The role of sperm proteasomes during sperm aster formation and early zygote development: implications for fertilization failure in humans

Vanesa Y. Rawe¹, Emilce S. Díaz², Roger Abdelmassih³, Cezary Wójcik⁴, Patricio Morales², Peter Sutovsky⁵ and Héctor E. Chemes⁶,⁷

¹Centro de Estudios en Ginecología y Reproducción (CECyR), Buenos Aires, Argentina; ²Faculty of Health Sciences, Department of Biomedicine, University of Antofagasta, Antofagasta, Chile; ³Clínica e Centro de Pesquisa em Reprodução Humana Roger Abdelmassih, São Paulo, Brazil; ⁴Department of Anatomy and Cell Biology, Indiana University School of Medicine, Evansville, IN, USA; ⁵Division of Animal Science, Department of Obstetrics and Gynecology, University of Missouri, Columbia, MO, USA; ⁶Laboratory of Testicular Physiology and Pathology, Center for Research in Endocrinology, National Research Council (CONICET), Endocrinology Division, Buenos Aires Children’s Hospital, Buenos Aires, Argentina

BACKGROUND: Sperm aster organization during bovine and human fertilization requires a paternally-derived centriole that must first disengage from the sperm tail connecting-piece. We investigated the participation of the 26S proteasome in this process. METHODS: Proteasome localization and enzymatic activity were studied in normal and pathological human spermatozoa by immunocytochemistry and enzyme-substrate assays. The role of proteasomes during bovine zygote development was investigated using a pharmacological proteasome-inhibitor, MG132, and with anti-proteasome antibodies delivered by Streptolysin O-permeabilization or with the Chariot reagent. Human zygotes discarded after ICSI failures (n = 28) were also examined. RESULTS: Proteasomes were localized in the sperm acrosome and connecting-piece, as well as in the pronuclei of bovine and human zygotes. Proteasomal enzymatic activities were decreased in defective human spermatozoa. Disrupted sperm aster formation and pronuclear development were found after pharmacological and immunological block of proteasomes in human/bovine spermatozoa and oocytes, as well as in 28 discarded human post-ICSI fertilization failures. CONCLUSIONS: Specific proteasome inhibition disrupts sperm aster formation and pronuclear development/apposition in bovine and human zygotes. Human spermatozoa with defective centriolar/pericentriolar structures have decreased proteasomal enzymatic activity. Release of a functional sperm centriole that acts as a zygote microtubule-organizing center probably relies on selective proteasomal proteolysis. These findings suggest an important role of sperm proteasomes in zygotic development.

Keywords: proteasomes; sperm aster; early zygote; fertilization; sperm pathology

Introduction

Paternally contributed centrosomal components play a crucial role in zygotic development of non-rodent mammals. In bovine and human zygotes, the microtubular network responsible for syngamy and mitotic spindle formation requires microtubule nucleation by the centrosome, a structure derived from the sperm centriole and oocyte pericentriolar material (Sutovsky and Schatten, 2000). The mechanism by which the complex structure of the sperm tail connecting piece disintegrates to release the proximal centriole after sperm entry into the oocyte is not fully understood, but sperm proteasomes have been implicated (Sutovsky et al., 2004; Rawe, 2005). The ubiquitin-proteasome pathway is the main cytosolic proteolytic system responsible for the regulated substrate specific degradation of most cellular proteins in eukaryotic cells (Glickman and Ciechanover, 2002). It takes part in the important events of fertilization in both invertebrates and vertebrates, including mammals. Fertilization is inhibited by anti-ubiquitin and anti-proteasome antibodies or by small molecule, peptide-based inhibitors of specific proteasome proteolytic activities (Matsumura et al., 1991; Sawada et al., 2002a,b; Wang et al., 2002; Sutovsky et al., 2003).

The present study explores the participation of sperm-borne proteasomes during early development of bovine and human zygotes. Experimental evidence is provided for their role during the post-fertilization disassembly of the sperm tail connecting piece, sperm aster formation, pronuclear development and apposition. Proteasomes have been localized by immunofluorescence in human and bovine spermatozoa, and their specific enzymatic
activities were measured in sperm samples from men suffering from centriolar deficiencies and abnormal head–tail attachment (Chemes et al., 1999; Rawe et al., 2002). Here, functional blockage of sperm or zygote proteasomes was induced by specific antibodies or pharmacological proteasome-inhibitor MG132. Finally, proteasome localization, pronuclear development and sperm aster formation rates were examined in abnormal human zygotes displaying post-ICSI fertilization failure.

