## OTHER RESEARCH

A. *In Vitro* Fertilization in Eutherian Mammals.

In the early 1950s it was recognized that mammalian spermatozoa must undergo physiological and structural changes as a prerequisite to fertilization. These changes in the spermatozoon were termed capacitation. Sperm capacitation normally occurs in the female genital tract but it could be reproduced in cell-free systems containing body fluids from several sources (e.g., follicular fluid, blood serum). For this reason, during the 1960s it was thought that a factor or factors were involved in inducing this phenomenon. In the early 1970s, our research was directed at demonstrating that sperm capacitation and fertilization could be achieved in vitro in a defined physiological solution free of macromolecules. To test these hypotheses, we used a heterologous *in vitro* fertilization system composed of guinea pig spermatozoa and zona-free hamster oocytes. Guinea pig spermatozoa underwent the acrosome reaction and other physiological changes consistent with capacitation after incubation for a few hours in a defined saline solution. Furthermore, these capacitated spermatozoa fertilized zona-free hamster oocytes both in a defined saline solution or in a saline solution supplemented with bovine serum albumin. At the resolution of the phase contrast microscope, fertilizing spermatozoa, fused with the oolemma, decondensed their chromatin and formed structures consistent with male pronuclei (Figs. 1, 2). These observations were corroborated later by others using transmission electron microscopy. Similarly, work by us and others demonstrated that the acrosome reaction and an increase/change in sperm motility (termed activation) obtained under these *in vitro* conditions were similar to that obtained during *in vivo* sperm capacitation (Fig. 3.). This work was conducted in the laboratory of my first mentor, Dr. Claudio Barros, while I was an undergraduate student at the Catholic University, Santiago.

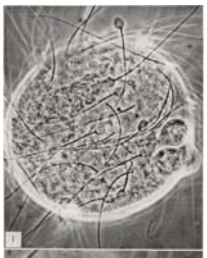

Fig. 1. A zona-free hamster oocyte penetrated by several guinea pig spermatozoa.

Fig. 2. Male pronucleus development within a zona-free hamster oocyte.

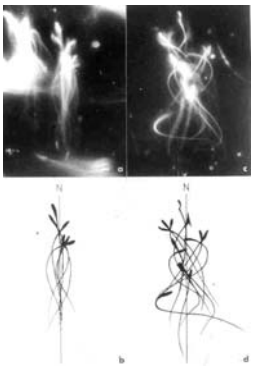

Fig. 3. Time-lapse dark field micrographs captured on a single photographic plate (top) and corresponding drawing renditions (bottom) of a not activated guinea pig spermatozoon (left) and an activated (capacitated) guinea pig spermatozoon (right).

B. Ultrastructure of the Acrosome of the *Octodon degus* Spermatozoon.

The acrosome of mammalian spermatozoa has been the subject of intense research. In the mid 1970s we became interested in investigating at what stage of spermatogenesis the acrosome conferred

spermatozoa the ability to fertilize eggs. To conduct these studies we selected the spermatozoon of a small hystrichomorph rodent *O. degus*. Mature spermatozoa from *O. degus* have several small protrusions in their acrosome (Fig. 4). The appearance of these protrusions may be used as markers for sperm maturation. Our first task was to capture the sequence of morphological events leading to a fully mature acrosome and thus a mature spermatozoon. Electron micrographs of late spermatids were assembled together to give a complete picture of the stages leading to the formation of a mature acrosome in this organism. This work was conducted in the laboratory of Dr. Claudio Barros while I was a graduate student at the Catholic University, Santiago.

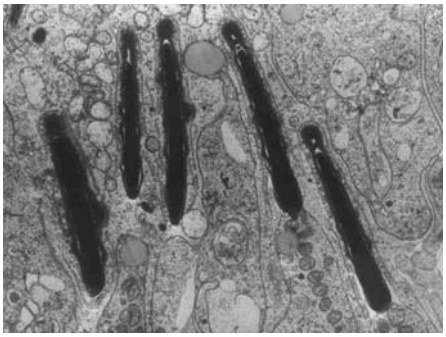

Fig. 4. *O. degus* late spermatids. Electron micrograph showing the final stages in the assembly of their acrosome.

