Zona Pellucida Surface of Immature and in Vitro Matured Mouse Oocytes: Analysis by Scanning Electron Microscopy

J. M. CALAFELL,[1,2] C. NOGUÉS,[1] M. PONSÀ,[1] J. SANTALÓ,[1] and J. EGOZCUE[1]

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Purpose: *The aim of this work was to determine the morphology of the zona pellucida surface of immature and in vitro matured mouse oocytes by scanning electron microscopy. For this purpose two groups of immature oocytes (germinal vesicle group and metaphase I group) were studied either before or after in vitro maturation.*
Results: *Before in vitro maturation, the germinal vesicle immature group showed mainly an unstructured zona pellucida surface with smooth cumulus cells. The metaphase I immature group showed a more structured zona pellucida with smooth or blebbing cumulus cells. After in vitro maturation, development of the zona pellucida toward a mature surface, related to the initial degree of oocyte maturity, was observed in both groups.*
Conclusions: *These observations show a correlation between the morphology of the zona pellucida surface and the degree of oocyte maturity; the in vitro maturation process can give rise to a proper development of this endowment when immature oocytes are used.*

KEY WORDS: scanning electron microscopy (SEM); zona pellucida; oocyte in vitro maturation; morphology.

INTRODUCTION

In vitro oocyte maturation has been widely studied in different mammalian species to improve the efficiency of in vitro fertilization (IVF) techniques as well as to understand the mechanisms that regulate the reinitiation of meiosis.

Nuclear oocyte maturation can be spontaneously achieved in vitro through the culture of immature oocytes free from the follicle, which inhibits meiotic maturation. This inhibitory effect was first observed by Pincus and Enzmann (1) using rabbit oocytes and later confirmed by Edwards (2) in other mammalian species.

However, in vitro fertilization of spontaneously in vitro matured oocytes and their subsequent development have been difficult to achieve (3–6). Thus arose the concept of cytoplasmic maturation defined by Thibault (4), according to which the oocyte needs to be nuclearly and cytoplasmically mature to be fertilized and give rise to a proper embryo development.

Nevertheless, the optimal fertilization of spontaneously in vitro matured mouse oocytes in percentages similar to those obtained with in vivo matured oocytes has been described (7). Similar percentages using rat oocytes (8) or human oocytes (9) have also been recently described.

Moreover in the in vitro maturation process, the mammalian zona pellucida (ZP) and the vitelline membrane have to be considered together with the nucleus and cytoplasm to understand the fertilizability of the oocytes obtained and the subsequent embryo development.

The structure of the ZP surface has been investigated by scanning electron microscopy (SEM) in mammalian oocytes according to their developmental stage. The general aspect of immature and mature mouse oocytes (10,11) as well as that of in vivo and in vitro aged mouse oocytes has been described, and a classification of the morphology of the ZP related to the maturity of the oocytes has been proposed (12). The comparative morphology of the ZP surface of unfertilized and fertilized human (13–15), mouse and hamster oocytes (16,17) has also been reported.

[1] Dept. Biologia Cel.lular i Fisiologia, Unitat Biologia Cel.lular. Fac. Ciències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
[2] To whom correspondence should be addressed.

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In this work we report the study by SEM of the ZP surface of immature and in vitro matured mouse oocytes, as a part of an extensive investigation of oocyte maturation that is being developed in our laboratory. The aim of this work was to determine if the in vitro maturation process can give rise to oocytes with a mature ZP surface, indicating that the oocyte had probably reached its nuclear and cytoplasmic maturity.

MATERIALS AND METHODS

Oocyte Collection

Immature and mature oocytes were obtained from $B6CBF_1$ prepubertal female mice (24–28 days old) superovulated by different procedures (see below). Oocyte–cumulus complexes were recovered by puncturing the follicles with a sharp needle under a stereomicroscope.

Five groups of oocytes were analyzed (depending on the gonadotropin treatment and their inclusion or not in the in vitro maturation process) and classified as follows.

