in vitro maturation of immature oocytes is possible and has a normal outcome.

In the cytoplasm of in vitro matured oocyte are present, but in low number, secretion vesicles, on the inner layer of zona pellucida are no longer present cytoplasmic „bridges”. In the periferic cytoplasm are present cortical vesicles, mitochondria and the oocyte has a lot of microvilli in the perivitellin space (Fig. 46).

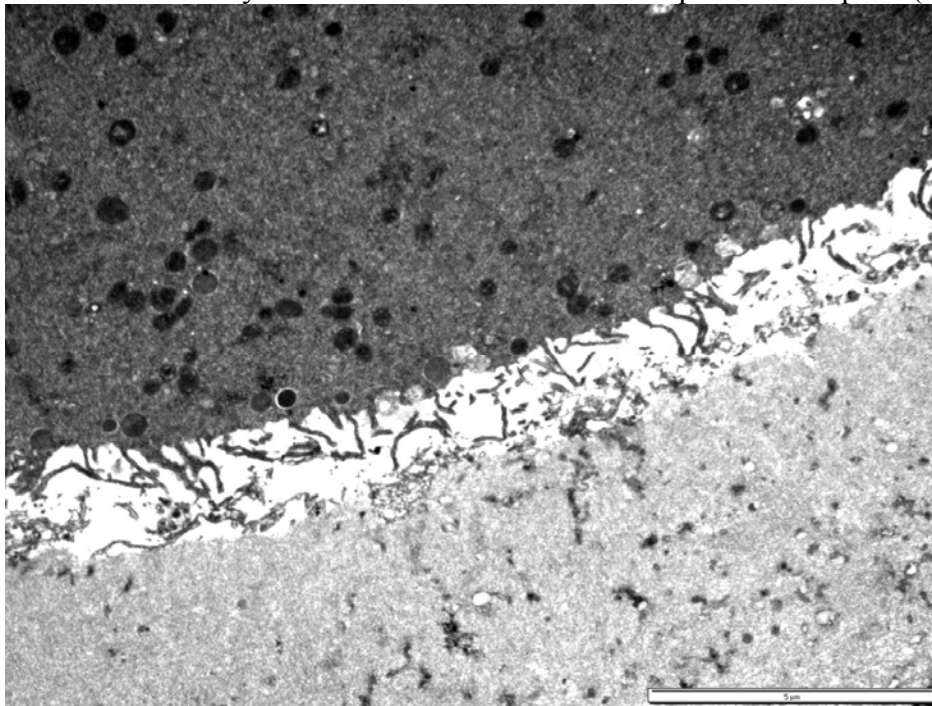


Fig. 46.

The ultrastructure of immature oocyte matured in vitro in presence of sperms shows at the inner layer of zona pellucida level a regular aspect. In the oocyte cytoplasm are present a large number of REN vesicles, mitochondria, but we were not able to find cortical vesicles. (Fig. 47).

We have no explication how the presence of sperms block the complete maturation of the oocytes, and why these oocytes are not able to be fertilized.

Due to the fact that we did not find any sperms attached to the zona pellucida we can say that ZP (receptor molecules) are not formed. Probably that hialuronidasis (the enzyme from acrosom) block the formation of ZP from zona pellucida.

In order to present a solid conclusion why the immature oocytes matured in vitro in presence of sperms are not fertilized needs further investigations. The number of oocytes from this category at our disposal was small.

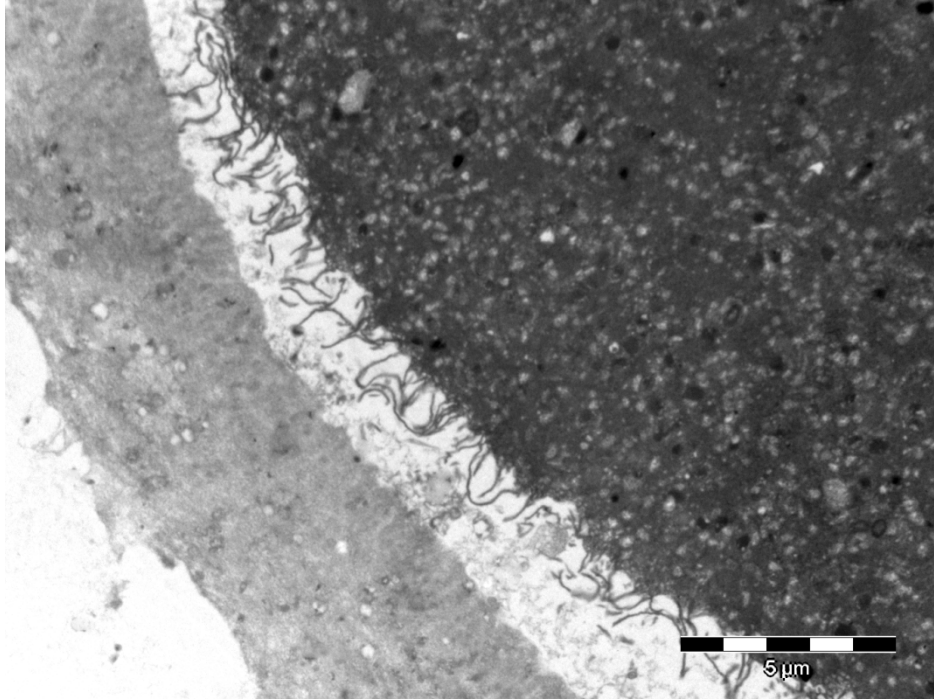


Fig. 47.

