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TECHNIQUES FOR STUDYING THE EFFECTS OF MICROGRAVITY ON MODEL PARTICLE/CELL SYSTEMS

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ABSTRACT

To study the direct effects of a low gravity environment on skeletal and cardiac muscle cells, experiments were initiated to determine whether skeletal and/or cardiac muscle cells would grow within the lumen of XM-80 hollow fibers (i.d. = 0.5 mm). Cells were prepared from skeletal or cardiac muscle tissue of 12 day embryos and were cultured for up to 7 days in the hollow fiber environment. Light microscopy revealed that cells proliferated to confluency over this period of time and fusion was apparent in the skeletal muscle cells. Once it had been verified that cells would grow to confluency, additional XM-80 fibers containing cells were placed in a Clinostat in the horizontal position at 100 rpm. Fibers were stretched by a built-in spring mechanism to hold the fiber tightly at the center of rotation. Under these conditions, the gravity vector approaches zero and the cells are in an environment that simulates microgravity. Examination of skeletal muscle cells by electron microscopy revealed that myoblast fusion and myofibril accumulation were extensive. Although data obtained thus far are preliminary, they suggest that myofibril organization in chicken skeletal muscle cultures is somewhat more poorly defined in Clinostat-rotated cultures than in controls that were not subjected to Clinostat conditions.

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BACKGROUND

The primary reasons for our initial interest in this research emanate from our experience in studying protein synthesis, degradation and gene expression in skeletal muscle cells, and from the extensive physical problems resulting from atrophy and weakness of skeletal and cardiac muscles following prolonged exposure to a low gravity environment (c.f., Morey-Holton and Wronski, 1981; Oganov et al., 1982; Grigor´yeva and Kozlovskaya, 1983; Leonard et al., 1983). Clearly, dramatic alterations in the balance between the rate of protein synthesis and the rate of protein degradation must accompany major changes in the quantity of muscle tissue, and it also seems exceedingly unlikely that loss of up to 25% of skeletal muscle mass could take place in the absence of switches in myofibrillar protein gene expression. The extent of skeletal muscle loss in humans and experimental animals after space flight is rather dramatic. To cite two specific examples, crew members of the first two Skylab missions maintained a negative nitrogen balance of approximately 4.5 g/day at the same time that total body potassium was also decreasing (Whedon et al., 1977). Much of this increased protein catabolism was due specifically to muscle protein degradation as evidenced by elevated rates of 3-methylhistidine excretion (Leach et al., 1979). Also, a pronounced decrease in mass and in myofibrillar cross sectional area of the soleus muscle in rats after exposure to a low gravity environment for 7 days has been reported (Goldspink et al., 1980; Riley et al., 1987).

An interesting and intriguing explanation for the effect of prolonged space flight on muscle atrophy is that a secretory defect for growth hormone (GH) may be occurring in pituitary cells (Grindeland et al., 1987a; Motter et al., 1987). Specifically, anterior pituitary cells isolated from rats flown for 7 days on the SL-3 mission secreted approximately half as much of a biologically active form of GH into culture media as ground-based controls. This observation has been qualitatively confirmed and extended by several independent approaches, including the finding that the serum concentration of GH is reduced by 50% in rats exposed to simulations of microgravity by hindlimb suspension (Motter et al., 1987).

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Additionally, Grindeland et al. (1987b) have concluded that muscles of rats exposed to microgravity are significantly more resistant to exogenous and circulating GH than the skeletal muscles of control rats, since administration of GH did not alleviate muscle atrophy in animals in which it was known that GH secretion rate and serum levels of GH were also decreased by 50%. These results imply that the defect in GH utilization may extend to skeletal muscles as well.

Thus, it seems clear that exogenous factors are responsible for at least a portion of muscle atrophy; however, it also seems possible that microgravity has direct intrinsic effects on the cytoskeletal and myofibrillar contractile systems. Examples of direct effects of microgravity on cells are rather limited, but available circumstantial data are consistent with the explanation that the cytoskeletal system is involved. For example, secretory processes in general, and by definition the secretion of hormones from the pituitary, are microfilament-dependent, and it is plausible that the reduced secretion of GH described above results from a direct effect on the cytoskeletal system. Moreover, if the defect in GH utilization extends to skeletal muscle cells, and if the Insulin-like Growth Factors (IGF's, which are regulated by GH and act directly on skeletal muscle) and their receptors are internalized by cytoskeletal-dependent processes into the cytoplasm of muscle cells, then this process may also be directly affected by microgravity. Since some of the contractile proteins in the highly organized myofibrillar protein arrays in sarcomeres are nothing more than different isoforms of some of the cytoskeletal contractile proteins, an effect on expression of one class of these proteins could logically be expected to have an effect on expression of the other. Further substantiation of this possibility results from the fact that the myosin heavy chain genes are members of a rather large multigene family that may have up to twenty members in some species such as chickens, and which is known to exhibit a significant level of plasticity in its ability to have different isoforms expressed under different tissue, developmental and environmental conditions. Most crucial, however, is the fact that neither the effect of actual microgravity nor the effect of simulated microgravity on the organization, synthesis/degradation or gene expression of the contractile proteins has ever been evaluated under critically controlled conditions. One of the only possible ways to evaluate this possibility in the absence of prolonged space flights is with cultured muscle cells grown within the fibers of a rotating Clinostat. As discussed below, this instrument has the effect of mimicing a low gravity environment, and therefore allows some of the above processes to be evaluated.