Materials and Methods

Chemicals and antibodies

All chemicals were obtained from Sigma Chemical Co (St Louis, MO, USA), unless otherwise stated. Free 20S and complete 26S proteasomes as well as other proteasomal forms are ubiquitous and exist in different proportions according to cell type, cell cycle phase and metabolic conditions. Because of this heterogeneity various antibodies were tested and those best suited for proteasome localization in a particular cell type (spermatozoa, oocytes, zygotes) were selected in pilot experiments. Rabbit polyclonal antibodies raised against multiple 20S proteasomal core subunits of α and β-type (anti-α/b), anti-LMP2 (inducible 20S subunit PSMB9/β-1i) and anti-MECL-1 (inducible 20S core subunit PSMB10/β-2i) were obtained from Biomol International, PA, USA, and used to label proteasomes in bovine and human oocytes and zygotes. To investigate the presence of proteasomes in bull and human spermatozoa, rabbit polyclonal antibody p31 against one of the subunits of PA700 was kindly donated by Dr George DeMartino (The Southwestern Medical Center at Dallas). For antibody-transfection experiments, a sodium azide-free rabbit polyclonal antibody best suited for immunohistochemical localization was obtained using fixation, the samples were permeabilized for 60 min in PBS containing 2 mg/ml of both Triton X-100 and 0.5% saponin. The samples were then washed with PBS and 0.5% saponin and incubated with 0.5% glutaraldehyde in the same buffer, post-fixed for 2 h in 1.3% Osmium Tetraoxide and embedded in Epon-Araldite. The blocks were cut in a RMC MT-7000 automatic ultramicrotome (RMC Inc. Tucson, AZ, USA). Thick 1 μm sections were cut with glass knives and thin sections (70–80 nm) displaying pale golden interference colors were obtained using diamond knives. Thin sections were double stained with uranyl acetate and lead citrate and studied on a Zeiss EM 109 electron microscope (Zeiss Obercochen, Germany).

Immunocytochemistry of ejaculated bull and human spermatozoa

Frozen bull semen (Juan de Bernardi Co., Buenos Aires, Argentina) was thawed, layered over a 45–90% Percoll gradient and centrifuged at 300g for 15 min to isolate live sperm fraction. Cryopreserved fertile human semen samples had been donated by consenting adult men at Centro de Estudios en Ginecología y Reproducción, washed with phosphate buffer (0.1 M, pH 7.4) and spermatozoa were separated by centrifugation at 1000g for 10 min. The pellets were fixed for 2–4 h with 3% glutaraldehyde in the same buffer, post-fixed for 2 h in 1.3% Osmium Tetraoxide and embedded in Epon-Araldite. The blocks were cut in a RMC MT-7000 automatic ultramicrotome (RMC Inc. Tucson, AZ, USA). Thick 1 μm sections were cut with glass knives and thin sections (70–80 nm) displaying pale golden interference colors were obtained using diamond knives. Thin sections were double stained with uranyl acetate and lead citrate and studied on a Zeiss EM 109 electron microscope (Zeiss Obercochen, Germany).

Biochemical determination of sperm proteasome enzymatic activities

Semen samples were donated by two consenting teratozoospermic men suffering from sperm centriolar deficiencies and abnormalities in the development of the sperm head–tail attachment (Chemes et al., 1999; Rawe et al., 2002). Semen samples from six fertile men were used as controls. The samples were kept frozen and used to prepare sperm extracts to study sperm proteasome enzymatic activities. The samples were thawed and diluted with 5 ml of modified Tyrode’s medium consisting of 117.5 mM NaCl, 0.3 mM NaH2PO4, 8.6 mM KCl, 25 mM NaHCO3, 2.5 mM CaCl2, 0.5 mM MgCl2, 2 mM glucose, 0.25 mM Na pyruvate, 19 mM Na lactate, 70 μg/ml of both streptomycin and penicillin, phenol red and 0.3% BSA (Sigma, A7030), centrifuged at 300g for 10 min and then resuspended in homogenization buffer (50 mM Hepes, 10% glycerol, pH 7.4), supplemented with 100 μM Nε-p-Tosyl-L-lysine-Chloromethyl-Ketone (TLCK, an acrosin inhibitor) at a concentration of 25 × 10–5 spectrophotometer per ml. The sperm suspension was then sonicated (Visonic, Gardiner, NY, USA) with six 60 watt bursts for 20 sec each, followed by centrifugation for 30 sec at 5000g in a Beckman microfuge to remove nuclear and flagellar material. The supernatant was used as the enzyme stock preparation. All these procedures were performed at 4°C. The protein concentration in sperm extracts, obtained using the Bradford method (Bradford, 1976), ranged between 0.3 and 0.8 mg/ml.