The fertilized oocytes (prezygotes) present 2 pronuclei, with 1 nucleol each. The periferic cytoplasm of fertilized oocytes has an uniform aspect and has a small number of mitochondria and secretion vesicles (Fig. 48)

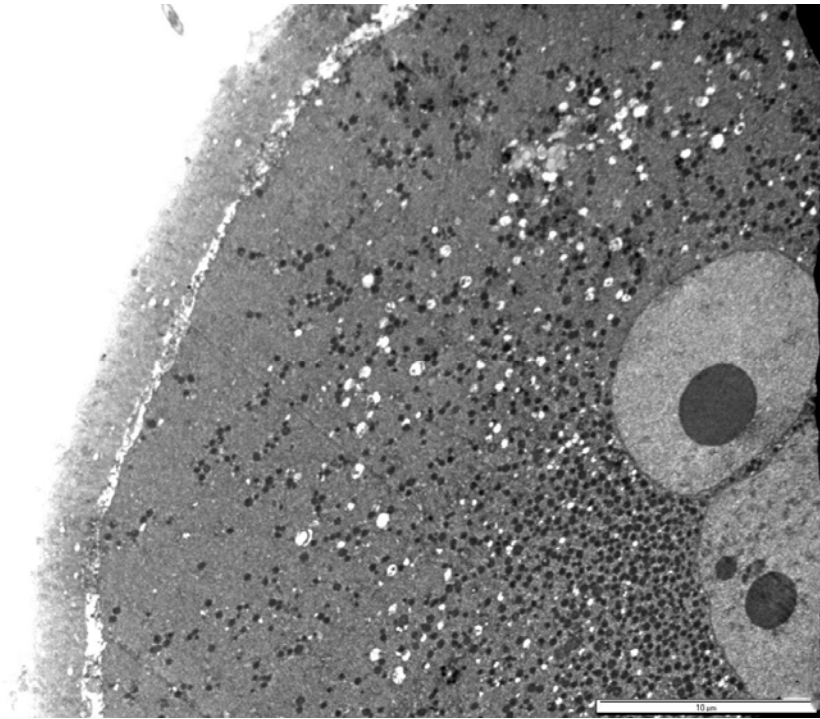


Fig. 48.

### **Conclusions upon oocytes.**

The oocyte is a unique cell in animal kingdom. Generally it has the same structure as any other eukaryotic cell, but the number and the disposition of organelles are unique for this cell.

The ultrastructural analysis of oocytes (found in different maturation stages) showed characteristic aspects, one of them are described in literature, others are new.

The immature oocytes have not been described in literature, and our findings are:

- the internal part of zona pellucida is not mature yet. At the oocyte plasmalemma level can be detected intense exocytosis processes.

- a large number of REN vesicles present in oocyte cytoplasm.

- low number of cortical vesicles in oocyte cytoplasm.

The premature oocyte presents degenerative processes, which explain why the embryos obtained from these type of oocytes are fragmented.

The immature oocytes matured in vitro in absence of sperms can be fertilized and form good quality embryos. The immature oocytes matured in vitro in presence of sperms are not fertilizable.

The ultrastructural investigations upon the 3 types of oocytes show that between those 3 types of oocytes are other intermediary types which are undetectable by optical microscopy. The conclusion is that the classification of oocytes using the corona radiata and cumulus aspect has a great dose of subjectivity. This finding can explain why we are not able to fertilize all the oocytes obtained at one follicular puncture and why the poor quality embryos obtained have low implantation chances.

We support the necessity of an excellent ovarian stimulation protocol use, which can provide the embryologist only mature oocytes, taking in account that in some cases (polycystic ovarian syndrome) the oocytes are all immature.

The IVF teams can follow two important therapeutic lines. The majority of teams tend to stimulate the ovary aggressively in order to obtain a large number of oocytes to obtain a large number of embryos to be transferred. From our data, in these cases the number of immature oocytes obtained are also large, so we do not support this kind of attitude.

From our results obtained from ultrastructural investigation we performed upon oocytes we support the use of moderate dosage of stimulation medication, which can help us to obtain few but good quality oocytes (mature oocytes).

**Final conclusions:**

1. The capacitated sperms, that spent 18 hours in culture medium loose their fertilization capacity due to the ultrastructural modifications present.
2. from clinical-practical point of view, our study shows the necessity of using only fresh sperms, sperms that spent maximum 6-7 hours in culture medium.
3. due to this finding, we shortened the insemination period and the fertilization rate raised with 10%.
4. the ultrastructural aspect of immature oocyte shows that exocytosis processes are present, which plead for an immature structure of the zona pellucida, which explain why these oocytes are unable to fertilize.
5. this aspect proves the importance of oocyte itself in zona pellucida building, also shows that if zona pellucida is not complete, the fertilization can not occur.
6. the ultrastructure of immature oocytes, matured in vitro is identical with the ultrastructure of mature oocytes, matured in vivo. This finding is an argument in favour of in vitro maturation of immature oocytes for assisted reproduction technique.
7. this findings upon oocytes allowed us to improve our cultivation techniques which led to more excellent quality embryos and finally led to an increase with 2% of pregnancy rate, similar with pregnancy rate from other european in vitro fertilization laboratories.
8. the ultrastructural aspects of oocytes described are strong arguments in favour of perfecting ovarian stimulation protocols in order to achieve good quality oocytes.
9. the statistical data we had obtained in 10 years are the first large data presented in Romania.

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