Final Report for Project NAG10-0064 entitled: "Microgravity Effects during Fertilization, Cell Division, Development, and Calcium Metabolism in Sea Urchins".

The overall objectives of this project are to explore the role of microgravity during fertilization, early development, cytoskeletal organization, and skeletal calcium deposition in a model developmental system: the sea urchin. While pursuing these objectives, we have also helped to develop, test, and fly the Aquatic Research Facility (ARF) system. This novel system has been tested successfully at the Kennedy Space Center and during a flight on the Endeavor STS-77 shuttle mission yielding a number of biomedically important findings relevant to promote the generation of valid scientific discoveries for space and for biomedical applications on Earth.

The goal of this research program is to explore and understand the effects of gravity on fertilization and early development using sea urchin eggs and embryos as a model system. Sea urchin development has several advantages for this project including the feasibility of maintaining and manipulating these cells during space flight, the high percentage of normal fertilization and synchronous early development, and the abundant knowledge about molecular, biochemical, and cellular events during embryogenesis which permits detailed insights into the mechanism by which gravity might interfer with development. Furthermore, skeletal calcium is deposited into embryonic spicules within a day of fertilization permitting studies of the effects of gravity on bone calcium deposition.

Space experiments require closed systems to contain the specimens. In addition to closed systems, other experimental constraints need to be considered. One challenging problem imposed by Space Shuttle operations is the delay between specimen loading and on-orbit experiment activation. As much as a twenty-four hour delay may be experienced between gamete collection and on-orbit fertilization. This is a drastic departure from standard laboratory practice where sea urchin eggs are typically fertilized within hours of collection. This delay does not pose a problem for sperm storage since they can be stored in concentrated form for days. In contrast, egg viability is affected when stored under normal laboratory conditions for prolonged lengths of time. Several viability parameters were tested and methods were determined during the progress of this project to maintain unfertilized eggs for up to 40 hours and preserve reliable fertilizability for fertilization conducted in space. Additional requirements to produce scientifically valid results was to maintain embryo cultures in biocompatible material that would allow culturing embryos up to the late pluteus stage of development. Results on this part of the project have been published in Steffen et al., 1992. The two most significant findings during these early studies are that gametes of sperm and eggs can be kept for at least 24 hours and up to 40 hours prior to fertilization when stored at 12^{o}C in biocompatible enclosure material. To generate scientifically reliable and reproducible results, sea urchin embryos need to be cultured in space under controlled experimental parameters such as steady temperature, humidity, illumination, and fixed at predetermined time points for accurate and reliable acquisition of data and analysis for the generation of valid scientific results. For the study of the effects of microgravity on aquatic systems in space, this project involved the development of an entire new hardware system called the Aquatic Research Facility (ARF). Together with the Canadian Space Agency and NASA this elaborate project was undertaken and successfully completed in Spring of 1996. The development of the ARF system will benefit many other investigations during future space flights and during experimentation on the space station. The development of the ARF system has overcome many of the earlier shortfalls to now conduct experiments with much higher scientific value under controlled conditions. Further advantages of the ARF system over previous containers used for experimentation in space is that it holds two centrifuges with one of them set at 1g to mimic conditions in gravity on Earth. This allows direct comparison of cells grown in microgravity with those at 1g in space and those cultured on Earth. In addition to developing the ARF system, this project also required the development of a hardware system that

would allow fertilization in space. A second novel piece of hardware was developed called the Fertilization Syringe Unit (FSU) that would allow storage of sperm and injecting sperm into the ARF system during flight for fertilization in space. The success of both newly developed hardware systems was demonstrated during the STS-77 mission with embryos obtained that had been fertilized in space with the NASA-designed FSU.

To explore the role of microgravity on cytoskeletal organization and calcium metabolism during fertilization, cell division, and early development, the sea urchin was chosen as a model developmental system with superb and natural synchronization during development representing a variety of aspects common to vertebrate and invertebrate fertilization and development including those of humans. Cells were fixed at predetermined specific time points to capture key events during development. Specifically, the events during fertilization, pronuclear fusion, cell division, cell differentiation, blastula, gastrula, and pluteus formation were analyzed using light, immunofluorescence, and transmission and scanning electron microscopy for the analysis of calcium-related events, membrane fusion and restructuring, chromosome organization, microfilament, microtubule, and centrosome reorganizations.