The chymotrypsin-like (CHTL) and peptidylglutamylpeptide hydrolyzing (PGPH) activity of the proteasome were assayed in duplicate using the fluorescent substrates N-Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLL-AMC, from Biomol International, PA, USA), and Carbobenzoxy-Leu-Leu-Leu-AMC (Z-LLL-AMC, from Peptides International, Louisville, KY, USA).

Aliquots of 50 μl of enzyme extract were incubated in a final volume of 1 ml containing 10 mM CaCl2, 50 mM Hepes, pH 7.4, 594-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) for 1 h and the DNA stained with 4',6-diamino-2-phenylindole (DAPI, Molecular Probes). Coverslips were mounted in VectaShield mounting medium (Vector Labs, Burlington, CA, USA) and sealed with nail polish. Samples were examined with an Olympus BX40 epifluorescence-equipped microscope operated with a DS-5Mc Nikon camera and ACT-2U software, or with a Nikon Eclipse 800 microscope equipped with CoolSnap CCD camera and MetaMorph software. Negative controls were performed by omitting the first antibody or by incubation with a non-immune rabbit serum followed by a fluorescently conjugated anti-rabbit immunoglobulin (Ig) G. Image acquisition times were similar in control and labeled samples. All experiments were repeated at least three times.
permeabilized sperm were preloaded with p31 anti-proteasome antibody supplement (Irvine Scientific, Santa Ana, CA, USA). SLO-permeabilized sperm were preloaded with p31 anti-proteasome antibodies for at least 1 h at 37 °C, washed and used for ICSI. Two aliquots of the SLO-permeabilized antibody loaded spermatozoa were analyzed to assess antibody penetration (by immunocytochemistry) and viability (by the hyposmotic swelling test, Jeyendran et al., 1984).

**Streptolysin O-permeabilization and sperm transfection with anti-proteasome antibody**

After swim-up, sperm concentration was adjusted to 5–10 × 10^6/ml. Permeabilization was accomplished as described by Yunes et al. (2000). Briefly, washed spermatozoa were resuspended in PBS containing 0.4 unit/ml of Streptolysin O (SLO) for 15 min at 48 °C. Cells were washed once with PBS and resuspended in human tubal fluid (Irvine Scientific, Santa Ana, CA, USA) supplemented with 15% of synthetic albumin-lactate-pyruvate (TALP) culture medium under mineral oil until the desired stages in zygote development. In some experiments, bull sperm mitochondria were prelabelled with the mitochondrial probe MitoTracker Green FM (400 nM) to identify the male pronucleus by its association with the sperm tail mitochondria.

Antibody transfection was carried out at 8 h post-insemination, around the time of sperm aster formation, using the Chariot reagent (Active Motif) according to Payne et al. (2003) and to manufacturer’s recommendations. To conduct transfections in live zygotes, the 1:10 dilution of Chariot reagent and 1:5 dilution of pre-immune IgG for 1 h later (18 h post-insemination).

**IVF of bovine oocytes, antibody transfection of zygotes and pharmacological inhibition of proteasomes**

Bovine oocytes obtained from a slaughterhouse were matured and fertilized in vitro according to standard protocols (Sirard et al., 1988). Mature bovine oocytes were placed into drops of Tyrode’s albumin-lactate-pyruvate (TALP) culture medium under mineral oil and inseminated with live bull spermatozoa at 39 °C under 5% CO₂ until the desired stages in zygote development. In some experiments, bull sperm mitochondria were prelabelled with the mitochondrial probe MitoTracker Green FM (400 nM) to identify the male pronucleus by its association with the sperm tail mitochondria.