C. Nuclear Assembly: Post-Fertilization Events in Primary Rabbit Oocytes.

Primary oocytes displaying a germinal vesicle (GV) cannot be fertilized normally; their ability to be fertilized is acquired during the resumption of meiosis just before ovulation. In the mid 1970s phase contrast microscopy showed evidence that seemed to confirm that spermatozoa reaching the perivitelline space could not fuse with and were not incorporated into the ooplasm of rabbit GV oocytes. In contrast, ultrastructural analyses of similar experiments in hamster GV oocytes revealed that they were able to incorporate fertilizing spermatozoa in a normal fashion. We became interested in evaluating, at the ultrastructural level, whether rabbit GV oocytes were indeed penetrated by spermatozoa and if post-fusion events like cortical granule breakdown, chromatin decondensation and pronuclear formation were also occurring normally. We conducted these experiments *in vivo* by first artificially inseminating the fallopian tubes of surrogate female rabbits with ejaculated spermatozoa. A few hours later, GV oocytes collected from the ovaries of non-stimulated donor female rabbits were also placed into the already inseminated fallopian tubes. After incubation in the upper female genital tract, oocytes were recovered from the fallopian tubes, fixed and prepared for transmission electron microscopy (Fig. 5). Ultrastructural analyses revealed that, spermatozoa do fuse with the oolemma of rabbit GV oocytes, but in contrast to what is observed in mature rabbit oocytes (metaphase II oocytes), cortical granule breakdown was only partial, the nuclear envelope of fertilizing spermatozoa remained intact and chromatin decondensation was delayed implying that resumption of meiosis may bring about functional changes to ooplasmic components. This work was conducted in the laboratory of Dr. Michael Bedford, while I was a research trainee at Weill Cornell Medical College, New York.

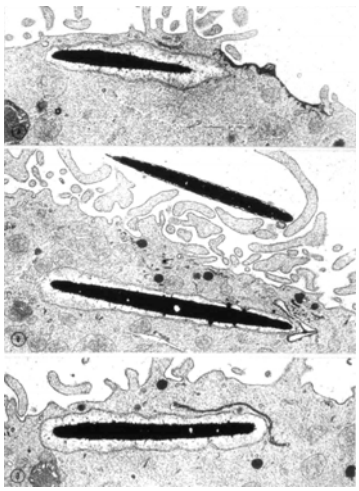

Fig. 5. Transmission electron micrographs of ultrathin sections through the nucleus of rabbit spermatozoa that have penetrated primary oocytes.

D. Nuclear Assembly in a *Drosophila* Cell-Free System.

During the early 1980s several groups developed cell-free nuclear assembly systems using extracts from activated *Xenopus* oocytes. These cell-free systems provided important new insights into the structure and function of the nucleus. In the late 1980's my laboratory began to develop similar *in vitro* nuclear assembly systems in the fruit fly *Drosophila melanogaster*. Specifically, we developed a heterologous nuclear assembly cell-free system that used demembranated *Xenopus* spermatozoa as a source of chromatin and low speed supernatants (cell-free extracts) from early *Drosophila* embryos as a source of ooplasmic nuclear components. This cell-free system was capable of decondensing demembranated spermatozoa from various species and assembled nuclei (male pronuclei) starting from demembranated frog spermatozoa (Fig. 6). Using indirect immunofluorescence microscopy, we demonstrated that these in vitro assembled nuclei were assembled in part from *Drosophila* embryo components originally stockpiled in oocytes. This work was conducted in my laboratory at Stony Brook.

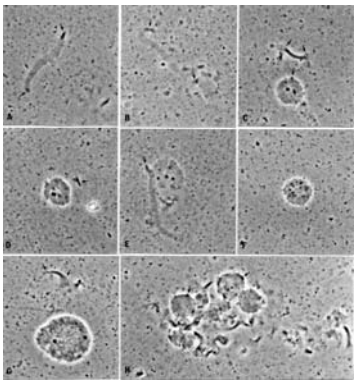

Fig. 6. Phase contrast micrographs showing the decondensation of demembranated *Xenopus* spermatozoa and the assembly of nuclei in cell-free extracts from *Drosophila* embryos.

