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**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**

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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

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Abstract

To explore the role of microgravity on cytoskeletal organization and skeletal calcium deposition during fertilization, cell division, and early development, the sea urchin was chosen as a model developmental system, and methods were developed to employ light, immunofluorescence, and electron microscopy on cultures which are being prepared for a flight on the Space Shuttle. For analysis of microfilaments, microtubules, centrosomes, and calcium-requiring events, our standard laboratory protocols needed to be modified substantially to meet the requirements for experimentation on the Space Shuttle. All manipulations were carried out in a closed culture chamber containing 35 ml artificial sea water as a culture fluid. Unfertilized eggs stored for 24 hours in these chambers were fertilized with sperm diluted in sea water and fixed with concentrated fixatives for final fixation in 1.1% formaldehyde, 1 μ M taxol, 10 mM EGTA, and 1 mM $MgCl_2 \cdot 6H_2O$ for 1-cell to 16-cell stages to preserve cytoskeletal structures for simultaneous analysis with light, immunofluorescence, and electron microscopy, and 1.5% glutaraldehyde, 0.4% formaldehyde for blastula and pluteus stages. The fixed samples were maintained in chambers without degradation for up to two weeks after which time the specimens were processed and analyzed with routine methods. Since complex manipulations are not possible in the closed chambers, the fertilization coat was removed after fixation using 0.5% freshly prepared sodium thioglycolate solution at pH 10.0 which provided reliable immunofluorescence staining for microtubules. Sperm/egg fusion, mitosis, cytokinesis, and calcium deposition during spicule formation in early embryogenesis were found to be without artificial alterations when compared to cells fixed fresh and processed with conventional methods.

Introduction

With the advent of space exploration, the cytoskeleton has experienced renewed attention since many of the metabolic imbalances experienced

by astronauts can be related to the cytoskeleton. The cellular and developmental effects of space biology^{1,2,3} indicates that several basic cell functions are altered by the gravity-free environment. Among these effects are muscle atrophy and loss of calcium.

Space flight experiments have demonstrated a profound effect of microgravity on a variety of cellular events including the early stages in the signal transduction cascade after the binding of growth factors to cell membrane receptors,⁴ and the cytoskeleton-mediated intracellular signals at the cell membrane and the nucleus. It was proposed that the cytoskeleton is involved in growth hormone secretion,^{5,6} and that secretory processes normally guided by the cytoskeleton are disturbed in a microgravity environment. The organization of the microfilament system was reported to be severely affected in tissue culture cells which were grown under simulated microgravity conditions.^{7,8} The muscle atrophy experienced by astronauts might be ascribed to irregularities in the molecular calcium-regulated cascade leading to proper dynamics of microfilaments, microtubules, and associated proteins in the muscle system. Microgravity has been shown to alter cartilage differentiation,^{9,10} and severe calcium loss is experienced by space travel, and fracture healing was impaired.¹¹ Other effects of exposure to microgravity are seen in the alteration of intermediate filament organization.¹²

Since the cytoskeleton also plays a crucial role during fertilization and development in most species, and since many of these events are regulated by calcium, we chose the sea urchin as a model system to explore cytoskeletal dynamics and calcium deposition under microgravity conditions. The sea urchin system is particularly suitable for those studies since the gametes can be obtained easily, the egg at fertilization performs virtually all the events of any higher cell, and the investigation of the sea urchin egg has paved the investigative path for much of cell and developmental biology. The unfertilized egg is stubbed with a lawn of microvilli, and the

metabolic activation of the unfertilized egg is triggered by an intracellular release of calcium ions, which also initiates the secretion of cortical granules^{13,14} leading to a modification of the cell surface. Sperm incorporation is mediated by an eruption of microvilli formed by the assembly of microfilaments.¹⁵ Once the sperm has entered the egg cytoplasm, microtubules assemble on the base of the spermhead, and when these microtubules contact the egg nucleus, the egg nucleus is drawn to the sperm nucleus to unite into the zygote nucleus.¹⁵ These motions and cytoskeletal alterations serve as a remarkable model in which to explore cytoskeletal behavior in a developmental system.

