Abnormal assembly of annulate lamellae and nuclear pore complexes coincides with fertilization arrest at the pronuclear stage of human zygotic development

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**BACKGROUND:** The assembly of nuclear pore complexes (NPC) and their cytoplasmic stacks, annulate lamellae (AL), promote normal nucleocytoplasmic trafficking and accompany pronuclear development within the mammalian zygote. Previous studies showed that a percentage of human oocytes fertilized in vitro failed to develop normal pronuclei and cleave within 40–48 h post insemination. We hypothesized that an aberrant recruitment of NPC proteins, nucleoporins and/or NPC preassembled into AL, might accompany human fertilization arrest. **METHODS AND RESULTS:** We explored NPC and AL assembly in unfertilized human oocytes, and fertilized and arrested zygotes by immunofluorescence with an NPC- and AL-specific antibody, mAb 414, and by transmission electron microscopy. Major NPC or AL assembly was not observed in the unfertilized human oocytes. Once fertilization took place, the formation of AL was observed throughout the cytoplasm and near the developing pronuclei with NPC. On the contrary, NPC assembly was disrupted in the arrested zygotes, whereas AL were clustered into large sheaths. This was accompanied by the lack of NPC incorporation into the nuclear envelopes. **CONCLUSIONS:** We conclude that the aberrant assembly of NPC and AL coincides with early developmental failure in humans.

**Key words:** fertilization failure/human zygotes/nuclear envelope/nuclear pore complex/pronuclear development

**Introduction**

One of the most striking features of the cell cycle is the stage-specific separation and blending of the nuclear and cytoplasmic compartments. The nuclear envelope (NE) of mitotic cells dissolves at the entry point of the M-phase (Newport and Spann, 1987) and reforms at M-exit, or interphase entry (Pfaller et al., 1991). Three steps are necessary for NE assembly (Boman et al., 1992; Macaulay and Forbes, 1996). First, membrane vesicles, which may contain receptors for the proteins of nuclear lamina (Collas et al., 1996), assemble around the chromatin and eventually fuse to form a continuous NE around it (Burke and Gerace, 1986; Pfaller et al., 1991). Second, the nuclear pore complexes (NPC), assembled from the specific set of O-glycosilated glycoproteins called nucleoporins (Davis and Blobel, 1986, 1987; Hanover et al., 1987; Snow et al., 1987), are incorporated into the NE and provide the channels for bi-directional nucleocytoplasmic trafficking, between the newly established nuclear and cytoplasmic compartments (Benavente et al., 1989; reviewed by Panté and Aebi, 1993; Görlich and Mattaj, 1996). Third, nuclear lamins are imported into the nucleus and form the scaffold of nuclear lamina underneath the NE (Newport et al., 1990).

The assembly of the NE following mammalian fertilization starts with membrane-free chromatin (Szöllösi et al., 1972; Longo, 1973) represented by two distinct entities: a set of maternal chromosomes and the sperm nucleus. Subsequently, the assembly of the NE takes place in a fashion similar to that seen in somatic cells, regardless of whether the sperm entered the oocytes during IVF or ICSI (Sutovsky et al., 1997, 1998).

Although the assembly and fusion of membrane vesicles during NE formation have been studied extensively in somatic cells and in a cell-free system (reviewed by Poccia and Collas, 1996), little is known about the pathways leading to the formation of NPC on the zygotic NE, especially in mammalian models. Previous studies in bovine have demonstrated that the fertilizing sperm triggers the assembly of annulate lamellae (AL), in parallel with NPC insertion into NE (Sutovsky et al., 1998). AL, the cytoplasmic stacks of NPC (reviewed by Kessel, 1992), cross-react with a nucleoporin-specific antibody mAb 414 in Xenopus egg extracts (Meier et al., 1995) and in
mammalian pronuclear zygotes (Sutovsky et al., 1998; Ramalho-Santos et al., 2000).

Despite the continuous improvement of IVF techniques, several levels at which fertilization can fail have been described (Asch et al., 1995; Rawe et al., 2000). The arrest at two-pronuclei (2PN) stage is one of frequently seen patterns. Although AL have been found in human metaphase II oocytes (Baca and Zamboni, 1967) and pronuclear zygotes (Hertig and Adams, 1967; Zamboni et al., 1966; Van Blerkom et al., 1987), it is not known how and when NPC are incorporated into NE of developing human pronuclei, or if NPC and/or AL play any role during human fertilization and zygotic development.

In the present study we used immunofluorescence (IF) with an NPC- and AL-specific antibody, mAb 414, to visualize the assembly of NPC and AL in the fertilized and arrested human zygotes. Ultrastructural features of fertilized and arrested human zygotes were characterized by electron microscopy (EM).