E. Myosin-like ATPases and the Nuclear Pore Complex.

Since the 1960s several groups attempted to identify the protein(s) responsible for an ATPase activity histochemically localized to nuclear pore complex-enriched fractions isolated from vertebrate cells. Although many attempts were made, definitive identification and characterization of the enzyme or enzymes associated with nuclear pore complex-enriched fractions were precluded by the inability to solubilize the ATPase in active form. As in vertebrate systems, in the early 1980s our attempts to purify the ATPase or ATPases associated with *Drosophila* nuclear pore complex-enriched fractions were frustrated by enzyme insolubility. To circumvent this problem, we used direct UV-photoaffinity labeling in tandem with protein purification under SDS-denaturation conditions. After UV-dependent cross-linking with [$\alpha^{32}$P]ATP, proteins from nuclear pore complex-enriched fractions were solubilized in SDS and subjected to SDS-PAGE and autoradiographic analyses. Using this strategy, a single radiolableld polypeptide band migrating at approximately 188-kD on SDS-PAGE gels was identified by autoradiography (Fig. 7). Specific antibodies were raised against the photolabeled polypeptide and used to biochemically characterize and localize the ATPase in situ by immunofluorescence and immunogold transmission electron microscopy. Results from these experiments revealed that the enzyme shared properties with myosin heavy chains and it was localized to nuclear pore complexes by both immunofluorescence and transmission immunoelectron microscopy (Fig. 8). Similar studies also revealed the presence of a myosin light chain-like polypeptide associated with *Drosophila* nuclear pore complex-enriched fractions. Antibodies directed against the myosin light chain-like polypeptide localized this subunit to *Drosophila* nuclear pore complexes (Fig. 9). This work was started in the laboratory of my doctoral thesis advisor, Dr. Gunter Blobel and was later continued in my own laboratory at Stony Brook.

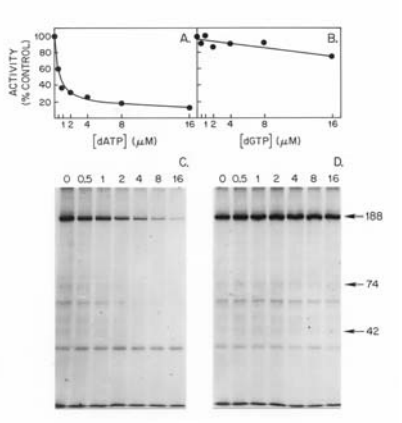


Fig. 7. Effect of "cold" dATP and dGTP on ATPase activity (A, B) and direct UV photoaffinity labeling (C, D) of a 188-kD polypeptide band associated with a *Drosophila* nuclear pore complex-enriched fraction.

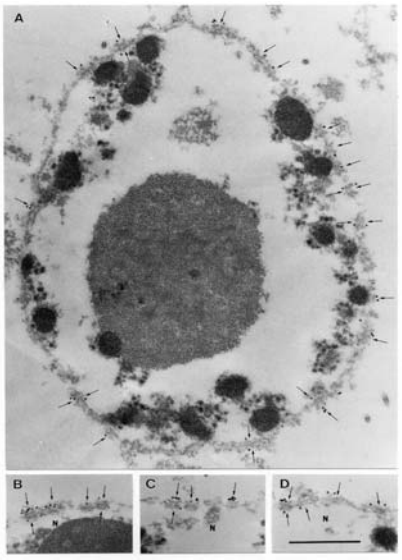


Fig. 8. Transmission electron micrographs showing immunogold labeling of *Drosophila* nuclear pore complexes (arrows) by antibodies directed against a 188-kD myosin heavy chain-like polypeptide associated with a *Drosophila* nuclear pore complex-enriched fraction. Bar: 50 nm.

