Arch. Biol. Med. Exp. *13*: 325-334, 1980

Unpacking of the Spermatozoal Nucleus at Fertilization*

Descondensación del núcleo espermático después de la fertilización

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(Recibido el 23 de julio de 1980)

BARROS, C., RHIM, A. Unpacking of the spermatozoal nucleus at fertilization. (Descondensación del núcleo espermático después de la fertilización). *Arch. Biol. Med. Exp. 13,* 325-334, 1980.

Mammalian gamete fusion in normal fertilization is soon followed by the dissolution of the nuclear membrane that preceeds the decondensation of the spermatozoal chromatin. Prior to or concomitant with this process it is possible to observe electron dense bodies in close association with the condensed chromatin. Additionally, near the area of chromatin decondensation appears a fine granular material that in more advanced stages can be seen in close association with electron dense bodies. The electron dense bodies might represent the release of DNA associated proteins that must be removed to allow chromatin decondensation. Full decondensation is observed about 1 hour after gamete fusion. However, the post-fusion events that experience the spermatozoon seem to be associated to the oocyte cell cycle and to the fertilized egg cell cycle. *In vitro* studies indicate that spermatozoal chromatin decondesation might be associated to the action of proteases and of agents that reduce S-S bonds.

I. *Ultrastructural aspects of spermatozoal chromatin dispersion*

Gamete membrane fusion is soon followed by the rapid loss of the spermatozoal nuclear membrane by a mechanism still to be established (1). The loss of the spermatozoal nuclear envelope heralds the spermatozoal chromatic decondensation. Fawcett (2) had suggested that the redundant nuclear envelope present in mature spermatozoa could be associated to the accomodation of the male pronucleus. This notion has, however been abandoned due to the fast loss of the spermatozoal nuclear envelope.

A series of complex events are observed in recently fused gametes prior to or concomitant with spermatozoal chromatin decondensation. In mammals, soon after gamete fusion it is possible to observe electron dense bodies of 0.1 to 0.2 μm in diameter coming out from the periphery of the still condensed spermatozoal chromatin (Fig. 1 and 3). The persistance of these bodies as visible structures is of short duration, however, structures of similar appearance can be put in evidence in later stages of fertilization and in association with a granular material. Later on, in the middle part of the spermatozoal nucleus and from the periphery to the center, it is possible to observe fine fibrils of chromatin (4, 5 and Figs. 2 and 3). The decondensation progresses from the middle part toward the caudal region of the spermatozoal head and last to the anteriormost region (Fig. 3), where a complex of membranes (Figs. 2 and 3), whose origin is still debated (see 1, 6, 7 for discussion on this point), move away from their close apposition to the spermatozoal nucleus exposing the perinuclear subacrosomal material. In the area lying between the spermatozoal chromatin and the externally located complex of membranes appears a finely

* Presented at the Symposium "Organization of the Spermatid Nucleus from Birth to Fecondation", Santiago, Chile. October, 1979, sponsored by the Sección de Biología Celular y Sección Biología de la Reproducción y del Desarrollo, Sociedad de Biología de Chile.

granular material of about 80 Å in diameter and grouped in clusters of 0.2 to 1.2 μm (Figs. 2 and 3). The chemical nature as well as the functional role of the dense bodies and the granular material are thus far unknown.

We believe that the dense bodies might represent the release of proteins from the condensed chromatin that is required for its decondensation. Our postulate is based on the following facts. During spermiogenesis there is a transition of nuclear proteins where the histones of preespermatid stages are replaced by more basic proteins rich in arginine, that remain complexed to DNA at least until fertilization. Ecklund and Levine (8) labelled mouse spermatozoa with ^{3}H – arginine and found that the label was associated to a specific basic protein named musculine. This protein was present in epididymal and uterine spermatozoa but barely present in testicular spermatozoa. Furthermore when labelled spermatozoa were used to fertilize eggs and studied by autoradiography it was found that spermatozoa appeared labelled when they were outside the egg, at the zona pellucida and in contact with the vitellus. However, when the spermatozoa had entered into the vitellus, the label remained associated to the spermatozoal nucleus until the time when the second anaphase begins. At later stages of fertilization, that is, during pronuclear formation no label was found associated to them. The time at which anaphasic chromosomal movement starts, coincides with the dissapearance of the dense bodies. However, the validity of the above proposed hypothesis needs to be proved using biochemical and high resolution autoradiography techniques.