Materials and methods

Patients and treatments

A total of 75 unfertilized, supernumerary, fertilized and arrested human oocytes and zygotes were studied using IF and transmission electron microscopy (TEM). The studied material was obtained from couples that had signed an informed consent undergoing IVF and ICSI in the Assisted Fertilization Programme of CEGyR, Buenos Aires, Argentina, where all studies were performed. Fertilized zygotes described as controls were supernumerary, discarded zygotes defined by the development of two pronuclei (2PN) ~16 h after insemination or sperm injection. At that time-point, these supernumerary zygotes had to be discarded upon the request of consenting donors. In the CEGyR programme, ~96% of the 2PN zygotes undergo mitosis. Supernumerary, pronuclear embryos that came from cycles with 100% cleavage rate were used as controls. Therefore, such embryos were considered to be normally fertilized. Couples that donated such zygotes indeed achieved pregnancy. Arrested oocytes/zygotes developed two apparently normal PN and did not cleave after ~40 h post insemination or sperm injection. Two of the studied arrested zygotes appeared to be normally fertilized and were cryopreserved in a previous attempt. These 2PN embryos did not cleave after 24 h and remained at PN stage. All unfertilized oocytes and fertilized zygotes used were discarded upon the request of consenting donors. Ethics committees of participating groups in this work have approved all procedures. Wives’ ovarian stimulation and IVF or ICSI procedures were performed as previously described (Rawe et al., 2000).

Nucleoporin and chromatin labelling

Nine prophase I (PI), 11 metaphase I (MI) and 10 metaphase II (MII) human oocytes were screened after the labelling of nucleoporins and chromatin by conventional epifluorescence microscopy. At the same time, a total of 21 supernumerary fertilized zygotes (11 from IVF and

![Figure 1. Nuclear pore complex (NPC) labelling in non-inseminated oocytes, visualized by immunofluorescence with nucleoporin-specific antibody mAb 414. (A) Continuous ring of NPC on the nuclear envelope of a human germinal vesicle (GV) stage oocyte. Diffuse, background labelling and some autofluorescent particles are seen in human oocytes after germinal vesicle breakdown (B; metaphase I) and at the second metaphase arrest (C; metaphase II). (D) Negative control labelling of a GV stage oocyte by the omission of the first antibody, mAb 414. (E) Negative control of a metaphase II oocyte, DNA counterstaining is shown in E’. Scale bars = 10 \( \mu m \).]
10 from ICSI) and 13 arrested fertilized zygotes (PN formation without further cleavage after ~40 h of insemination or injection, six from IVF and seven from ICSI) were screened for nucleoporins and chromatin labelling using a modified protocol of Messinger and Albertini (1991). For this purpose, zona pellicula was removed by a brief incubation with acid Tyrode’s medium, and denuded oocytes/zygotes were fixed and permeabilized for 20 min at 37°C in a microtubule stabilizing buffer (0.1 mol/l PIPES, pH 6.9, 5 mmol/l MgCl₂, 2.5 mmol/l EGTA containing 2.0% formaldehyde, 0.5% Triton X-100, and 1 µmol/l taxol). Fixed oocytes/zygotes were blocked for 1 h at 37°C with 2% bovine serum albumin (BSA), 2% powdered milk, 2% normal goat serum, 0.1 mol/l glycine and 0.01% Triton X-100 in PBS. If necessary, oocytes/zygotes were stored for up to 3 days at 4°C in this solution. To identify NPC, oocytes were incubated for 40 min at room temperature with 1:250 dilution of antibody mAb414 (BabCo; Berkeley, CA, USA) (Davis and Blobel, 1986, 1987; Meier et al., 1995; Sutovsky et al., 1998) in PBS containing 0.1% BSA and 0.02% sodium azide (PBS + BSA). After a short wash in blocking solution, oocytes were incubated in 1:500 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG), for 1 h at room temperature. Finally, treated oocytes were washed three times in PBS + BSA, counterstained with Hoechst 33258 (1 µg/ml) for 30 min at room temperature, washed again in PBS + BSA and mounted between a microscopy slide and a coverslip for examination using Olympus BX-40 epifluorescent microscope. Images were photographed using Kodak Ektachrome film (1600ASA) and processed using Adobe Photoshop 5.0 software (Adobe System Inc.). For control staining, PBS + BSA alone replaced the specific antibody solution. Secondary antibodies and reagents used were purchased from Sigma (St Louis, MO, USA).