Cell division, involving the chromosomal movements during mitosis and the physical separation of the cell during cytokinesis also requires the sequential activity of microtubules and microfilaments. Though the precise mechanisms are still unclear, intracellular membranes, competent to sequester and release intracellular calcium, play a significant role during these cytoskeletal reorganizations. The centrosome, one of the main microtubule organizing centers, is believed to play an important role in the establishment of the axis for first division and further differentiation which becomes most obvious during the 16-cell stage when micromeres, mesomeres, and macromeres are formed.

For space travel related to bone structure, one particularly interesting stage during development is the pluteus stage with the formation of spicules which occurs as a result of the deposition of calcium carbonate on an organic matrix as well documented by Benson et al.¹⁶ The utility and speed of this example of the formation of an extracellular skeletal array will serve as a significant model in which to evaluate the effects of gravity on calcium deposition, particularly with quantitative analytical scanning transmission electron microscopy. The loss of calcium by astronauts (up to 460 mg/day) is significant and may help us understand factors contributing to osteoporosis on earth.

Past experimentation has resulted in determining optimal incubation parameters for a space flight experiment.¹⁷ These include incubation temperature, egg concentration, containment material, and tolerable delay periods. The studies presented here address questions related to specimen preparation so that the experiments conducted on Earth can be applied to Space Shuttle Missions.

Materials and Methods

Past studies have shown that *Strongylocentrotus purpuratus* gametes can be stored under specific conditions for 24 hours post spawning and still remain viable.¹⁷ For *Lytechinus pictus*, we have seen that eggs and sperm remain viable even after 48 hours of storage at 4°C.

Gametes from the sea urchin *Lytechinus pictus* were cultured in artificial sea water (ASW)(113.28g NaCl, 3.08g KCl, 21.64g MgCl₂·6H₂O, 28.52g MgSO₄·7H₂O, 4.72g CaCl₂·2H₂O, 0.8g NaHCO₃, 4.0ml penicillin-streptomycin dissolved into 4.00L of distilled water) that had been aerated for at least 2 hours. Sea urchins were injected with 0.55M KCl to induce release of gametes, and sperm were collected "dry" on ice into plastic petri dishes. Eggs were collected into artificial sea water kept at 12°C by a water bath and were allowed to settle. Sea water was removed and replaced with fresh filtered sea water multiple times, and eggs were only used if they were not misshapen. Sperm were also diluted and observed, and were not used if misshapen or immotile. A test fertilization was then performed, and gametes were not used unless at least 80% of the eggs were fertilized.

The controls and experimental chambers were then set up (the culture chambers used are described elsewhere; they are part of the Aquatic Research Facility (ARF) system sponsored by NASA). In each experiment there was a 24 hour period between incorporation of eggs into the chamber and the injection of sperm. Precaution was taken to insure that no air was trapped in the chamber during the initial incorporation of the eggs. In each case, eggs were centrifuged to cause delicate packing and the desired volume of packed eggs was delivered to each chamber with a micropipet. "Dry" sperm was diluted (50 µl in 2.5 ml ASW) and stored in covered glass centrifuge tubes. Gametes were stored at 4°C until insemination. After 24 hours, chambers were removed from the 4°C incubator and the sperm solution was injected via a syringe. This syringe was then cycled 3-5 times to insure adequate mixing of the gametes. Chambers were then placed in a 12°C incubator for desired length of time.

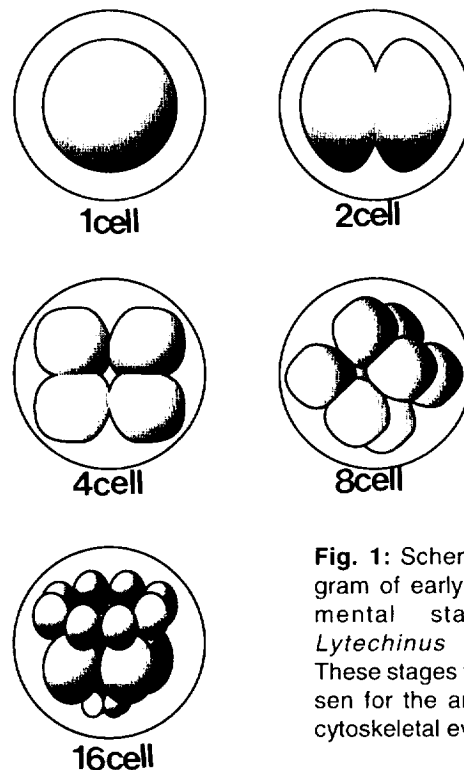
In one set of experiments, 150-200µl of packed eggs were placed into the 35ml chambers. For these experiments, approximately 150µl of the diluted sperm suspension was used for insemination. After fertilization, embryos were cultured in the 12°C incuba-

tor for 30 minutes to 6 hours. In another set of experiments, 10-20 μ l of packed eggs were placed into the 35ml chambers to analyze early stages of development, fertilized with approximately 50 μ l of the sperm suspension, and kept in the 12 $^{\circ}$ C incubator for 24 hours-7 days to achieve development into blastulae and plutei.