The objectives for the experiments were to ask four specific questions.
1. How does microgravity affect the early stages at fertilization including signal transduction events, cortical granule secretion, and calcium sequestration, and how does it affect the cytoskeletal system including microfilaments, microtubules, and centrosomes during fertilization and cell division? Fertilization and cell division employ similar molecular mechanisms as muscle does during contraction and relaxation. Calcium sequestration, secretion, and cytoskeletal organization play crucial roles during these processes. The hypothesis was tested that these processes will undergo changes and adapt to the microgravity environment. This hypothesis is based on findings that the muscle system which employs similar molecular mechanisms for function as cells do during fertilization and cell division, is altered in the microgravity environment. Our experiments could determine that the secretion and microfilament organization is affected by microgravity which results in altered cortical granule exocytosis and microfilament behavior. The centrosome-microtubule organization during cell division was affected resulting in 4% of cells with abnormal cell divisions. 2. How is cell differentiation at the 16-cell stage affected by microgravity? The hypothesis was tested that cell differentiation depends on a functional centriole-centrosome complex. As during cell division, our results revealed that a small percent of cells was affected by microgravity which might indicate that the centriole-centrosome complex might need gravity for proper orientation but that cells are able to undergo adaptations while proliferating under microgravity conditions. 3. How is cilia movement and embryo motility and development regulated in the absence of gravity? We hypothesize that the swimming behavior of the blastula embryo is gravity sensitive and that the centriole-centrosome complex at the base of cilia in conjunction with striated fibers are the organelles defining the coordinated beating of cilia and swimming bahavior of the embryo. Calcium and phosphorylation events may be altered during cilia beating in microgravity as a result of changes for energy requirements. The results of this stage of development are currently under investigation. 4. Is spicule formation during the late gastrula/early pluteus stage affected by microgravity? The hypothesis is being tested that calcification of spicules is affected by microgravity similar to the effects of microgravity on bone structure which results in osteoporosis and bone structure alterations. This question addresses calcium regulation under microgravity conditions and will help us analyze the onset and causes which lead to osteoporosis on Earth.

To address the specific questions for each developmental stage, cells were fixed at preselected time points to preserve the structures and organelles of interest with regards to cell biology events during development. The protocols used for the analysis of the results had been developed during the earlier part of this research and were applied for post-flight analysis (published in Chakrabarti and Schatten, 1995) using light and (immuno)fluorescence microscopy, scanning electron microscopy, and transmission electron microscopy. The structures of interest are: microtubules during fertilization, cell division, and cilia movement; microfilaments during cell surface restructuring and cell division;

centrosomes and centrioles during cell division, cell differentiation, and cilia formation and movement; membranes, Golgi, endoplasmic reticulum, mitochondria, and chromosomes at all stages of development; and calcium deposits during spicule formation in late-stage embryos.

Several forms of microscopy were applied for post-flight analysis that includes light microscopy of whole and sectioned embryos, confocal immunofluorescence microscopy, high resolution scanning electron microscopy, transmission electron microscopy, and x-ray microanalysis. Routinely available poly- and monoclonal antibodies to detect cytoskeletal structures included anti-actin for the detection of microfilaments, anti-tubulin for the detection of microtubules, anti-centrosomal antibodies for the detection of centrosomes, and antibodies against calcium regulatory proteins such as calmodulin and Ca^{++}-ATPase activity.

In addition to further explore aspects important for living in space, several aspects of this research are also aimed at understanding diseases that affect humans on Earth which may be accelerated in space. The loss of bone calcium will result in bone structure alterations and osteoporosis. Changes in calcium metabolism will lead to muscular diseases or muscular atrophy which is severely experienced by astronauts and cosmonauts traveling in space. Our studies on the centriole-centrosome complex during cell division will directly benefit research on cancer which is the result of uncontrolled cell divisions that might be deregulated at the centrosome level. Since the cytoskeleton is significantly important for many processes in the cell including signal transduction, cell surface restructuring, hormone secretion, organelle transport, cell shape changes, fertilization, cell division, cell polarity, and many more, these studies will be a benefit directly or indirectly to human health on Earth and contribute significantly to biomedical research on Earth related to infertility, aging, cancer, osteoporosis, and neuronal disorders including Alzheimer's disease. Benefits from studies exploring cytoskeletal organization and calcium metabolism will come in form of generating database information and identifying target sites for pharmaceutica to correct pathological conditions during the many diseases related to improper cytoskeletal function and calcium metabolism.