- Immature oocytes obtained from females primed only with pregnant mare serum gonadotropin (PMSG) and recovered 53 hr later. In a parallel way, cytogenetic analysis showed that 87.3% were at the germinal vesicle (GV) stage, 11% at metaphase I, and 1.7% at metaphase II. This sample is referred to as the GV immature group.
- Immature oocytes obtained from females primed with PMSG and human chorionic gonadotropin (hCG) 48 hr apart and recovered 5 hr post hCG injection. Cytogenetic analysis showed that 100% were at the metaphase I (MI) stage. This sample is referred to as the MI immature group.
- Oocytes from the GV immature group matured in vitro for 15–16 hr in M_{16} medium supplemented with 5 IU of hCG. This sample is referred to as the GV matured group.
- Oocytes from the MI group matured in vitro during 15–16 hr in M_{16} medium supplemented with 5 IU of hCG. This sample is referred to as the MI matured group.
- A control group of freshly ovulated oocytes obtained 13–14 hr after hCG injection from PMSG–hCG-primed females.

Culture Media

We used M_2 medium (18) containing 4 mg bovine serum albumin (BSA)/ml to collect immature oocytes. M_{16} medium (19) containing 4 mg BSA/ml, supplemented with 10% fetal calf serum (FCS) and 5 IU hCG/ml, was used as the in vitro maturation medium.

Processing for Scanning Electron Microscopy

After removal of cumulus cells with 0.1% hyaluronidase solution, immature, control, and in vitro matured oocytes were fixed for at least 1 hr in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer, postfixed for 1 hr in 1% osmium tetroxide in distilled water, and dehydrated through an ethanol–isoamil acetate series of increasing concentration (12). After dehydration, they were critical point-dried using CO_2 in a polythene chamber with the top and bottom substituted by 37-nm plancton grids, mounted on the specimen holder, coated with gold, and observed in a Hitachi S-570 SEM. Oocytes were observed at ×1900 and zona pellucida details at ×8000.

Statistical Analysis

A chi-square test was used, with Yates' correction for continuity when necessary.

RESULTS

A total of 407 oocytes obtained from the different groups (Table I) was analyzed by SEM. Seventy-six corresponded to the GV immature group, 87 to the GV matured group, 62 to the MI immature group, 68 to the MI matured group, and 114 to the control group. Of the total analyzed, 19 were classified as degenerated.

The oocytes analyzed were classified into five types according to the general characteristics of the zona pellucida surface. This classification follows that of Nogués *et al.* (12), to which two new morphologies corresponding to immature oocytes have been added. Moreover, the general aspect of the cumulus cells surrounding the oocyte has been analyzed at fixation time.

Table I. General Characteristics of the Sample Studied by SEM and Zona Pellucida Classification[a]

Denomination	Hormonal treatment	Total oocytes	Degenerated oocytes	Type of zona pellucida				
				Y	Z	A/B	C	D
GV immature group	PMSG/—	76	0 (0)	**47 (61.8)**	6 (7.9)	4 (5.3)	1 (1.3)	18 (23.7)
GV matured group	PMSG/—	87	1 (1.1)	1 (1.1)	**53 (61.0)**	29 (33.4)	1 (1.1)	2 (2.3)
MI immature group	PMSG/hCG	62	0 (0)	18 (29.0)	**30 (48.4)**	3 (4.8)	0 (0)	11 (17.8)
MI matured group	PMSG/hCG	68	6 (8.8)	0 (0)	0 (0)	**46 (67.6)**	1 (1.5)	15 (22.1)
Control group	PMSG/hCG	114	12 (10.5)	0 (0)	0 (0)	**84 (73.7)**	8 (7.0)	10 (8.8)
Total		407	19	66	89	166	11	56

[a] Figures in parentheses are percentages referred to the total number of oocytes analyzed. Figures in **boldface** reflect the type of ZP most frequently observed for each group.

Zona Pellucida Surface Classification

Y-Type. This is an unstructured, amorphous zona, without or with only a few pores of small diameter and completely covered with cellular debris. SEM images seem to indicate that these debris have originated from the ZP itself. The oocytes with a Y-type ZP usually have a compact cumulus (Figs. 1a and b).

Z-Type. The zona begins to be more structured, with shallow pores of small diameter. Fewer cellular debris are observed in the zona surface compared to Y-type ZP. This zona show less and more expanded cumulus cells in comparison with Y-type ZP (Figs. 2a and b).

A/B Type. The A-type ZP is characterized by a fibrous network (more than in type B), with numerous pores with a large diameter and deeper than in types Y or Z. Types A and B are associated with fully mature oocytes (12), and they are referred to as A/B-type ZP. In general, no cellular debris is observed in this type of zona and the cumulus cells have an expanded appearance (Figs. 3a and b).