Transmission electron microscopy
Six arrested zygotes (2PN stage) and five control zygotes were fixed for 1 h in a fixative composed of 2.5% glutaraldehyde and 0.6% paraformaldehyde in 0.25 mol/l cacodylate buffer (pH 7.2), then washed in 0.1 mol/l cacodylate buffer containing 0.2 mol/l sucrose and post-fixed for 1 h in 1% osmium tetroxide. Following dehydration by ascending ethanol series (30–100%), oocytes were infiltrated by a post-fixed for 1 h in 1% osmium tetroxide. Following dehydration by ascending ethanol series (30–100%), oocytes were infiltrated by a paraformaldehyde solution. Secondary antibodies and reagents used were purchased from Sigma (St Louis, MO, USA).

Transmission electron microscopy of control and arrested zygotes
Transmission electron microscopy of control and arrested zygotes
To provide more support for the observations of fertilized oocytes using IF, we explored the distribution of NPC and AL (Figure 2B, Table I). A differential pattern of AL assembly was seen in the cytoplasm of these arrested zygotes compared with controls. AL formed large patches assembled near the pronuclei (Figure 2C and C'), which in some cases became fragmented into multiple small karyomeres (Figure 2B). No differences were seen in the abnormal distribution pattern observed between IVF and ICSI zygotes. It is noteworthy that two of the studied fertilized zygotes were cryopreserved in a previous attempt. These zygotes were normally fertilized, i.e. formed two pronuclei of normal appearance. Once thawed, these 2PN zygotes did not cleave after 24 h and remained at PN stage. The patterns observed in this material were identical to the previously observed clustering of AL near both pronuclei (Figure 2D and D'). Surprisingly, while the arrested zygotes showed two pronuclei in light microscopy (Figure 2 E), and their overall appearance was similar to control 2PN zygotes (Figure 2F), epifluorescence revealed DNA condensation and fragmentation.

Table I. Fluorescence patterns of unfertilized and fertilized oocytes studied after nucleoporin labelling

<table>
<thead>
<tr>
<th>Category</th>
<th>Number</th>
<th>NPC labelling pattern</th>
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<tbody>
<tr>
<td>GV oocytes</td>
<td>9</td>
<td>Regular ring of NPC on NE</td>
</tr>
<tr>
<td>MI oocytes</td>
<td>11</td>
<td>Diffuse cytoplasmic/background</td>
</tr>
<tr>
<td>MII oocytes</td>
<td>10</td>
<td>Diffuse cytoplasmic/background</td>
</tr>
<tr>
<td>Normal zygotes</td>
<td>21</td>
<td>Mostly peri-pronuclear/punctate pattern of AL in cytoplasm</td>
</tr>
<tr>
<td>Arrested zygotes</td>
<td>13</td>
<td>Large cytoplasmic clumps</td>
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Normal fertilized zygotes were defined by the development of two pronuclei (2PN) ~16 h after IVF and ICSI. Arrested fertilized zygotes developed two apparently normal PN and did not cleave within 40 h post IVF or ICSI. NPC = nuclear pores complexes; NE = nuclear envelope; GV = prophase I/germinal vesicle-stage oocytes; MI = metaphase I oocytes; MII = metaphase II oocytes; PN = pronuclei; AL = annulate lamellae.

Results
Nucleoporin and DNA patterns in the non-inseminated oocytes
All studied oocytes were informative after processing the material. The nuclei (germinal vesicles, GV) of human prophase I oocytes displayed a continuous ring of NPC labelling on their NE, while some auto-fluorescence and occasional punctate-specific fluorescence were found in the cytoplasm (Figure 1A). After the resumption of meiosis (GV breakdown) the nuclear envelope labelling disappeared and a diffuse background-like labelling with occasional punctate autofluorescence in the cytoplasm was seen in the MI and MII oocytes (Figure 1B, C respectively). The numbers of oocytes and zygotes studied as well as the labelling patterns are summarized in Table I.

Distribution of nucleoporins and DNA in fertilized zygotes
Normal fertilization
A distinct punctate pattern of labelling with mAb 414 appeared throughout the cytoplasmic AL after 16–18 h of insemination and/or sperm injection (Figure 2A), suggestive of the assembly of cytoplasmic AL. In most cases, this cytoplasmic labelling was accompanied by the presence of NPC in the nuclear envelope of the fertilized zygotes (Figure 2A, Table I). The presence of a bright signal on the surfaces of both pronuclei can be seen in Figure 2A.