Cultures were fixed at 30 minutes, 60 minutes, 6 hours, 24 hours, and 7 days after fertilization, and the fixative was stored for the same length of time as the gametes/embryos. Fixative was injected to achieve a final concentration of 1.1% formaldehyde, 1 μ M taxol, 10mM EGTA, 1mM MgCl₂·6H₂O for 1-cell to 16-cell stages. For blastula and pluteus stages, the final fixative concentration was 1.5% glutaraldehyde, 0.4% formaldehyde. The containers were opened 7-10 days after fixation, and the fertilization coat was removed by incubating the specimens with 0.5% freshly prepared sodium thioglycolate solution, pH 10.0 for 5-10 minutes. The embryos were then washed and passed several times through a pasteur pipette to remove the fertilization coat. After washing in ASW, embryos were placed onto poly-L-lysine coated coverglass and washed extensively in PBS. Samples were fixed in chilled methanol (-20 $^{\circ}$ C) for 5-10 minutes and hydrated in PBS. For long-term fixation experiments, samples were treated with 0.2M ethanolamine (pH 7.5) for 2-8 hours at room temperature to block free reactive aldehyde and washed in PBS. Samples were stained with primary antibody for 48-72 hours at 4 $^{\circ}$ C. The mouse monoclonal antibody E7 was used for characterization of microtubules, and the human antibodies SPJ or 5051 or mouse antibodies Ah6 or 4D2 for the characterization of centrosomes. After three washes in PBS for 20 minutes each, embryos were incubated with secondary antibody for 48-72 hours at 4 $^{\circ}$ C. After repeated washes in PBS, DAPI was added to the PBS buffer at a concentration of 4 μ g/ml, and after 15 minutes, washes in PBS were followed by mounting the samples in mivial (Calbiochem) containing 2.5-5% DABCO.

For electron microscopy of 1-16-cell stages, the embryos that had been fixed for 8-10 days in the previously described fixative cocktail were post-fixed in 1% OsO₄ dissolved in ASW for 90 minutes and then rinsed in ASW. These specimens were then dehydrated in ethanol followed by propylene oxide, and embedded in soft Spurr's resin.¹⁸

Plutei were fixed for subsequent quantitative x-ray microanalysis to determine the calcium deposition



in the spicules.

Fig. 1: Schematic diagram of early developmental stages of *Lytechinus pictus*: These stages were chosen for the analysis of cytoskeletal events.

Results

Lytechinus pictus embryos were fixed at 1-cell (30 minutes and 60 minutes), 16-cell (6 hours), blastula (24 hours), and pluteus (7 days) stages after culture in the described chambers for the analysis of microtubules and centrosomes (fig. 1). Figure 2 shows images of a blastula after 24 hours (top) and pluteus after 7 days (bottom) taken from cultures which had been stored at 4 $^{\circ}$ C for 24 hours in the culture chambers, fertilized, and maintained at 12 $^{\circ}$ C.

Since fertilization will be performed under microgravity conditions, gametes must be stored separately for 24 hours at 4 $^{\circ}$ C before being fertilized in space. Therefore, we tested the fertilizability of the gametes and examined the condition of microtubules and centrosomes after extended storage. Fertilization was normal (data not shown), and microtubules and centrosomes were also normal when compared to the controls (fig. 3).

Since complex manipulations are not possible inside the closed chambers, the hardened fertilization coat was removed after its formation. To accomplish this task, we selected sodium thioglycolate,

which was found to effectively remove the fertilization coat at any stage after fertilization. Microtubules were normal when compared to controls (fig. 4).

We anticipate that the Space Shuttle mission will be approximately two weeks in length, and as a result, embryos must be kept for extended periods of time in the fixative cocktail. In long term fixation (8-10 days), we have found that microtubules are quite normal, and the taxol in the fixative cocktail produced no artificial effects (fig. 5).