C-Type. This is a rough ZP surface with few pores and without cellular debris. Fewer cumulus cells are adhered to the ZP compared to the types described above (Figs. 4a and b). This type of ZP has been described in aged oocytes (12).

D-Type. This is a flat, unstructured, and amorphous surface without cellular debris or cumulus

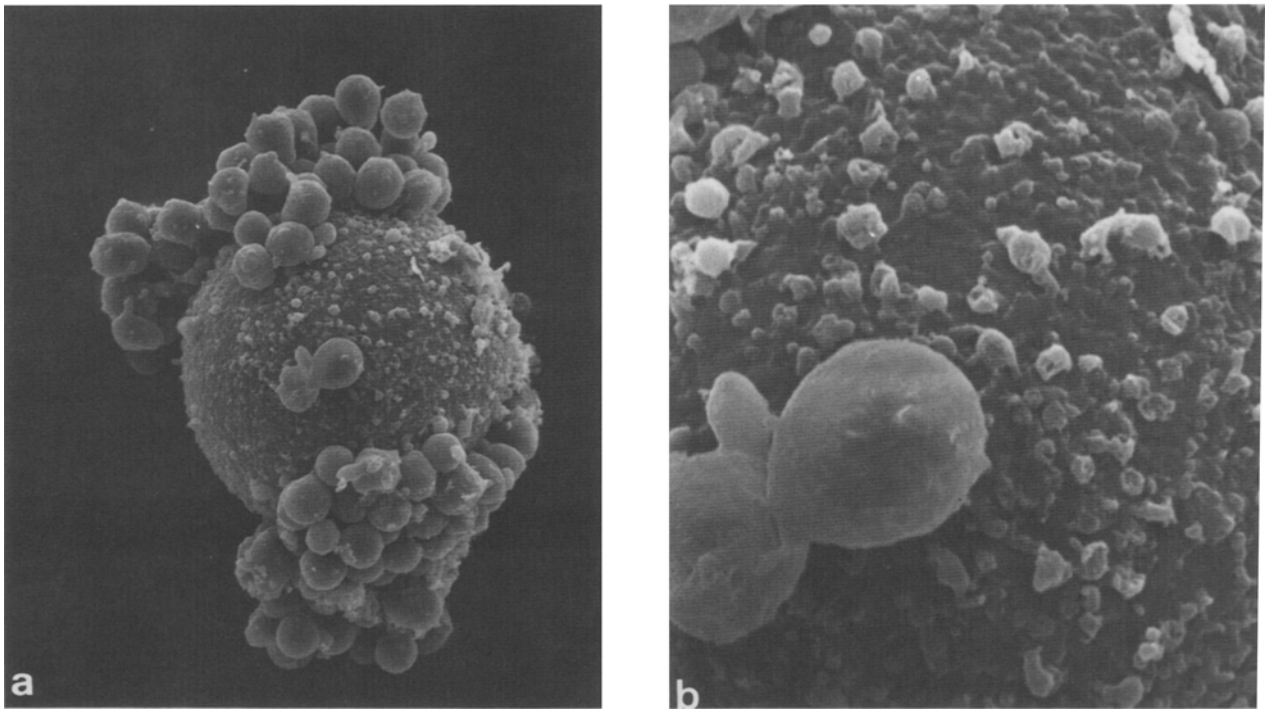

Fig. 1. Y-type ZP.