Antibody transfection was carried out at 8 h post-insemination, around the time of sperm aster formation, using the Chariot reagent (Active Motif) according to Payne et al. (2003) and to manufacturer’s recommendations. To conduct transfections in live zygotes, the 1:10 dilution of E446 antibody was used. Chariot–antibody binding was accomplished by incubating a 20 μl volume mix containing a 1:10 dilution of ChariotTM reagent and 1:5 dilution of pre-immune IgG or 1:2 dilution of E446 antibody. For additional control experiments, Chariot reagent and antibody E446 were also used alone. Following this binding step, each 20 μl volume was added to one well of a 96-well plate containing 20 zona- and cumulus-free zygotes suspended in 80 μl of serum-free TALP medium. The samples were incubated at 39 °C for 1 h, after which an additional 100 μl of serum-containing TALP medium was added to the well. Zygotes were cultured and fixed for immunocytochemistry 10 h later (18 h post-insemination).

Pharmacological inhibition of proteasomal activities during fertilization was achieved by using MG132, a specific and reversible inhibitor of proteasomal proteolytic activity (Biomol International, PA, USA). IVF was performed as described earlier. Specific proteasomal inhibitor MG132 (10 μM) was added either to oocytes at the time of insemination or by pretreating oocytes or spermatozoa with MG132 before insemination. Control IVF was performed without the inhibitor, by using vehicle solution (100% ethanol) at concentration identical to vehicle concentration in MG132-treated cells. Samples were further cultured until 20–24 h post-insemination, at which time they were fixed and analyzed by immunofluorescence.

For immunocytochemistry, bovine cumulus cells and zonae pellucidae were removed from oocytes and zygotes by short incubations with 1 mg/ml hyaluronidase and 2 mg/ml pronase, respectively. Zona-free bovine oocytes, zygotes and embryos were then gently pipetted onto poly-L-lysine-coated coverslips in Ca^2+^-free TALP medium, fixed for 40 min in 2% formaldehyde and permeabilized in 10 mM PBS+0.1% Triton X-100 for an additional 40 min. After fixation and permeabilization, oocytes and zygotes were processed for immunocytochemistry with anti-proteasome antibodies and imaged using an Olympus spectral confocal microscope, with laser lines at 488, 568, 594 and 633 nm wavelengths, or by using a conventional epifluorescence Nikon Eclipse 800 microscope, equipped with CoolSnap CCD camera operated by MetaMorph software. Negative controls included preimmune sera or anti-MECL-1 antibody immunosaturated with a synthetic MECL-1 peptide. Nuclear envelopes were labeled with anti-nucleoporin antibody MAb414 (Covance).

**ICSI of human oocytes: injection with p31 antibody-loaded human spermatozoon and spontaneous fertilization failures**

A total of 12 metaphase II (MII) human oocytes, donated for this research by consenting patients, were injected with SLO-permeabilized spermatozoa. Eight of them received SLO+p31 antibody-treated spermatozoa (see above). The other four oocytes were used as controls and injected with human spermatozoa permeabilized with SLO without antibodies. All injected human oocytes were fixed at 16–18 h after ICSI, processed by immunocytochemistry and studied by epifluorescence or confocal microscopy as described earlier.

Thirty-two discarded human oocytes (no pronuclei visualized 18–20 h after sperm injection) were also studied. These zygotes with post-ICSI fertilization failure were fixed, processed for immunocytochemistry and studied by confocal microscopy.

All supernumerary human oocytes were donated for research with informed written consent by couples undergoing ICSI at the Clinic of Human Reproduction Roger Abdelmassih (Sao Paulo, Brazil). No part of work with human gametes or zygotes was performed at the University of Missouri-Columbia, USA. Normal and abnormal human zygotes that failed to cleave within 40 h after ICSI and supernumerary oocytes not used for infertility treatment or cryopreservation were donated for research by consenting, adult female donors (less than 35 years old) with medical indication of ICSI because of male factor. All volunteer donors remained anonymous to researchers involved in this project. None of the presumed normal ova donated for this project were allowed to cleave in vitro or be transferred to recipients after ICSI. These ova were going to be discarded if not donated for research. All animal and human procedures were approved by CEGYR and Abdelmassih Internal Review Board and Ethic Committee accordingly.

**Statistical analysis**

Each experiment was repeated at least three times. To compare percentages of fertility between control and treated groups, contingency tables were constructed and analyzed by means of Chi-squared test. Odds ratios were calculated and 95% confidence intervals were obtained. Software used was InfoStat (Universidad Nacional de Cordoba, Argentina). In all cases, less than 0.01 error probability was considered highly significant. For each experiment, representative images are shown for oocytes, zygotes and embryos.