The spermatozoal chromatin is fully decondensed at about one hour after gamete fusion and no nuclear membrane is seen around it (Figs. 4 and 5). Thus, the area where the male chromatin is located appears similar to the ground cytoplasm and its limits are demarcated by an array of cell organelles (Fig. 5). The complete absence of nuclear membrane during mammalian spermatozoal chromatin decondensation contrasts with that found in sea urchin where nuclear envelope breakdown is incomplete and portions of the nuclear membrane remains over the apical and caudal regions of the head (9).

II. *The egg cell cycle and spermatozoal chromatin decondensation*

Mature mammalian spermatozoa can fuse with immature (10-13), mature, fertilized (11, 14) mammalian eggs and with blastomeres of cleaved eggs (11). However, the post-fusion process regarding the spermatozoal nucleus is different and seems to be related to the stage of the cell cycle of the oocyte and of the fertilized egg.

a. *Immature oocyte*

Golden hamster spermatozoa fused with immature oocytes show a rapid loss of the nuclear envelope, however, the nucleus remains highly condensed (11, 15) even after three hours after gamete fusion. Some degree of spermatozoal chromatin decondensation is observed in oocytes that have started prometaphase I; at metaphase-anaphase I most of the spermatozoa show full decondensation of the chromatin. The fastest decondensation and development of functional pronucleus occurs when the mature oocytes are at the second meiotic metaphase. Guinea-pig immature oocytes can also fuse with homologous and heterologus spermatozoa followed by the loss of the nuclear envelope, but the chromatin remains highly condensed (Figs. 6 and 7). In the rabbit, spermatozoa fused with immature oocytes also show no chromatin dispersion, but in contrast to the hamster and guinea-pig the nuclear membrane persists around the condensed chromatin (1, 13). An interesting exception to the above studied species is the dog whose egg is ovulated at the GV stage and can be fertilized at any time after ovulation. Spermatozoa entering oocytes at the GV stage show chromatin decondensation as it occurs in fertilization of oocytes ovulated at the second meiotic metaphase (16). At this point of the discussion several questions are appropriate such as: Is the GV breakdown required for spermatozoal chromatin decondensation? or Is it nec-

essary the cytoplasmic maturation the only requirement for chromatin dispersion? Are GV breakdown and cytoplasmic maturation two phenomena that are required for spermatozoal chromatin decondensation? The analysis of studies done in invertebrates and in amphibians might be of use in the understanding of this problem.

At the turn of this century Delage (17) working with starfish oocytes found that when they were cut in two at the GV stage, development was possible only in the half that contained the GV, however, if the operation was performed after the GV breakdown, both halves were able to support development after fertilization. Delage's conclusion was that GV breakdown was required for what he called "cytoplasmic maturation". Over half a century later, Detlaff, Nikitina and Stroeva (18) working with amphibian oocytes showed the importance of the admixture of GV contents with the cytoplasm to support cleavage. In their experiments they transplanted nuclei from blastula into enucleated oocytes. Development was normal when enucleation was performed after GV breakdown, but if enucleation was performed before GV breakdown, eggs failed to cleave. Subsequent works showed that oocytes enucleated before progesterone stimulation and incubated *in vitro* can undergo all the maturational changes observed in nucleated controls, such as cortical granule breakdown, elevation of the vitelline membrane, rotation within the perivitelline space, increase in egg turgidity and changes in the appearance of the egg surface (19). On the other hand, the enucleated oocytes loss the ability to cleave when they were injected with blastula nuclei. The capacity to cleave was partially restored when GV material from progesterone stimulated or non-hormone treated eggs was injected into the enucleated and progesterone stimulated eggs. It seems then that cytoplasmic maturation can proceed in the absence of GV, and the release of GV material into the cytoplasm would be necessary to support cleavage (19, 20). A further insight into our discussion can be obtained from the work in which toad spermatozoal nuclei were injected into toad oocytes. Moriya and Katagiri (21) studied the behavior of toad spermatozoal nuclei injected into mature and maturing oocytes. When the spermatozoa, pretreated with 0.025% Triton X– 100 to rid off the membranes, were injected into the cytoplasm or nucleoplasm before GV breakdown of immature toad oocytes, no change in shape was observed. When the injections were performed at different times after the progesterone treatment, the spermatozoal nuclei experienced changes that were in synchrony with the oocyte nucleus. It appears then that the nucleoplasm of the GV does not have in itself the property of dispersing the spermatozoal chromatin but rather its admixture with the oocyte cytoplasm brings about this property. In fact, Katagiri and Moriya (22) showed that when toad oocytes were enucleated up to six hours after progesterone stimulation and then inseminated, the spermatozoal nucleus remained intact or slightly swollen, even after 90 minutes, time at which the nucleated controls showed a well developed pronuclei. Enucleations performed 7 – 8 hours after progesterone stimulation resulted in a small percentage of oocytes undergoing cleavage, however, enucleations done at that time tended to cause rupture of the GV, producing a mixing of GV material with oocyte cytoplasm. Similar results were obtained when spermatozoal nuclei were injected into the oocytes. The evidence strongly indicates that admixture of GV material with the cytoplasm is required for spermatozoal chromatin decondensation.