One surprising finding was that centrosomal components could not be detected with antibodies to centrosomes (SPJ, 5051, Ah6, 4D2) after removal of the fertilization coat with sodium thioglycolate. To analyze the normalcy of centrosomes in controls and in cells which will be exposed to microgravity conditions, analysis will be performed with transmission electron microscopy and on samples which are thick sectioned without removal of the fertilization coat and stained with the described antibodies for detection of centrosomal material.

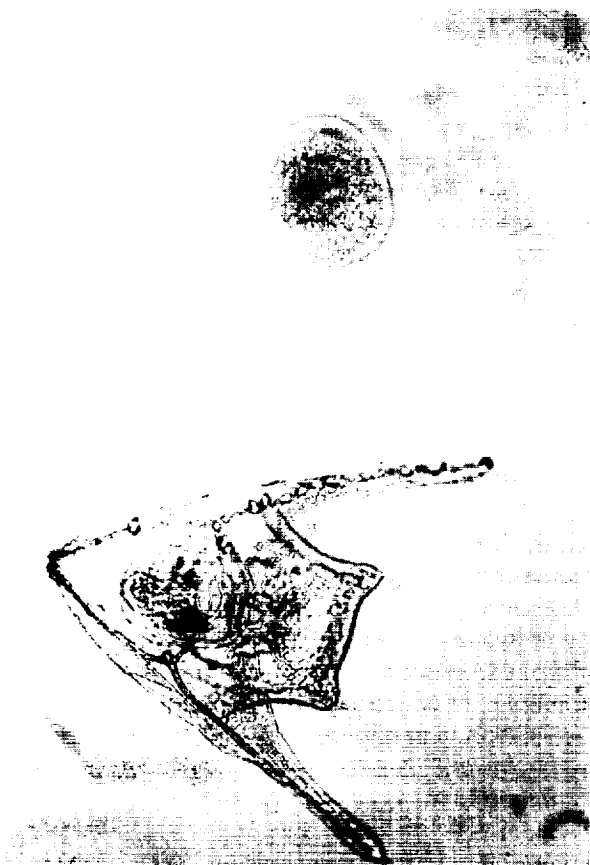


Fig. 2: Light microscopic photographs of *Lytechinus pictus* cultured inside the chambers: blastula (above, 24hr.), and pluteus (7 days).

Discussion

The sea urchin system displays a number of cytoskeletal changes during fertilization and early embryogenesis which are fundamental events in cell and developmental biology. These include signal transduction,^{19,20} cytoskeletal organization involving microfilaments, microtubules,²¹ and centrosomes,²² chromosome and centrosome cycling,²³ the cascade of ionic events and calcium sequestration,^{24,25} secretion processes,^{13,14} and a variety of complex molecular processes with regard to fertilization, cell cycle events, and later development.¹⁷ The sea urchin provides an ideal model system to study the fine regulation of cytoskeletal dynamics and stages of development within a short period of time. The short microvilli of the unfertilized egg surface elongate during fertilization and engulf the entering sperm. Microtubules forming from the centrosome around the entering sperm are responsible for uniting egg and sperm nuclei into the zygote nucleus. Microtubules and microfilaments reorganize for cell division to occur, and unequal divisions at fourth cleavage start the process of developmental differentiation, followed by blastula, gastrula, and pluteus stages. Calcium becomes deposited on an organic matrix during these later stages to form the spicules well visible in the light microscope during late gastrula and pluteus stages. The synchrony during this development is remarkable and facilitates the exploration of cytoskeletal participation which is needed to lead to each one of the described stages.

The ARF flight calls for in-flight fertilization and fixation at prescribed timepoints. The indicated times were chosen to capture specific events in the development of the urchin embryo. For the experiments planned on a shuttle flight mission, these fixed specimens will be examined using light, immunofluorescence, and scanning and transmission electron microscopy. The specimens will be examined and compared to ground controls and to controls conducted on the Space Shuttle centrifuged at 1g. Specimen container development has been divided into four areas: incubation, sperm dilution, fertilization, and fixation. Maintaining a complete separation of eggs and sperm and later embryos and fixative until the proper time is critical to mission objectives. As a result, all engineering efforts up to this time have concentrated on these problems.