**Results**

**Acrosomal and flagellar localization of proteasomes in bull and human spermatozoa**

Figure 1 depicts mature bull (Fig. 1A) and human spermatozoa (Fig. 1B) labeled with p31 anti-proteasome antibody. The labeling reveals that each spermatozoon is labeled at two discrete locations: there is a larger labeling in the acrosome and a smaller punctuate pattern overlying the sperm proximal centriole and the flagellar connecting piece. In electron micrographs, the...
connecting piece appears as a dense structure formed by the capi-
tulum and the striated columns that enclose the sperm proximal
centriole, a cylindrical organelle formed by nine microtubular tri-
plets deeply embedded in the center of the dense connecting piece
(Fig. 1C). In head–tail junction-defective human spermatozoa
(Chemes et al., 1999) acephalic forms exist in which the proximal
centriole and proximal centriolar adjunct display normal archite-
cture and are also embedded in the center of the dense structures of
the connecting piece (Fig. 1C').

**Decreased enzymatic proteosomal activity in head–tail
junction-defective human spermatozoa**

Specific proteosomal enzymatic activities were quantified in
six fertile (control) men and two infertile patients with centrio-
lar anomalies and defects of the head–tail attachment. The
semen of these men displayed a mixture of acephalic sperma-
toza and forms in which heads and tails were not aligned
along the same axis. In both patients, contamination with
round spermatogenic or somatic cells was negligible. Sperma-
toza from these two patients exhibited a marked decrease in
both ChTL and PGPH activities of the proteasome below the
20% and 40% values of control samples (Fig. 2).

**Localization of proteasomes in the pronuclei
of bovine zygotes**

Specific immunolocalization of proteasomal subunits LMP2,
MECL1 and other α-type and β-type proteasomal subunits
was performed in bovine pronomural zygotes at various stages
of pronuclear development. Early after sperm incorporation
into ooplasm, the fully condensed sperm nucleus was free of
detectable proteasomes (Fig. 3A). Proteasomal subunits were
first detected in the male and female pronuclei at the initial
stage of sperm nuclear decondensation. Proteasomal labeling
was present within both pronuclei prior to pronuclear apposition
and reached highest density inside the apposed, full-sized pro-
nuclei (Fig. 3B and C). No nuclear/chromosomal labeling
with anti-proteasomal antibodies was observed in the ova that
remained unfertilized 20 h after insemination (not shown).
Negative control using anti-MECL1 antibody saturated with
synthetic MECL1 peptide did not show any signal (Fig. 3D).

**Pharmacological inhibition of bovine zygotic
proteasomes using MG132**

The role of zygote proteasomes in early post-fertilization events
was explored in bovine oocytes subjected to IVF using bull
spermatozoa and treated with 10 μM MG132 (a specific proteasome inhibitor) after fertilization. The average fertilization rate in ethanol-treated control zygotes was 75.7%. Incubation with 10 μM MG132 significantly reduced fertilization down to 38.2% (P < 0.0001, Table I). Pre-incubation of ova with 100 μM MG132 prior to fertilization had no effect on the import of proteasomal subunits into pronuclei (not shown).

Pronuclei inside the fertilized ova were visualized by the monoclonal antibody mAb 414 recognizing a group of related NPC proteins. Nucleoporin labeling was chosen based on our previous observation that it is an informative marker of nuclear envelope integrity and functionality throughout all stages of pronuclear/zygotic development (Sutovsky et al., 1998).

The patterns of pronuclear development in the ova that were fertilized in the presence of 10 μM MG132 were abnormal. Failed pronuclear formation due to premature chromosome condensation (PCC) was the most common abnormality (Fig. 3E and F, respectively). Partial scattering of chromosomes in PCC (E) and the assembly of nucleoporins around the condensed chromatin (F). Note the partial scattering of chromosomes in PCC (E) and the clustering of NPC proteins (arrow head in F) around the condensed chromatin. Bull spermatozoa carry only a residual amount of nucleoporins located in the redundant nuclear envelopes in the sperm tail connecting piece (red, G).

Table I. Percentages of fertilized bovine oocytes in control conditions and under treatment with MG132 or Chariot-antibody complex.

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Control</th>
<th>Treated</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG132</td>
<td>75.7 (n = 87)</td>
<td>38.2 (n = 107)</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Chariot-antibody complex</td>
<td>67.2 (n = 299)</td>
<td>21.6 (n = 315)</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

Differences in percentages were analyzed by the Chi-squared test. n, total number of oocytes in each experimental condition.