b. *Mature oocyte*

Mammalian oocytes are considered mature when meiosis is at the stage of metaphase II and the cortical granules have reached a location immediately beneath the plasma membrane. Fertilization occurs at this stage and spermatozoal chromatin decondensation progresses very rapidly. Mature oocytes have been used to study the behavior of the spermatozoal nucleus placed in contact with the oocyte cytoplasm by artificial means. Using this experimental design it has been found that

isolated nuclei from hamster spermatozoa injected into mature hamster oocytes develop into a morphologically normal male pronucleus. Moreover, freeze-dried and freeze-thawed human spermatozoal nuclei can also develop into morphologically normal male pronucleus, (23). Later, it was learned that for pronuclear development it was necessary to activate the oocyte, being the morphological signs of activation, cortical granule breakdown and resumption of meiosis (24). Another point of interest was the finding that spermatozoa from the testis and caput epididymis could disperse within the non-activated oocytes cytoplasm, however, if they were injected into the activated cytoplasm only testicular spermatozoa could form pronuclei, as do cauda epididymal one (24).

c. *Fertilized and cleaved eggs*

Artificial removal of the zona pellucida of fertilized hamster eggs recovered from mated females allows them to fuse with additional spermatozoa of the same species (11, 14) and with spermatozoa of the mouse and of the guinea-pig (25, 26). After fusion, the evolution of the nucleus of the refertilizing spermatozoa will depend on the stage of fertilization of the egg. Thus, hamster spermatozoa fused with fertilized eggs before pronuclear formation, show full spermatozoal chromatin decondensation with dissolution of the nuclear envelope. As the host pronuclei develops, the chromatin of the refertilizing spermatozoon stays in the cortical cytoplasm and the chromatin remains highly condensed. A nuclear envelope can be seen around the chromatin and it is supposed to be a newly formed envelope (11). When refertilization was done just before the first cleavage division, the spermatozoal nucleus decondensed fully and formed a well developed pronuclei. On the other hand, spermatozoa fused with blastomeres at the two and four cell stage were observed to remain in the condensed state. Nuclear envelope was seen in some spermatozoa while in other was absent (Figs. 22 and 23 of Usui and Yanagimachi, 11). No studies have been carried out thus far to find out whether before the second and third cleavage the cytoplasm of the blastomeres is endowed with the ability to induce spermatozoal chromatin decondensation.

Experiments carried out using mouse and guinea-pig spermatozoa instead of hamster spermatozoa have shown that mouse spermatozoa can fuse with fertilized hamster eggs for a period of up to six hours. Full decondensation was observed during the first three hours. When the host pronuclei reached the stage characterized by the presence of many small nucleoli the mouse spermatozoal chromatin displayed only partial dispersion. Guinea-pig spermatozoa can fuse with fertilized hamster eggs for a period of about 14 hours and full decondensation (Fig. 8) was observed for a period of six hours, however, when the host pronuclei reached the stage characterized by the presence of large and few nucleoli, the guinea-pig spermatozoal chromatin remained condensed (Fig. 9). The disagreement between the reported results of homologous and heterologous refertilization might be more apparent than real, since Usui and Yanagimachi (11) stated only that "fertilized eggs at the pronuclei stage failed to decondense the chromatin...", without referring to the exact stage of the host pronucleus. Moreover, since *in vivo* fertilization is a phenomenon that occurs within a period of two or more hours, a given population of fertilized eggs might include eggs with pronuclei at different stages of development, that might explain the apparent disagreement.