The research described in this paper has overcome the problem of employing different fixatives to pre-

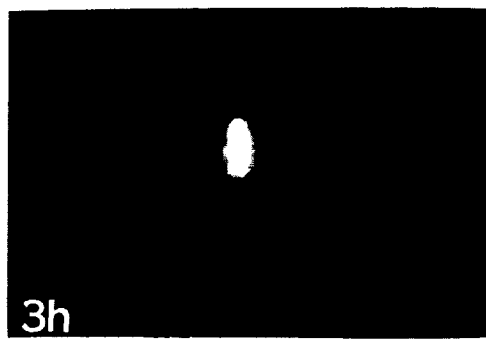
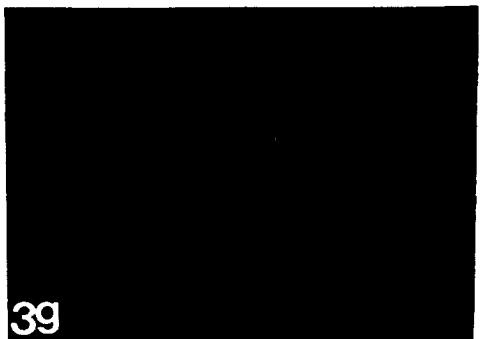
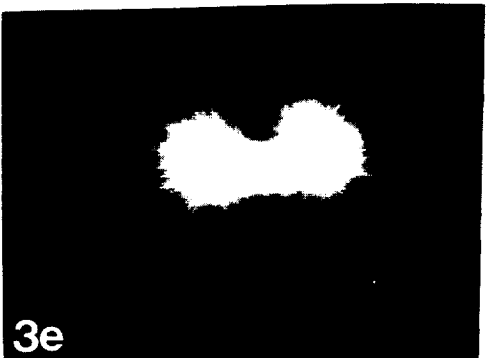
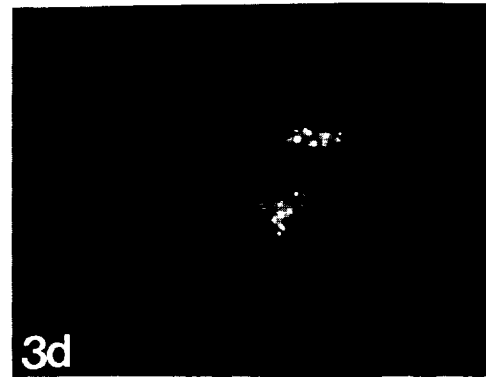
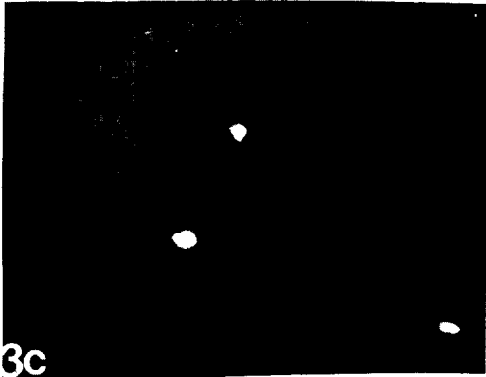
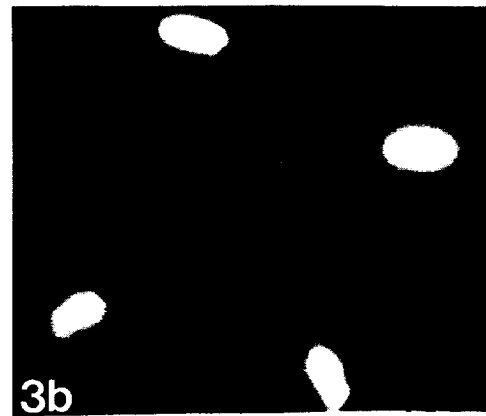
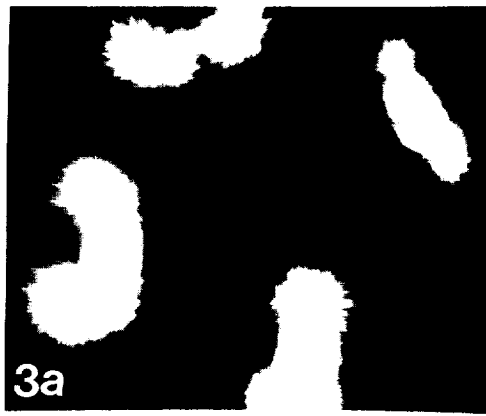