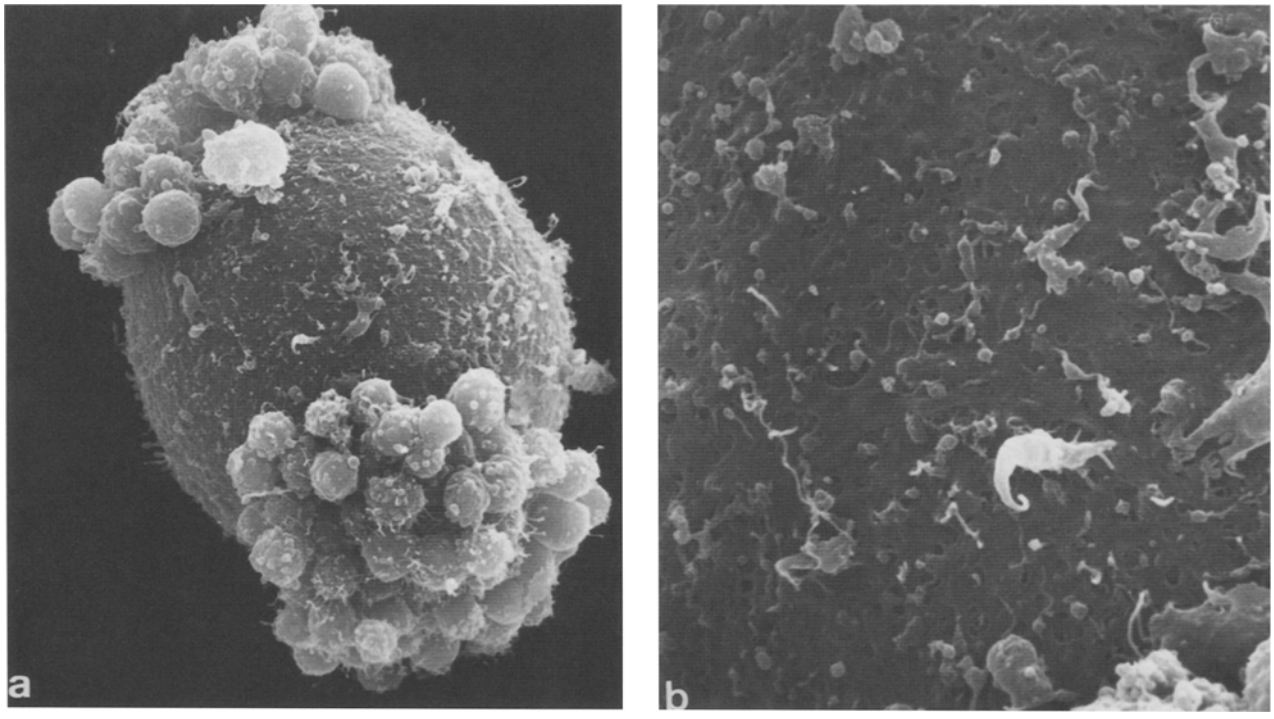

Fig. 2. Z-type ZP.

cells adhered to it (Figs. 5a and b). This type of ZP has been described in degenerated oocytes (12).

Cumulus Cell Characteristics

Smooth Cumulus Cells. These are spherical cells, smooth, compacted, and usually found in oocytes with a Y-type ZP (Fig. 1a).

Blebbing Cumulus Cells. These are spherical cells, blebbed, and less compacted, giving rise to a cumulus with a spongy appearance. This type of cumulus cells has been observed among smooth cumulus cells in oocytes with a Z-type ZP. Oocytes with A/B- or C-type ZP show only blebbing cumulus cells, with a higher degree of blebbing (Figs. 2a, 3a, and 4a).

Incidence of the Different Types of ZP Observed

Scanning electron microscopy analysis of the GV immature group showed a high percentage of oocytes with the Y-type ZP (61.8%) compared to the other types of ZP (7.9, 5.3, 1.3, and 23.7% for Z, A/B-, C-, and D-types, respectively) (Table I).

After the in vitro maturation process of oocytes arrested at the germinal vesicle stage (GV matured group), the Z-type ZP was the most frequently observed (61.0%) in comparison with the rest of the ZP types (1.1, 33.4, 1.1, and 2.3% for Y, A/B, C, and D-types, respectively). Thus after this process, a significant increase in the Z-type ZP (7.9 vs 61.0%; $P \leq 0.001$), has been observed together with a significant increase in the A/B-type ZP (5.3 vs 33.4%; $P \leq 0.001$) and significant decreases in types Y and D (61.8 vs 1.1 and 23.7 vs 2.3%, respectively; $P \leq 0.001$). No significant differences were observed in the frequency of C-type ZP.

Referring to the MI immature group, SEM observations showed that the Z-type ZP was the most frequently observed type (48.4%) compared to the other types (29.0, 4.8, 0, and 17.8% for Y, A/B, C, and D-types, respectively).

After the in vitro maturation process (MI matured group) the A/B-type ZP was the more often observed type (67.6%) compared to the others (0, 0, 1.5, and 22.1% for Y, Z, C, and D-types, respectively). In this group, we have observed a significant increase in A/B-type ZP (67.6 vs 4.8%; $P \leq 0.001$) together with a significant decrease in Y-type ZP (29.0 vs 0%; $P \leq 0.001$) and Z-type ZP (48.4 vs 0%; $P \leq 0.001$).