The behavior of the spermatozoal nucleus has also been studied in the cytoplasm of somatic cells, using inactivated Sendai virus to promote cell fusion. Thus, rabbit spermatozoa were fused with mammalian somatic cells, such as rabbit embryonic kidney and fibroblast cells and W1-38 human cells and TH-1 turtle cells. The mammalian cells formed 90% of heterokaryons while the turtle cells formed only 4 to 7%. However, in none of the heterokaryons formed, was observed chromatin decondensation or DNA synthesis as measured by H^3-thymidine incorporation (27). On the other hand, human

spermatozoa were fused with various fibroblast cell lines and 30 to 40% of heterokaryons were obtained. In these heterokaryons the acrosome and nucleus appeared swollen but only 2% of the spermatozoal nuclei were decondensed. Nuclei decondensation was accompanied by a shift from a protamine to histone content and induction of DNA and RNA synthesis (28). Spontaneous cell fusion between mouse spermatozoa and chinese hamster fibroblast cell lines resulted in a subsequent transfer of DNA from the spermatozoon to the host cell nucleus that was detected by the presence of mouse cell specific immunofluorescence reaction (29). However, the photomicrographs presented by the authors to validate cell fusion are not convincingly enough and it seems mere phagocitosis rather than cell fusion. The spermatozoal nucleus remained highly condensed in the cell cytoplasm.

From the data analyzed above, it has became evident that the conditions required for spermatozoal cromatin dispersion are not present at all times within the cytoplasm. These conditions, whatever they might be, become active only during certain stages of the egg cell cycle. The effect of the cytoplasm upon nuclear activities and its relation with the cell cycle has been extensively studied in somatic cell fusion. Sendai virus mediated cell fusion between a hen erythrocyte and HeLa cells results in the formation of a heterokaryon in which the genetically inactive erythrocyte nucleus becomes activated as judged by ^{3}H-thymidine and ^{3}H-uridine incorporation (30). Moreover, Bolund, Ringertz and Harris (31) found that 41 to 47 hours after hen erythrocytes were fused with HeLa cells, the amount of DNA present in the erythrocyte nucleus was similar to that found in G_2 phase of the cell cycle. Furthermore, it has been established that *in vitro* treatment of erythrocyte nuclei with supernatant of homogenized L, TLT (Taper liver tumor cells) or mouse liver cells, induces an active DNA synthesis. The rate of DNA synthesis was directly correlated to the type of cells used; thus tumor cells that are normally more active in DNA synthesis, induced a higher rate of synthesis than other less active cells (32). The reactivation of the hen erythrocyte nucleus is preceeded by the swelling of chromatin and a massive influx of proteins (33).

The behavior of the nucleus in the foreign cytoplasm, depends closely on the phase of the cell cycle of both, the nucleus and of the cytoplasm. Thus, a HeLa cell in S phase will induce the S phase in a foreign nucleus in G_1 phase. Similarly, a HeLa cell in G_2 phase will induce the S phase in a foreign nucleus in G_1. Moreover, a cell in G_2 phase shortens the length of the S phase of the foreign nucleus as well as a cell in S phase shortens the length of the G_1 phase of the foreign nucleus. The initiation of mitosis of a nucleus in G_2 phase can be retarded when introduced in the cytoplasm of a cell in S phase (34).

III. *In vitro studies*

The great technical difficulties of performing biochemical studies with mammalian zygotes at the stage of spermatozoal chromatin decondensation has made it necessary to study this phenomenon under *in vitro* conditions with the hope that it might help in the understanding of such a basic and fundamental phenomenon.