Fig. 3: Immunofluorescence images of cultures showing normal development after gamete storage for 24-48 hours prior to insemination. Here cells are at mitosis showing microtubules, centrosomes, and DNA. Conventional methanol fixation was performed after removing the fertilization coat with 3-amino-1,2,4-triazol (After 24 hours of storage: 3a, 3b show MTs and DNA; 3c, 3d show centrosomes and DNA. After 48 hours of storage: 3e, 3f show MTs and DNA; 3g, 3h show centrosomes and DNA).

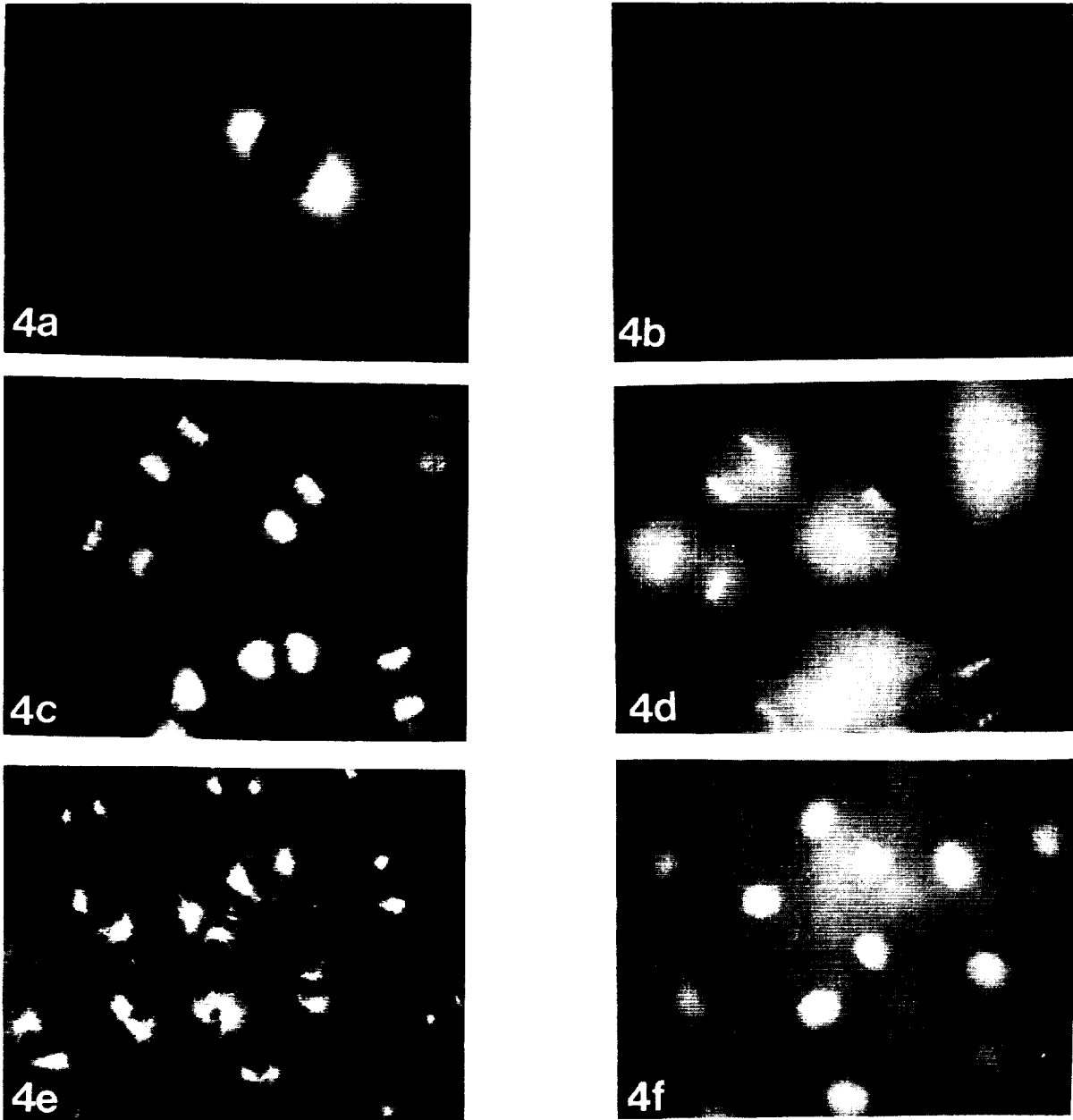


Fig. 4: Immunofluorescence images of cells showing microtubules and DNA 60 minutes (4a, 4b) and 6 hours (4c, 4d, 4e, 4f) after fertilization. Conventional methanol fixation was performed after removing the fertilization coat with sodium thioglycolate.