Immunological inhibition of bovine zygotic proteasomes by Chariot-mediated antibody transfection

To explore the possible role of proteasomes in the late fertilization events leading to sperm aster formation, we transfected newly fertilized bovine oocytes with E446 anti-proteasome antibodies coupled to the Chariot reagent. Antibodies were introduced into bovine zygotes at 8 h post-insemination, at the time pronuclei begin to form in most IVF zygotes. These were allowed to develop until 20 h after insemination when full pronuclear formation is achieved under control conditions. Co-localization of proteasomes (green fluorescence), tubulin (red fluorescence) and DNA (TOTO-3; blue fluorescence) was verified by immunocytochemistry.
Inhibition of proteasomal activity by immunological neutralization during bovine IVF (Chariot™), SLO experiments and in human fertilization failures after ICSI

(A) Normal pronuclear development after the incubation of bovine zygotes with Chariot alone (control). (A') Proteasomes (green) were assembled inside male and female pronuclei. Normal sperm aster formation is seen (red, tubulin) coinciding with fully apposed pronuclei. (B) After zygote transfection with Chariot coupled to anti-proteasome antibody E446, oocytes show failure of sperm aster formation and lack of pronuclear apposition. An intense green labeling of proteasomes is observed close to the sperm tail connecting piece in the majority of transfected zygotes (arrow in boxed area). (B') Absent sperm aster formation and aberrant pronuclear development. The inset shows the merging of B and B' boxed areas. Note the condensed sperm nucleus (blue) and proteasome recruitment over the sperm tail connecting piece (green). In C and D, high magnification details of the sperm nucleus region of two zygotes can be appreciated: (C) ICSI zygote obtained by injection of a SLO-permeabilized human spermatozoon loaded with p31 anti-proteasomal antibody. Note proteasome accumulation (green, arrows) to one side of the sperm pronucleus (blue). Absence of the sperm aster formation (red) and pronuclear development/apposition. Black and white panels to the right show proteasome accumulations (upper insets) and paternal DNA labeling (lower insets). (D) After human ICSI failure, proteasomes (green, arrow) accumulated near the sperm nucleus (blue). Complete lack of sperm aster formation (red). Black and white panels showing the area of proteasomal ‘cloud’ (upper inset) next to the sperm nucleus (lower inset) can be discerned separately. Scale in A, A', B and B' is 20 μm (represented in A). Scale bar in C and D correspond to 5 μm.

Figure 4A and A' (control) show normal pronuclear development and apposition after using either Chariot alone or Chariot pre-incubated with non-immune IgG (fertilization rate 67.2%). High density of proteasomes was observed inside the apposed pronuclei and in lesser amounts throughout the cytoplasm (Fig. 4A'). Tubulin was organized in a fully developed sperm aster that originated close to the male pronucleus (Fig. 4A'). Inset shows the intranuclear localization of proteasomes in a control human zygote with fully formed pronuclei and sperm aster. After transfection with antibody E446 (incubation of zygotes with the Chariot-E446 antibody complex), only 21.6% of inseminated bovine oocytes fertilized ($P < 0.0001$, Table I). In affected zygotes, pronuclei did not form or were insufficiently developed and the pronuclear proteasome labeling was either absent or aberrant (Fig. 4B'). Cytoplasmic proteasomes were heavily concentrated over the sperm tail connecting piece in a cloud-like configuration (Fig. 4B' and inset). There was also incomplete polymerization of tubulin (absence or abnormalities of sperm aster formation, Fig. 4B') and failure of pronuclear apposition.

**Immunological inhibition of sperm proteasome functions in SLO-permeabilized spermatozoa**

The above data demonstrated that antibody transfection of the bovine fertilized ova efficiently inhibited the function of proteasomes involved in the disassembly of the sperm tail connecting piece and formation of the zygotic sperm aster. However, when zona pellucida-free bovine oocytes were incubated with bull spermatozoa pretreated with the Chariot-p31 antibody complex, normal rates of pronuclear development, apposition and sperm aster formation were observed (data not shown). These negative results raised the question of the lack of penetration of the antibody complex into spermatozoa. In order to ensure antibody accessibility to the connecting piece, we used human spermatozoa that were first permeabilized with SLO and subsequently loaded with function-blocking p31 antibodies. After microinjection of SLO+p31 antibody-treated spermatozoa into eight MII human oocytes, only three of them developed two pronuclei (37.5% fertilization rate) compared with a fertilization rate of 100% in four control oocytes (injected with sperm treated with SLO but no antibodies). Control zygotes showed a fully developed sperm aster and proteasomes localized over the well formed pronuclei. Conversely, non-fertilized oocytes (5/8, 62.5%) completely lacked sperm asters and pronuclear development/apposition. Proteasomes did not assemble inside the pronuclei and were concentrated as a dense cloud around the sperm tail connecting piece on one side of the sperm nucleus (Fig. 4C).