The spermatozoal nucleus of eutherian mammals and that of some low vertebrates is highly resistant to disruption and requires very strong chemical treatments to free the chromatin (35). However, *in vitro* decondensation can be obtained by agents that break non-covalent bonds, such as sodium dodecyl sulfate and those that reduce S–S bonds, such as dithiothreitol, mercaptoethanol or cysteine (36-38). Decondensation can also be induced with inorganic sulphides and strong bases, but not with strong acids (38). In sea urchin spermatozoa it was found that HCl treatment of spermatozoa did not in itself induce chromatin decondensation, but it was necessary to remove basic proteins that allowed a further treatment with egg cytoplasm that induced dispersion (39). The thiol induced chromatin decondensation was the base to suggest that cellular thiols of low molecular weight such as reduced glutathione and some specific SH-dependent enzymes might be involved in

decondensation during fertilization (38). Moreover, Yanagimachi (15) had suggested that the nucleolus of the GV of immature oocytes could be the storage place of the spermatozoal chromatin decondensing factor. His suggestion was based on the grounds that the nucleolus gives a strong reaction with the ferric ferrycyanide reduction technique that could be due to the presence of sulfhydryl groups.

Another interesting finding is that spermatozoal histones are extensively degraded when isolated bull spermatozoal chromatin is incubated under the appropriated conditions. Furthermore, from the spermatozoal chromatin it was extracted a protease of trypsin-type that it was found to be electrophoretically indistinguishable from the acrosomal protease (40). These findings have been correlated with the events that occur during fertilization and it has been proposed that proteolysis of histones could play an important role in spermatozoal chromatin dispersion during fertilization. A slightly different viewpoint has been suggested by Young (41) who could obtain rabbit spermatozoal cromatin dispersion only when the preparation included the acrosomal enzymes, but not when they were excluded from the *in vitro* system. He concluded that dispersion could not be due to chromatin proteolytic activity, but instead to acrosomal enzymes bound to the inner acrosomal membrane. However, the anteriormost region of the spermatozoal head which is covered by the inner acrosomal membrane is the last to be incorporated into the egg cytoplasm and by that time chromatin dispersion is already well in progress (5). Another interpretation for a release of the proteins associated to DNA is that phosphorylated proteins bind less strongly to DNA (42, 43). In the mouse has been found that immediately after fertilization there is a high level of protein phosphorylation (44) that could well be associated to the unpacking of the spermatozoal chromatin.

Disruption of the plasma membrane seems to be also necessary to obtain chromatin dispersion, since in most *in vitro* studies agents were used that strongly altered the plasma membrane. This idea finds support in studies of cell fusion, because better results of chromatin dispersion were obtained when fusion was induced with lysolecitin that when Sendai virus were used (45). In the experments of microinjection of spermatozoal heads in oocytes, it is likely that the freeze-thaw treatment of the spermatozoa (23), might have altered the plasma membrane to allow chromatin dispersion. This might be the reason for the lack of chromatin dispersion when spermatozoa are phagocytized by somatic cells.

CONCLUSIONS

1. Gamete fusion is soon followed by the rapid loss of the spermatozoal nuclear membrane.
2. Prior to or concomitant with the beggining of spermatozoal chromatin decondensation is possible to observe electron dense bodies that emerge from the highly condensed chromatin. It is theorized that the presence of dense bodies might represent the release of DNA bound proteins.
3. Near the area of spermatozoal chromatin decondensation, is possible to observe a fine granular materia. In more advanced stages of fertilization this material is seen in association with electron dense bodies.
4. The post-fusion changes that undergoes the spermatozoal nucleus seem to be associated to the oocyte and to the fertilized egg cell cycles.
5. Thiols and proteases are believed to be involved in the post-gamete fusion spermatozoal chromation decondensation.
6. Full decondensation is reached about one hour after gamete fusion.

ACKNOWLEDGMENTS

This work was supported in part by Grants #45/80 from DIUC and #17/79 fron UNESCO. Grateful acknowledgments are due to Professor Luis Izquierdo for reading and commenting on the manuscript and also to Drs.. C. Doggenweiler and V. Monasterio of the Faculty of Sciences, University of Chile for allowing us the use of the electron microscope.