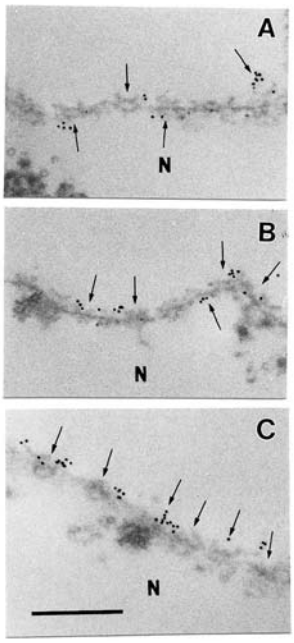


Fig. 9. Transmission electron micrographs showing immunogold labeling of *Drosophila* nuclear pore complexes (arrows) by antibodies directed against a myosin light chain-like polypeptide. N: nucleoplasm. Bar: 300 nm.

F. Site-directed photo-induced digestion of proteins *in vivo* and *in situ*.

Photosensitizers (PS) have been used extensively in biology and medicine to elucidate cellular processes, macromolecular interactions, localize macromolecular targets and as therapeutic agents for a number of diseases. Photosensitization occurs when a PS absorbs a photon of light and is promoted from its ground state to a singlet excited state. Subsequently, the singlet excited state PS undergoes intersystem crossing to a triplet excited state capable of reacting with a substrate either through an oxygen mediated mechanism (Type II) or radical formation (Type I). The type of products formed upon reaction of a PS with cellular macromolecules depends on a number of factors, including the nature of the target (i.e., nucleic acids, proteins), the reactivity of the PS, the distance between PS and target, and the concentration of oxygen in the system. Photochemically induced reactions targeting nucleic acids and proteins include cleavage to smaller fragments and crosslinking to substrates. Photosensitized reactions have also been exploited for their therapeutic value. Photodynamic therapy (PDT) takes advantage of the ability of certain photosensitizers to accumulate in tumor cells and, upon irradiation with visible light, selectively kill malignant cells with minimal effect on surrounding normal tissue. The promise of PDT as an alternative cancer therapy carrying minimal side effects and collateral damage to normal cells has spurred on the development of a number of promising PS for potential use in PDT, as well as a variety of techniques for better targeting malignant cells.

Visible light dyes have been conjugated to proteins to site-specifically induce the cleavage of DNA and proteins. In collaboration with Dr. Kimberly Conlon, we used Rose Bengal (RB), a xanthene dye with a λmax of absorption at 560 nm in water to initiate visible light photoreactions. Moreover, the reaction of a hexanoic acid derivative of RB containing an N-hydroxysuccinimide group targeting specific antibodies afforded the creation of conjugates that could site direct photochemically induced cleavage reactions. To test this methodology we chose anti-myosin antibodies and skeletal myosin heavy chain (MHC) polypeptide. The photo-induced digestion of MHC was accomplished using both a direct and an indirect photochemical method. In the direct method, MHC was incubated with RB-conjugated anti-myosin antibodies prior to photolysis. In the indirect method, MHC was incubated with anti-myosin antibodies, followed by an additional incubation with RB-conjugated anti-mouse IgG antibodies. The resulting immunoconjugates were then irradiated with visible light. SDS-PAGE and immunoblot analyses confirmed the existence of photodigestion products with the direct or indirect method. SDS-PAGE and immunoblot analyses revealed that MHC was not digested when similar reactions were performed with either free RB or unconjugated anti-myosin antibodies.

We used similar in situ photochemical reactions to demonstrate the site-directed disruption of the cytoskeleton. For this purpose, two different proteins that specifically bind actin and filamentous actin (f-actin), anti-actin antibodies and heavy meromyosin (HMM) respectively, were conjugated to RB. *In situ* photochemistry was performed on actively growing mouse embryo fibroblasts. Cells were incubated with RB-conjugated anti-actin antibodies or RB-conjugated HMM, and irradiated with visible light. After irradiation, cells were probed with fluorescently-labeled phalloidin to evidence the disruption of the actin cytoskeleton. Fluorescent microscopy revealed the disruption of the actin cytoskeleton by these RB-conjugates. *In situ* photoreaction with these RB-actin binding conjugates was specific for the actin cytoskleleton since neither free RB nor RB-conjugated antibodies directed against non-components of the actin cytoskeleton were able to disrupt it. Later, using similar photochemical methods we demonstrated the association of DNA base excision repair enzymes with *in vitro* assembled microtubules and with microtubule networks during interphase and mitosis.