On the other hand, the A/B-type ZP was the most frequently observed in the control group (73.7%)

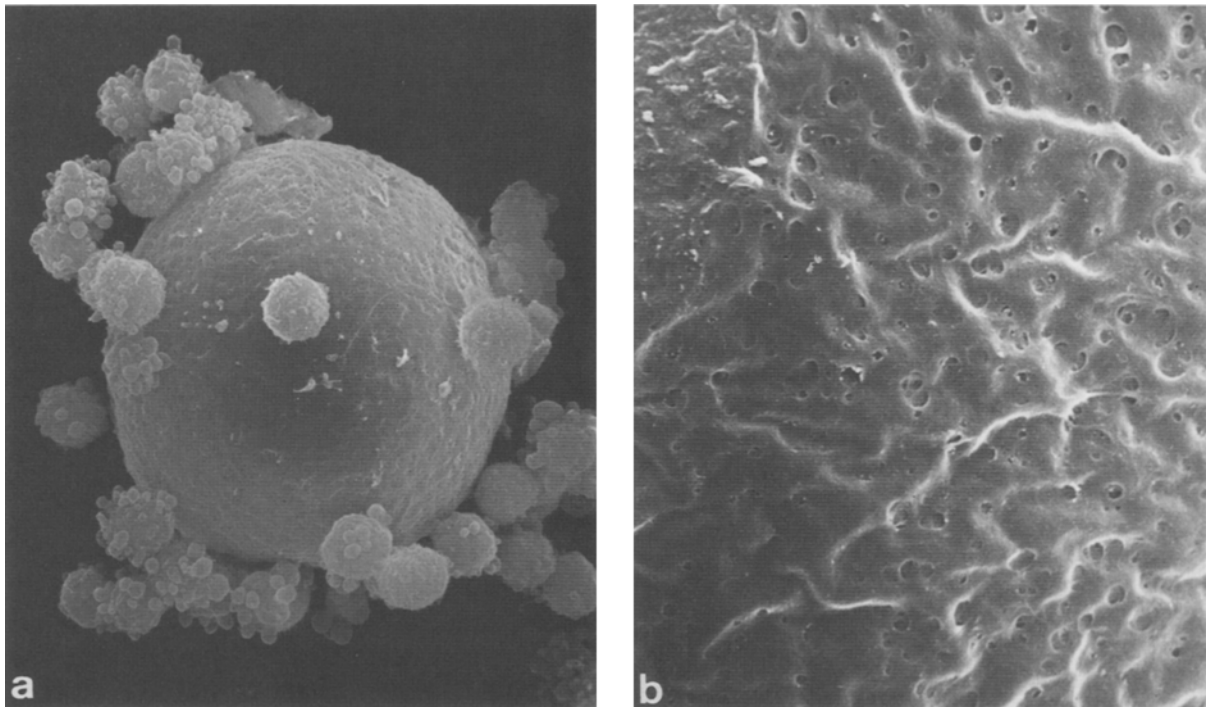

Fig. 3. A/B-type ZP.

compared to the rest of the ZP types (0, 0, 7.0, and 8.8% for Y, Z, C, and D-types, respectively) as would be expected.

DISCUSSION

The high percentage of oocytes with an A/B-type ZP and blebbing cumulus cells obtained in the control group (73.7%) indicates that this type represents the characteristic surface of cytoplasmic and nuclear mature oocytes (metaphase II) ready to be fertilized. These results are in agreement with previous SEM reports in the mouse (10,12), hamster (17), and humans (13–15,20).

The significantly high percentage of oocytes with the Y-type ZP obtained in the GV immature group (61.8%) compared with that obtained in the MI immature group (29.0%), suggests that this type of ZP would correspond to a very immature oocyte degree, since hCG administration is known to induce oocyte maturation. The low percentages of Z-type ZP (7.9%) obtained in the GV immature group compared with that in the MI immature group (48.4%) indicates that the Z-type of ZP reflects a more advanced degree of maturation before the normal degree represented by the A/B-type ZP.

This idea is supported by the results obtained after in vitro maturation of these oocytes (GV matured group and MI matured group). The frequencies obtained suggest that oocytes with a Y-type ZP reach the Z- and A/B-types ZP after in vitro maturation, and those with the Z-type ZP advance until the A/B-type ZP. These data also indicate that the in vitro maturation process is a reliable method to obtain mature oocytes, at least when referring to the ZP surface.