Arrested fertilization
Fertilization arrest at 1-cell stage coincided with an abnormal distribution of NPC and AL (Figure 2B, Table I). A differential pattern of AL assembly was seen in the cytoplasm of these arrested zygotes compared with controls. AL formed large patches assembled near the pronuclei (Figure 2C and C'), which in some cases became fragmented into multiple small karyomeres (Figure 2B). No differences were seen in the abnormal distribution pattern observed between IVF and ICSI zygotes. It is noteworthy that two of the studied fertilized zygotes were cryopreserved in a previous attempt. These zygotes were normally fertilized, i.e. formed two pronuclei of normal appearance. Once thawed, these 2PN zygotes did not cleave after 24 h and remained at PN stage. The patterns observed in this material were identical to the previously observed clustering of AL near both pronuclei (Figure 2D and D'). Surprisingly, while the arrested zygotes showed two pronuclei in light microscopy (Figure 2 E), and their overall appearance was similar to control 2PN zygotes (Figure 2F), epifluorescence revealed DNA condensation and fragmentation.

Transmission electron microscopy of control and arrested zygotes
Transmission electron microscopy of control and arrested zygotes
To provide more support for the observations of fertilized oocytes using IF, we explored the distribution of NPC and AL by EM. At the ultrastructural level (Figure 3), the presumed normally fertilized zygotes displayed small stacks of AL
distributed through the cytoplasm and adjacent to the NE (Figure 3A). In contrast, large assemblies of AL were predominantly found in the vicinity of the male and female pronuclei in the arrested zygotes (Figure 3B, C). An interesting feature of arrested zygotes was the presence of numerous mitochondria with accumulation of the electron-dense material in mitochondrial matrix (Figure 3C). On serial ultrathin sections, such mitochondria were found to be distributed evenly throughout the zygotic cytoplasm, but were not detected in the control zygotes. In the supernumerary fertilized zygotes (controls), the EM study revealed an apparently normal pronuclear development (Figure 3D). While the NE of such zygotes contained numerous NPC (Figure 3E), the number of NPC was greatly diminished in the arrested zygotes (Figure 3F, sections).

Figure 2. Nuclear pore complex (mAb 414/NPC) and DNA configuration in control and arrested oocytes. (A) Normally fertilized zygote at the time of pronuclear apposition. Note the punctate pattern of annulate lamellae labelling throughout the cytoplasm, and a distinct labelling of the nuclear envelope of both male and female pronuclei. (B) Large assemblies of NPC near the decondensed pronuclei after fertilization arrest (B, C and C'). (D and D'). Note that the nuclear envelopes are not labelled. A similar pattern of aberrant NPC and annulate lamellae distribution is seen in a fertilized zygote arrested at the pronuclear apposition stage after cryopreservation and thawing. Scale bars = 10 µm in A, B, C and D, and 5 µm in C' and D'. (E) Phase contrast micrograph of an arrested human zygote 40 h after insemination. (F) A control zygote 16 h after insemination.
Table II. Internalization of AL into both pronuclei, described previously (Zamboni et al., 1966; Van Blerkom and Motta, 1989) in presumably normal zygotes, and the presence of NPC-free stretches of NE were recorded in the arrested zygotes (Figure 3F), but not in the control ones.

Discussion

The present results indicate that the assembly of cytoplasmic AL in humans is triggered by fertilization and accompanies pronuclear development. Although seldom observed in GV stage, MI and MII oocytes, the AL were assembled in the oocyte cytoplasm after sperm penetration. This event was evidenced by the presence of punctate labelling of cytosolic AL, recognized by mAb 414 (Meier et al., 1995; Sutovsky et al., 1998) after fertilization. Accordingly, AL have been described in human oocytes at all stages ranging from GV oocytes to pronuclear zygotes (Adams and Hertig, 1965; Zamboni et al., 1966; Baca and Zamboni, 1967; Hertig and Adams, 1967; Van Blerkom and Motta, 1989). Based solely on ultrastructural observations, these studies did not allow for a comparison of AL density in the fertilized versus non-fertilized oocytes. An abnormal male and/or female PN development, observed after ICSI in rhesus monkey oocytes (Sutovsky et al.,...
1996), is also accompanied by accumulation of AL and reduced recruitment of NPC to the male pronuclear NE (Ramalho-Santos et al., 2000). Related to the deviant turnover of AL in the arrested zygotes could be the absence of NPC from large areas of pronuclear NE. This irregular NPC insertion pattern was not seen in the control, fertilized zygotes.