Preliminary reports of these studies have been provided during the Pre Science Verification Test in September 1995, during the Payload Verification Test in January 1996, in annual progress reports, and in the Aquatic Research Facility Crew Familiarization Package. Several reports have either been published or are in preparation for publication which include the following selections.

1. Steffen, S., Fiser, R., Simerly, C., Schatten, H., and Schatten, G. (1992). Microgravity effects on sea urchin fertilization and development. Adv. Space Res. Vol 12, No. 1, pp(1)167-(1)173.
2. Chakrabarti, A., Stoecker, A., and Schatten, H. (1995). Modification of experimental protocols for a space shuttle flight and applications for the analysis of cytoskeletal structures during fertilization, cell division, and development in sea urchin embryos. AIAA, 95-1095, 1-10.
3. Hedrick, J., Chakrabarti, A., and Schatten, H. (1995). Effects of microgravity simulated with clinostat rotation on cytoskeletal structures of *Drosophila* KC23 cells in culture. ASGSB abstracts, 1995
4. Schatten, A., and Chakrabarti, A. (1996). Sea urchin development from egg to embryo in the newly developed ARF hardware system. In preparation for Adv. Space Res.
5. Schatten, H., Fiser, R.,. Franz, J., Zoran, S., Simerly, C., and Chakrabarti, A. (1996). Fertilization during an aircraft parabolic flight and hardware development for culture of sea urchin embryos in space. In preparation for Adv. Space Res.
6. Schatten, H., and Chakrabarti, A. (1996). Contributions to unravel the enigma of centrioles. Are they gravity-sensing organelles and evolutionary remnants of the cell? In preparation for Physiologist.
7. Hedrick, J., Chakrabarti, A., and Schatten, H. (1996). Comparison of *Drosophila* KC23 cells subjected to microgravity as simulated with clinostat rotation with cells during aging. In preparation for Physiologist.

New Technology Report

A. ARF Hardware System

1. ARF HARDWARE

The Aquatic Research Facility (ARF) is a novel hardware system that has been developed during the conduct of this project. The Aquatic Research Facility was specifically designed to provide controlled conditions to conduct scientific experiments requiring controlled temperature, humidity, illumination, and fixation at predetermined time points. In previous experiments using similar animal systems as used for our project in grant NAG10-0064, experiments in space suffered from the lack of controlled experimental conditions which made the interpretation of scientific data obtained difficult if not impossible. The development of the ARF system has overcome many of the earlier shortfalls to now conduct experiments with much higher scientific value.

Further advantages of the ARF system over previous containers used for experimentation on the space shuttle is that it holds two centrifuges with one of them set at 1g to mimic conditions in a gravity environment on Earth. This allows direct comparison of cells and embryos grown in microgravity with those grown at 1g which then can be compared with cells and embryos cultured on ground. The other centrifuge in the system will turn slowly once every three minutes providing a microgravity environment which ensures that all specimens are exposed equally to any variations in temperature or lighting occuring within the ARF. The ARF system was used for the first time during the STS-77 mission on the Endeavor space shuttle to successfully contain and allow the culture of sea urchins, starfish, and muscles in space.

2. STANDARD CONTAINERS

The Standard Container Units (SCU's) are referred to as miniaquariums that hold the sea urchin cultures used during this project. Two Standard Container Assemblies (SCA's) side by side are the individual compartments of the SCU that hold 33ml of embryo cultures each. Each SCA is equipped with a fixative block assembly that is actuated by computer control at preset time points to release fixative into the SCA's which will fix the sea urchin embryos at preselected time points.

3. SOFTWARE

The software written to accomodate these experiments under controlled temperature, humidity, lighting, video recording, and fixation conditions, is described in the manuals provided by MPB Technologies, Montréal.

B. Fertilization Syringe Unit

The Fertilization Syringe Unit (FSU) was designed by NASA to store prediluted sea urchin sperm for fertilization in space. The design for this unit is described in more detail in protocols written by NASA. The Fertilization Syringe Unit was employed for the first time during the STS-77 mission on Endeavor to fertilize sea urchin eggs contained in the ARF system successfully resulting in embryos that were fertilized and cultured in space.