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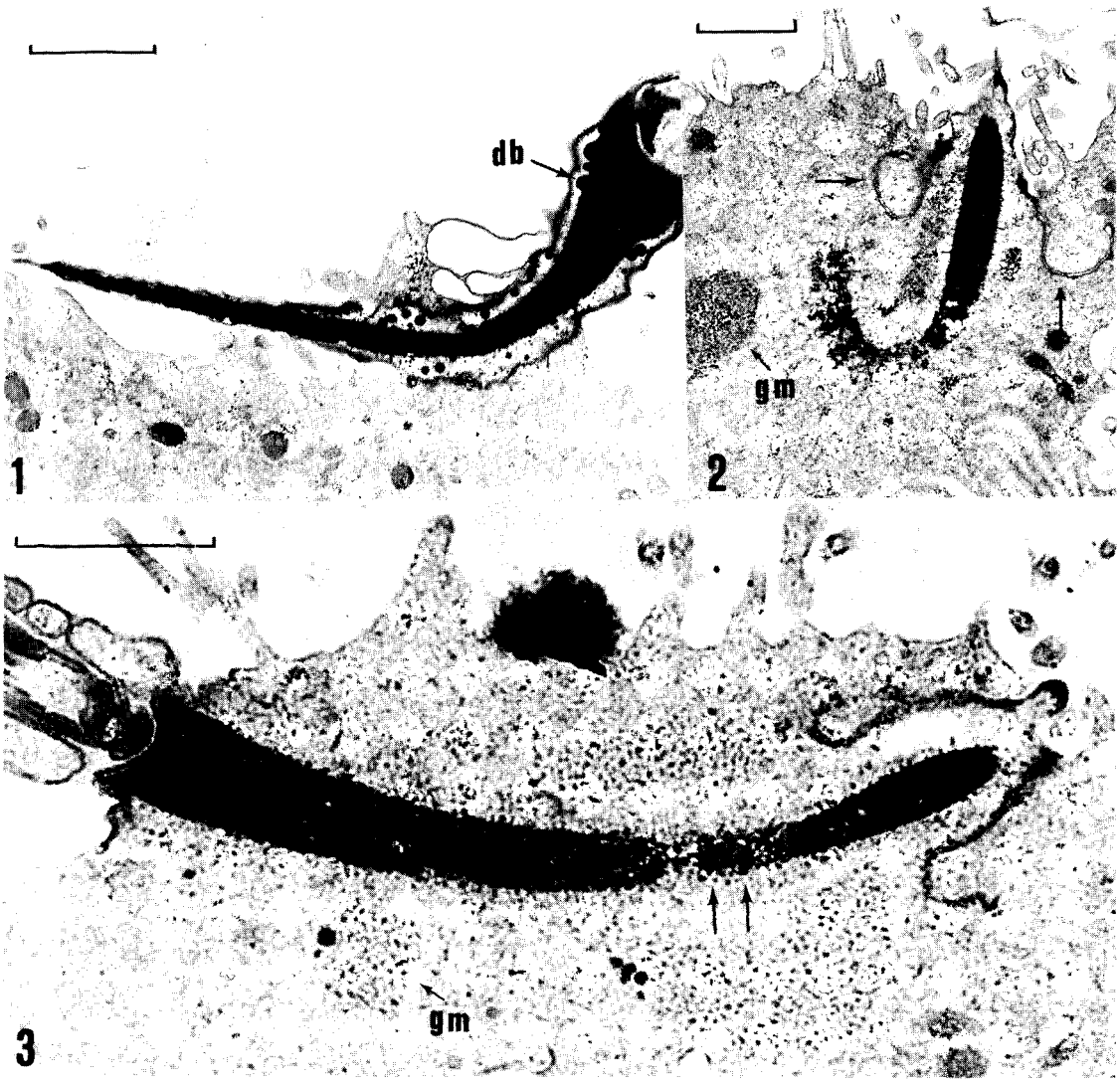

Fig. 1. Spermatozoon recently fused with an oocyte. Electron dense bodies (db) can be seen in close association with the spermatozoal chromatin.

Fig. 2. Spermatozoon within the egg citoplasm. The complex of membranes (arrows) have moved away from their close relationship to the nucleus. An accumulus of granular material (gm) can be seen near the decondensing chromatin.

Fig. 3. A tangential section of a spermatozoon, within the egg cytoplasm, that shows decondensation of the middle part of the nucleus (arrows). The granular material (gm) can be seen near the decondensing chromatin.