Taking into account these data and the cytogenetic analyses performed in a parallel way to the maturation process, we suggest that Y-type ZP basically corresponds to oocytes arrested at the GV stage, the Z-type ZP mainly to those arrested at MI, and the A/B-type ZP to mature oocytes arrested at metaphase II.

The C- and D-types of ZP correspond to the ZP surface prior to degeneration as described by Nogués *et al.* (12). The low percentage of D-type ZP obtained in the GV matured group could result from oocyte lysis after a long period of in vitro culture and as a consequence of a degeneration process. This phenomenon has not been detected in the MI matured group, probably because the more structured ZP surface in these oocytes would confer more protection in front of oocyte lysis.

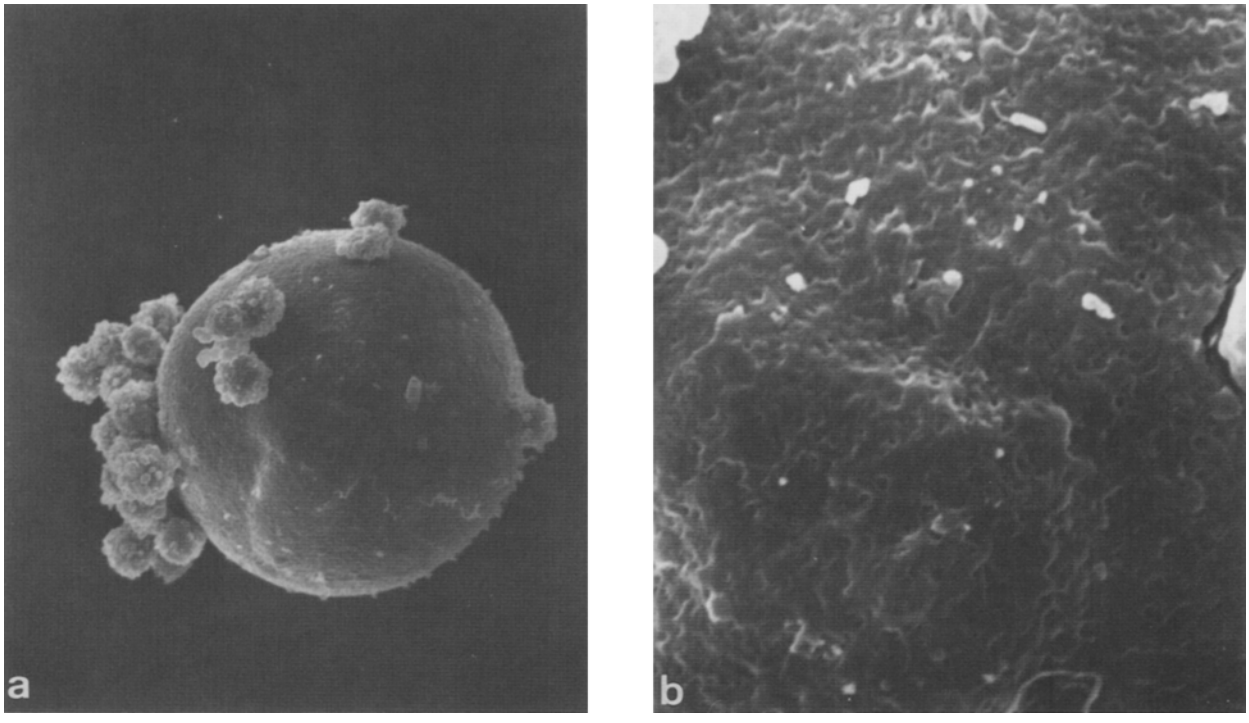

FIg. 4. C-type ZP.