serve cytoskeletal structures for the analysis of data with immunofluorescence and electron microscopy. To preserve cytoskeletal components in sea urchins, the best fixation protocol for electron microscopy is using a 1% osmium solution in 0.4M sodium acetate, pH 6.1,²⁶ and methanol for the preservation of microtubules and centrosomes employing immunofluorescence microscopy.^{27,28} To employ these fixation protocols for experiments on the Space Shuttle is not feasible, and to overcome this complexity, sev-

eral approaches were necessary to find a compromise solution for preserving cytoskeletal structures for simultaneous analysis with electron and immunofluorescence microscopy. With these methods in hand, we now have reliable methods for the analysis of cytoskeletal structures during fertilization and development in the sea urchin model.

The importance of studies on the cytoskeleton in a developmental system lies in the involvement of the

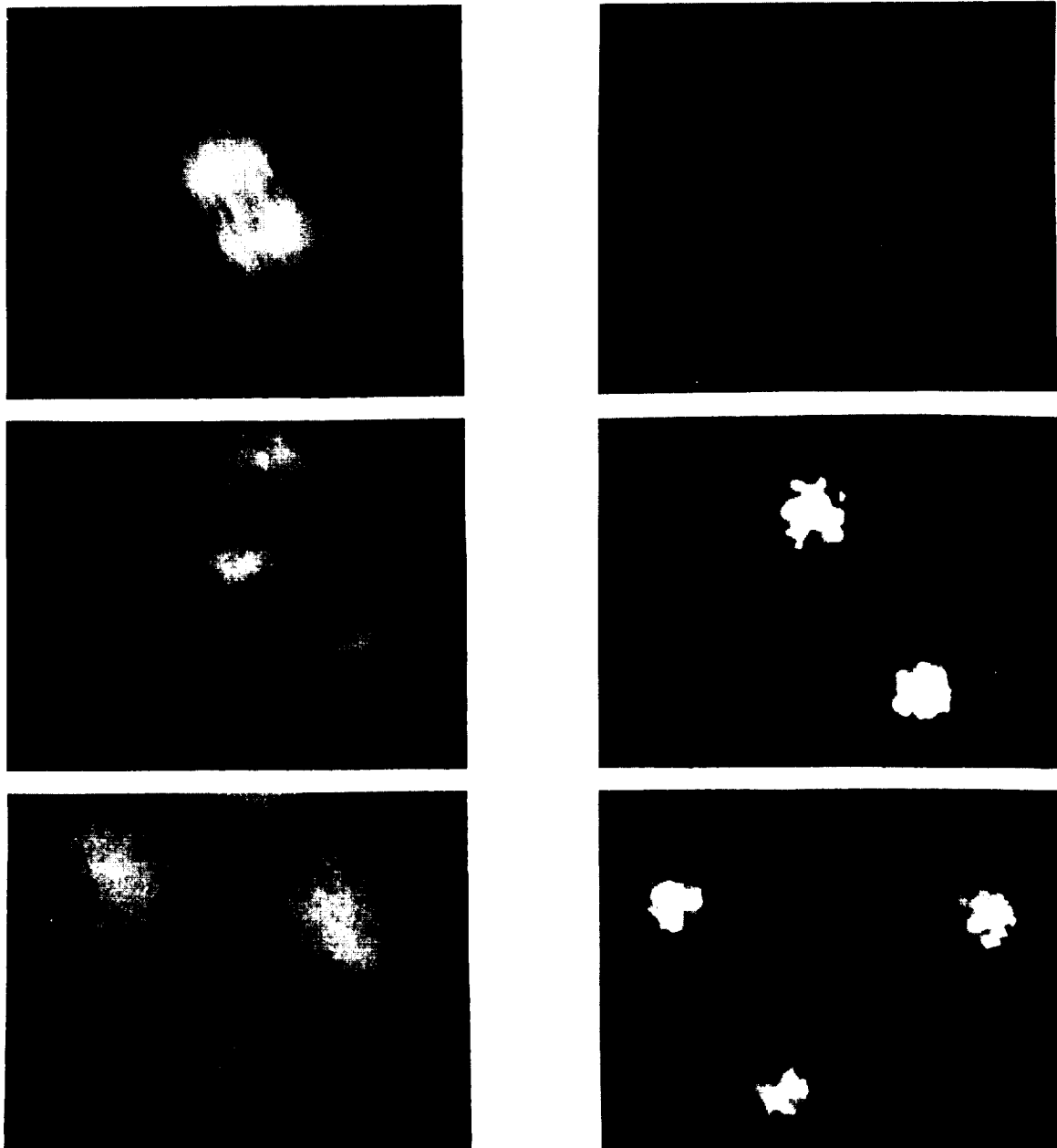


Fig. 5: Immunofluorescence images of cells treated with sodium thioglycolate after long term fixation (8-10 days) in modified fixative medium. Cells at different stages of development showing microtubules (left panels) and DNA (right panels).

cytoskeleton in most processes necessary for proper development. Preliminary studies on a KC-135 flight revealed that microtubules in the sperm tail may be affected by microgravity.²⁹ During the brief window of microgravity on a KC-135 flight, sperm exposed to short-term microgravity exhibited reduced motility, and mixing between eggs and sperm did not occur readily and therefore had to be enforced through mechanical mixing.

Gravity may play a role in early development, but studies performed so far are not conclusive and may vary in different systems. Clinostat and sounding rocket studies suggest that in *Xenopus*³⁰⁻³³ altered gravity environments may alter normal fertilization and embryogenesis. Past spaceflight,³⁴ clinostat,^{35,36} centrifuge, and random motion studies³⁷ using *Xenopus* embryos subjected to centrifugation throughout development showed axis formation dif-

ferent from controls.³⁸

Sounding rocket experiments performed by Ubbels et al. involved fertilizing *Xenopus* embryos in microgravity and fixing some of them after six minutes while returning the rest live to continue development in full gravity on Earth for up to 5 days.³⁹ Subsequent analysis revealed abnormalities in the embryos subjected to microgravity.