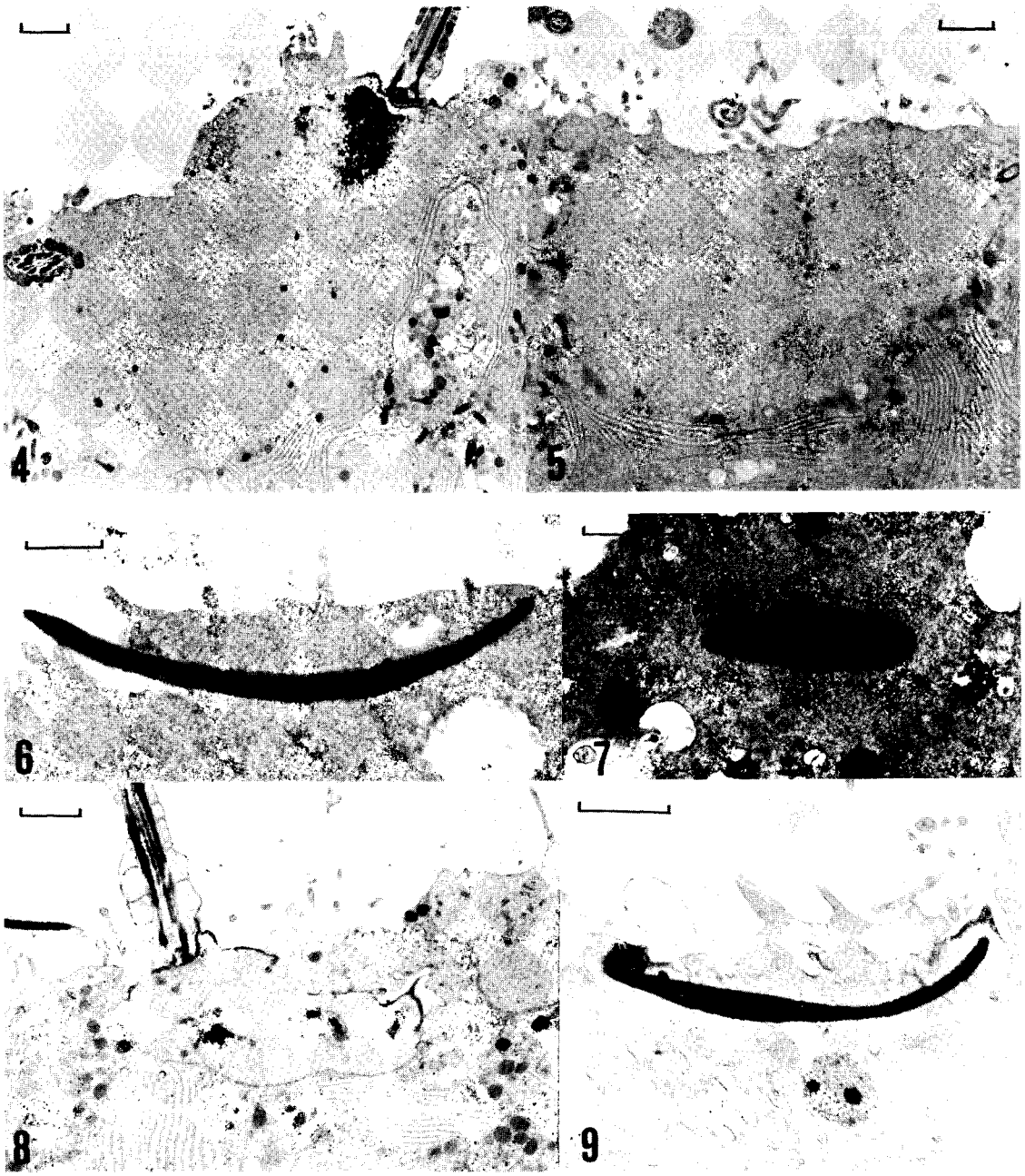

Fig. 4. An advanced stage of decondensation of the spermatozoal chromatin. Note the absence of nuclear membrane and that the area where the decondensed male chromatin is located, appears similar to the ground cytoplasm.

Fig. 5. Fully decondensed male chromatin. Note the absence of nuclear membrane and the array of cell organelles that limits the area of dispersion.

Bars equals 1μm

Fig. 6. Guinea-pig spermatozoa fused with an immature guinea-pig oocyte. Note the highly condensed chromatin.

Fig. 7. Hamster spermatozoal fused with an immature guinea-pig oocyte. Note the highly condensed chromatin.

Fig. 8. Guinea-pig spermatozoa, within a fertilized hamster egg, that shows an advanced degree of decondensation.

Fig. 9. Guinea-pig spermatozoa, within a fertilized hamster egg, that shows a slight degree of decondensation.

Bars equals 1μm