The effects of E446 antibodies transfected into bovine zygotes using the chariot reagent and those of p31 antibodies introduced into SLO-permeabilized spermatozoa previous to ICSI suggest that both p31 and E446 interfere with normal proteasome function and therefore can be referred to as function-blocking antibodies.

**Proteasome localization in the discarded human zygotes that failed to cleave after ICSI**

As described in the previous section, injection of p31 antibody-loaded human spermatozoa into human oocytes or incubation of bovine zygotes with either Chariot-E446 antibody complexes or MG 132 resulted in post-fertilization failures. To test whether a similar phenomenon could be traced after spontaneous ICSI-failure in humans, 28 zygotes discarded because of fertilization failure were processed for
immunocytochemistry. Sixteen out of 28 (57%) of the studied fertilization failures displayed dense accumulations of proteasomes close to the non-decondensed male pronucleus (proteasomal cloud). Failure of pronuclear development/apposition and lack of sperm aster formation were also observed in these 16 abnormal zygotes (Fig. 4D). This pattern was identical to the pattern we had previously documented in bovine zygotes treated with Chariot-E446 antibody, MG132 or in human ICSI with SLO-permeabilized p31 antibody-loaded spermatozoa.

Discussion
Cytoskeletal organization in bovine and human zygotes is similar as both require the participation of a paternally derived centriole that organizes the sperm aster responsible for progression of pronuclear apposition and syngamy. The mechanism by which this centriole is released from the dense structures of the tail-connecting piece has not been clarified to date, but it has been suggested that proteasomes may be involved in this process (Sutovsky et al., 2004; Rawe, 2005). Proteasomes are macromolecular protease complexes found in most eukaryotic cells where they participate in regulated, substrate-specific proteolysis of polyubiquitinated substrates. Proteasomes take part in multiple steps of fertilization such as acrosome exocytosis, zona pellucida penetration, sperm mitochondrial sheath degradation and chromatin remodeling in the zygote (Wójcik et al., 2000; Bialy et al., 2001; Sutovsky et al., 1996, 2003; Haraguchi et al., 2004; Berruti and Martegani, 2005). Recent reports show the presence and proteolytic activity of proteasomes in the nuclear compartment of somatic cells and porcine zygotes (Chen et al., 2002; Sutovsky et al., 2004). Our present findings extend these observations to bovine and human zygotes and demonstrate that proteasome translocation from the cytoplasm to pronuclei is efficiently blocked by specific proteasomal inhibitors such as the MG132.

While proteasomes are abundant in nuclei and cytoplasm, they are particularly enriched at the centrosomes of somatic cells, where they form the ‘proteolytic center’ (Wójcik et al., 1996; Wojcik, 1997a,b; Wigley et al., 1999; Fabunmi et al., 2000). To date, little is known about the functional significance of proteasomes associated with the centrioles of the sperm tail connecting piece (Lin et al., 2000; Fabunmi et al., 2000; Mochida et al., 2000; Wójcik et al., 2000; Berruti and Martegani, 2005). This location suggests novel functions for centriole-associated proteasomes. Failure of converting the reduced sperm centriole into an active zygotic centrosome may be one of the reasons for post-fertilization developmental arrests affecting couples treated at IVF clinics (Chemes et al., 1999; Hewitson et al., 2000; Chemes and Rawe, 2003). This possibility became very clear to us after the study of a case of fertilization failure due to centriolar dysfunction in patients with abnormal head–tail connecting piece attachment (Chemes et al., 1999; Rawe et al., 2002). Since proximal centrioles are structurally normal in these patients, one of the objectives of the present study was to elucidate the nature of the centrosomal dysfunction. The diminished sperm proteasome enzymatic activity reported here in two patients with abnormalities of the head–tail attachment indicates that they are endowed with abnormal proteolytic machineries and are probably unable to disassemble the connecting piece and therefore release the functional sperm centriole into the oocyte cytoplasm. To further test this hypothesis, we studied proteasome localization in human and bovine spermatozoa, oocytes and early zygotes, carried out pharmacological or immunological inhibition of proteasomes and examined proteasomal distribution in cases of human post-ICSI fertilization failures.