Short-term preliminary studies using clinostats to simulate a reduced gravity environment⁴⁰ or sounding rockets to apply temporary microgravity conditions concluded that altered gravity could not detect morphological changes on sea urchin fertilization or very early embryogenesis.^{41,42} However, when cells which were fertilized in microgravity conditions and then cultured further at 1g in the laboratory, slight morphological abnormalities were noted at the pluteus stage.⁴³ These experiments deserve further investigation since the effects might be observed to be more severe if cells are grown under constant microgravity conditions. It also indicates that some events at early stages may have windows in which microgravity affects further development.

Many questions remain open since these studies were not aimed at investigating cytoskeletal dynamics which have been reported to be affected by microgravity in other systems, and it is of interest to investigate a) whether or not there is truly no effect of microgravity in this system, b) if adaptation to new environments takes place, or c) if there are effects if microgravity is experienced for a longer time than previous studies could employ. Since the sea urchin represents a fast developing species, one could speculate that the cytoskeletal dynamics are affected but that adaptation is achieved before any severe effects are noted. The experiments to be conducted on the Space Shuttle are aimed towards exploring possible alterations in cytoskeleton organization. If there is no effect on this cytoskeletal level, then these studies are still of value since one might conclude that although the basic cytoskeletal functions are not affected, secondary or indirect factors and parameters are responsible in more complex systems to account for the cytoskeletal abnormalities reported so far which might be the basis for muscle atrophy and calcium loss in astronauts.

The ARF project will provide valuable insights for scientists studying cell division and development in and out of microgravity. The baseline knowledge may well be essential in learning to maintain ani-

mals in a microgravity environment for extended periods of time. This sort of information is essential with regards to space stations and long space flights. Plant or animal reproduction will be necessary in microgravity for these endeavors to fill nutritional and environmental requirements. Using the sea urchin system to investigate these areas may well provide the valuable insights needed to fulfill future space missions and a phenomenal opportunity to study reproduction itself.

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