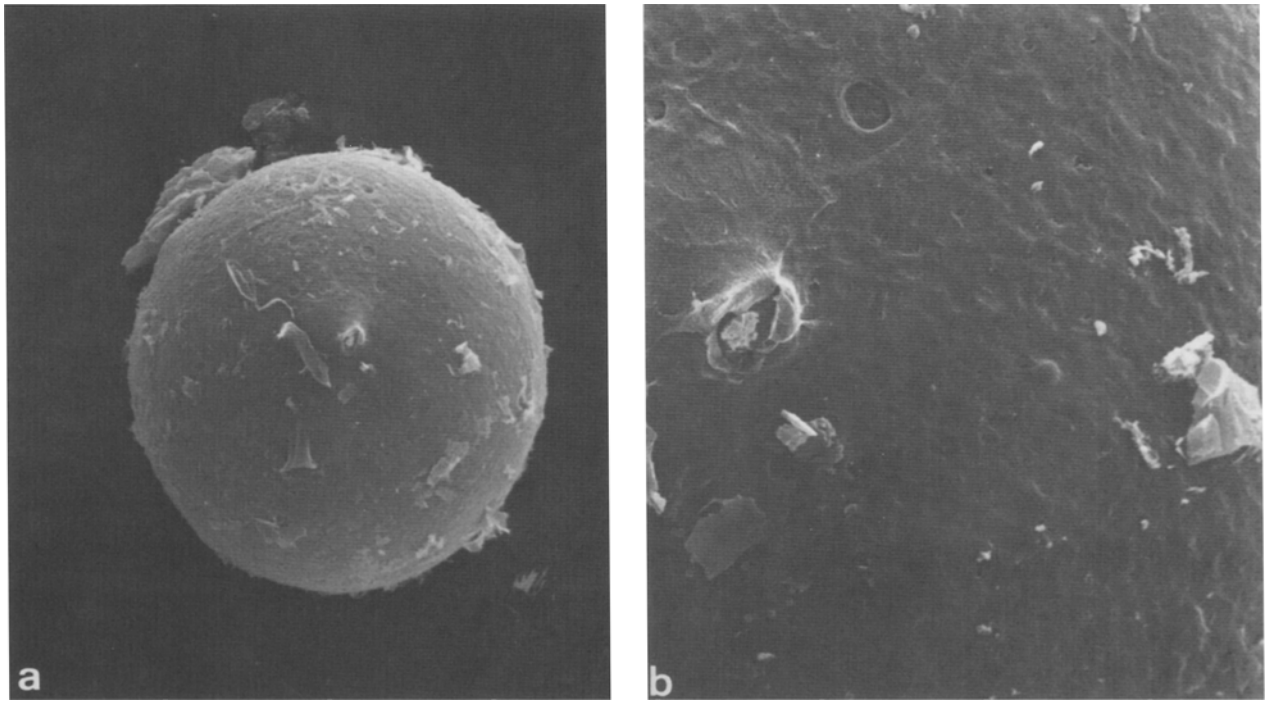

Fig. 5. D-type ZP.

The correct development of the ZP surface observed after the in vitro maturation process is in good agreement with the in vitro fertilization percentages obtained in our laboratory after insemination of in vitro matured oocytes (unpublished data). High fertilization levels were found in the MI matured group (over 90%) as can be expected considering the high rates of A/B-type ZP surface (fully mature) observed in this group. Lower fertilization rates (around 45%) were found in the GV matured group, as should be expected considering that the Z-type ZP surface (still immature) has been observed in high percentages. These data are in agreement with those obtained in human oocytes by Familiari *et al.* (13), where after the in vitro fertilization process, mature oocytes showed numerous spermatozoa attached to the ZP, while in immature oocytes the presence of spermatozoa was lower.

Together with the ZP surface, we have observed morphological differences in the cumulus cells through the in vitro maturation process. Immature oocytes showed smooth cumulus cells that became blebbing cells in mature oocytes as has been reported in rat oocytes (21).

Our results obtained by SEM confirm the morphological characteristics of the ZP surface and the cumulus cells of immature and mature mouse oocytes as well as their correct development through the in vitro maturation process depending on the initial meiotic stage of the oocyte.

CONCLUSIONS

(i) Immature oocytes arrested at the GV stage show an unstructured ZP surface (Y-type ZP), without or with few pores, and smooth cumulus cells. Cellular debris can also be observed in the ZP surface.

(ii) The ZP surface of immature oocytes arrested at the MI stage is characterized by the presence of shallow pores of small diameter and little cellular debris (Z-type ZP). The cumulus is composed of smooth and blebbed cells.

(iii) The in vitro maturation process gives rise to a correct development of the ZP surface of immature oocytes toward a mature ZP surface. This mature ZP shows a porous appearance without cellular debris and an expanded cumulus (A/B-type ZP).

(iv) In vitro fertilization rates obtained after the in vitro maturation process of immature oocytes are in correlation with the degree of ZP development.

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