Bovine and human spermatozoa display two distinct proteasome populations, one located over the acrosome and the other one as distinct punctuate densities on the connecting piece. This is in accordance with the few previous reports available (Wójcik et al., 2000; Sutovsky et al., 2004). Early in fertilization, most cytoplasmic proteasomes translocate to the newly formed pronuclei where they possibly take part in active chromatin remodeling prior to syngamy. The present data show that the inhibition of proteasomes by MG132 in early stage pronuclei results in PCC and incomplete pronuclear development. These findings suggest that degradation mediated by proteasomes is required for the release of oocytes from MII block and full pronuclear progression.

To explore the role of proteasomes in the release of the centriole from the complex structure of the sperm tail connecting piece, we incubated bovine IVF zygotes with proteasomal inhibitor MG132 or with Chariot coupled to E446 anti-proteasome antibodies. Also, donated human oocytes were injected with SLO permeabilized spermatozoa preloaded with function-blocking anti-proteasome antibody p31. In all experiments, a common pattern of fertilization failure emerged. Proteasomes accumulated around the sperm tail connecting piece in a dense cloud-like configuration and there was absent or defective sperm aster formation and pronuclear development/apposition. Identical findings were present in the zygotes from couples with spontaneous post-ICSI fertilization failure that also displayed dense proteasome aggregations overlying the sperm connecting piece and lack or deficiencies in sperm asters and pronuclei. We propose that the proteasome recruitments observed after experimental inhibition of proteasomes and in spontaneous post-ICSI fertilization failures represent abortive attempts of the zygotes to overcome the impaired release of the sperm centriole caused by experimental or spontaneous proteasome deficiencies.

Upon proteasome inhibition, proteasomes and polyubiquitinated proteins aggregate around the centrosomes forming an inclusion body or ‘aggresome’ in the area of the proteolytic center (Wójcik et al., 1996; Wójcik, 1997a,b; Johnston et al., 1998; Wójcik and DeMartino, 2003). The proteasome clusters reported by us are strikingly similar to aggresomes, suggesting that their formation is a general response of cells when proteasomes are unable to adequately degrade ubiquitinated proteins (Johnston et al., 1998). Thus, the sperm centrosome appears to be a site of proteasome-regulated proteolytic activity just like their counterparts in somatic cells. Altogether, these data support our hypothesis that proteasomes are involved in proteolysis of the sperm tail connecting piece. Failure of fertilization after microinjection of p31 antibody-loaded human spermatozoa indicates that the sperm centriole-associated proteasomes could function as a first-wave degradation mechanism of the structures surrounding the sperm-borne proximal centriole. After this
priming of the sperm tail connecting piece by proteasomes, the abundant set of oocyte proteasomes could contribute to degrading and re-structuring the connecting piece to ensure the release of a sperm functional centriole and its conversion to the zygotic centrosome, the organelle that nucleates a sperm aster responsible for bringing the parental genomes together prior to first mitosis.

Acknowledgements
We would like to thank Dr George DeMartino (The Southwestern Medical Center at Dallas) for the gift of the anti-proteasome antibodies and Mrs Maria de Luján Calcagnio for expert assistance with statistical methods. Special thanks belong to Florencia Nodar, Mariano Lavolpe and Gisella Maggiotto for their dedication in collecting the donated human gametes. We are very grateful to anonymous, consenting donors who kindly donated their supernumerary gametes for this research. The technical assistance of Ana Paula Reis, Soraya Abdelmassih and Roberto Fernández (Confocal Microscopy at the University of Buenos Aires) is fully acknowledged.

Funding
CEGyR Foundation and Grants from CONICET (PICT 2565); ANPCyT (PICT 9591); Fondecyt 1040295.

References
Sirard MA, Parrish JJ, Warburton C, Leibfried-Rutledge ML, First NL. The culture and re-structuring of the connecting piece to ensure the release of a sperm aster connecting piece by proteasomes, the abundant set of oocyte proteasomes could contribute to degrading and re-structuring the connecting piece to ensure the release of a sperm functional centriole and its conversion to the zygotic centrosome, the organelle that nucleates a sperm aster responsible for bringing the parental genomes together prior to first mitosis.

Payne C, Rawe VY, Ramalho-Santos J, Simery C, Schatten G. Cytoplasmic dynein/dynactin association with nucleoporins and vimentin mediates geno-