While the arrested zygotes showed 2PN in light microscopy, and their overall appearance was similar to control 2PN zygotes (Figure 2E, F), epifluorescence revealed DNA condensation and fragmentation in the arrested zygotes. It is possible that DNA within the pronuclei of arrested zygotes became fragmented and condensed while the NE remained intact, thus maintaining 'normal' appearance in light microscopy. This is supported by the observation that all arrested zygotes screened by immunofluorescence showed 2PN configurations indistinguishable from normal zygotes when examined by light microscopy. DNA fragmentation and disappearance of NPC from NE of the arrested zygotes could be signs of abortive apoptotic process.

Previous studies suggested that NPC are involved in selective nucleocytoplasmic transport in the pronuclear bovine zygotes (Sutovsky et al., 1998). The anomalies of AL assembly were shown to accompany fertilization arrests after ICSI in rhesus monkey (Ramalho-Santos et al., 2000). NPC are removed from the sperm NE during spermatid elongation in the testis (Sutovsky et al., 1999) and the NE of mature sperm is removed completely during fertilization in order to make the sperm chromatin accessible to oocyte cytoplasmic factors necessary for the remodelling of the sperm nucleus (Perreault et al., 1984; Poccia and Collas, 1996; Sutovsky et al., 1996, 1997; Sutovsky and Schatten, 1997, 2000; Usui et al., 1997; Wright, 1999).

After fertilization arrest at 1-cell stage, the AL- and NPC-specific antibody mAb 414 (Meier et al., 1995; Sutovsky et al., 1998) revealed the accumulation of AL in the cytoplasm of arrested zygotes. AL formed large patches assembled near the fragmented pronuclei, as previously observed in some rhesus monkey ICSI oocytes (Sutovsky et al., 1996; Ramalho-Santos et al., 2000). It is possible that the pronuclear development and nucleocytoplasmic communication were impaired in these zygotes. Consequently, the pronuclear development was not completed and the mitotic division had not taken place. This is consistent with the observation that the microinjection of an NPC antagonist, wheat germ agglutinin, prevented embryo development in bovine (Sutovsky et al., 1998). Similarly, the S-phase entry was blocked in rhesus monkey embryos with deviant PN and AL appearance after ICSI (Ramalho-Santos et al., 2000). Studies of Xenopus oocytes suggest that one of the NPC components, RanBP2 (also called Nup358), is a key factor in the interphase nucleocytoplasmic transport, but also in the control of mitotic events, including spindle assembly during metaphase and the reformation of the NE during telophase (Greber and Carafoli, 2002).

Alternatively, the abnormal biogenesis or function of the zygotic centrosome, documented previously in some cases of human fertilization failure (Asch et al., 1995; Van Blerkom, 1996), could affect the recruitment of NPC and cytoplasmatic trafficking of AL in some of the arrested, fertilized zygotes, mainly in those not showing pronuclear apposition. The nocodazole-induced disruption of the microtubules within fertilized bovine oocytes was indeed shown to derail the pronuclear recruitment of NPC, PN development and cytoplasmic movement of AL in fertilized bovine oocytes (Sutovsky et al., 1998).

EM studies confirmed the pathological reorganization of NPC and AL in arrested zygotes, previously observed by IF. Large assemblies of AL have been found in the cytoplasm of arrested 2PN zygotes, while smaller stacks of AL were seen in the control ones. A similar pattern of AL assembly was observed by IF in normally fertilized zygotes that did not cleave after cryopreservation/thaw. Cytoskeletal damage caused by cryopreservation (Sathananthan et al., 1988; Van der Elst et al., 1992; Park et al., 1997; Wei-Hua et al., 2001; Boiso et al., 2002) may affect the pattern of AL distribution. The association of AL with microtubules has been demonstrated previously (reviewed by Kessel, 1992; Sutovsky et al., 1998). The electron-dense deposits observed in the mitochondrion throughout the cytoplasm of arrested zygotes were reminiscent of calcium phosphate deposits in preimplantation embryos of mouse t-mutant (Hillman and Tasca, 1973). In our studies, such mitochondria were distributed randomly throughout the cytoplasm of arrested zygotes, and could be suggestive of apoptotic process, known to start with alterations of the mitochondrial membrane and matrix. Furthermore, it might be interesting to speculate that the release of activated caspases by these abnormal mitochondria could lead to the digestion of many cellular proteins responsible for cell cycle regulation, DNA damage recognition and repair and regulation of the cellular structure (for review see Nalepa and Zukowska-Szczeczkowska, 2002).

The results presented in this study document that the patterns of NPC and AL distribution in human oocytes that failed to fertilize do not mimic those seen in normal zygotes. Such observations suggest a differential distribution of NPC and AL in the normal and arrested pronuclear zygotes. These findings have implications for understanding the aetiology and cellular basis of early developmental failure in humans.

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