OBJECTIVES

The general objective of this research was to assess the effects of exposure to simulated microgravity using a rotating Clinostat on morphological aspects of the contractile system in chicken skeletal muscle cells. Specifically, cell morphology, fusion, and patterns of contractile filament organization in skeletal muscle cell cultures grown in hollow fibers of a rotating Clinostat were evaluated. Primary techniques for this work were light microscopy and electron microscopy. Several different cell types were examined in preliminary experiments to determine the best cell type for this and subsequent projects. These cell types included chicken muscle cells, chicken cardiac muscle cells, and fetal bovine skeletal muscle cells.

METHODS AND PROCEDURES

1. Chick skeletal muscle cell cultures

Thigh muscle from 12 day broiler chick embryos was removed and disaggregated into individual cells by vortexing the muscle in growth medium on a vortex mixer at maximum speed for 20-30 seconds (Young et al., 1981). The suspension was then filtered through nylon mesh to remove connective tissue and bone, and the cells were recovered by centrifugation. Following resuspension in an appropriate amount of growth medium (Eagle's Minimum Essential Medium containing 5% chick embryo extract, 10% horse serum, 50 units/ml penicillin, 50 ug/ml streptomycin, 2.5 ug/ml fungizone) to give a concentration of 1.5×10^{10} cells/ml, the cell suspension was injected into a 70 mm long piece of 0.5 mm (inner diameter) XM-80 hollow fiber using a 1cc syringe and a 26 3/8 gauge needle. Both ends of the fiber were sealed with hot wax, and the fiber was loaded into a glass tube containing 5 ml of complete media. The fiber was held taut by a spring so that it would always be at the exact center of rotation of the Clinostat, and the glass tube was then sealed and the entire assembly loaded into the Clinostat.

Muscle cell cultures prepared as described above proliferate, fuse and begin to synthesize myofibrillar protein within 2-3 days in culture, and under most experimental conditions we have employed so far, they attain a maximum and constant quantity of myofibrillar proteins by approximately 7 days. Because the synthesis rate and the degradation rate must be exactly equal to each other in order to maintain a constant quantity of protein at steady-state, and because perturbations in either synthesis or degradation rates will result in a net change in the quantity and/or organization pattern of myofibrillar proteins, these cells will provide an excellent model for studying the dynamics of muscle protein accumulation and loss.

2. Microscopic Evaluation of Cells Grown in the Clinostat

A. Light Microscopy

Fibers were removed from Clinostat cultures and fixed in a 5% neutral buffered formalin solution for a minimum of 24 hours. The fibers were dehydrated through a graded ethanol series of 70%, 95%

and absolute ethanol, followed by a final treatment in xylene. The fibers were soaked in hot paraffin for 4-8 hours, embedded in the paraffin in plastic molds and sectioned with a microtome. The thin sections were rehydrated through a reverse graded alcohol series (100%, 95%, 70%) and deionized water, and subsequently stained with a hematoxylin/eosin or PAS myofibril stain using standard staining procedures. Slides were viewed and photomicrographs made at 45X and 100X. These light micrographs were useful for routine monitoring of the rate of cell growth and differentiation under the different experimental treatments.

B. Transmission Electron Microscopy

Fibers containing the cell cultures from the Clinostat were fixed in 4% buffered glutaraldehyde, followed by 1% osmium tetroxide fixation. The fibers were partially dehydrated in ethanol (25% and 50%) and stained with 1% uranyl acetate. After complete dehydration through a graded ethanol series (85%, 95% and absolute) the fibers were embedded in Spurr embedding medium and thin sectioned using a microtome. Sections were viewed and photographed with a Philips Model 201C transmission electron microscope. These electron micrographs were analyzed to determine if simulated microgravity had direct ultrastructural effects on the sarcomeric and/or cytoskeletal system in muscle cells.

RESULTS

The fast rotating Clinostat used for these experiments was designed at MSFC and generously loaned to us by Dr. Robert Snyder. Briefly, the Clinostat is made up of a culture chamber which rotates about a horizontal axis, and an XM-80 hollow fiber containing cells is mounted in the center of rotation. Depending on the density difference between the particles and the liquid in which they are suspended, the particles may settle within circular trails. At high enough speed of rotation, cells become motionless with respect to the gravity vector and microgravity can be simulated (Brieglieb, 1983). The simulation approaches 100% for particles in suspension where centrifugal forces and Brownian motion offset each other. In the hollow fibers containing muscle cells, however, the diameter of the fiber is small enough that the centrifugal force is only approximately 0.006 x g at 100 rpm of rotation. Operation of the Clinostat in the horizontal position simulates microgravity, and operation in the vertical position serves as a control since the gravity vector is always constant in the vertical position. Additional control experiments consisting of cells in hollow fibers lying horizontally in a sealed tube are also conducted to ensure that a horizontal, non-rotating control is always available. Photographs of the Clinostat are shown in the horizontal (Figure 1) and vertical positions (Figure 2). The cells are placed inside the small hollow fiber inside the rotating chamber in the foreground in Figure 1, the ends are sealed with wax, and the fiber is held taut by the spring loaded mount. This ensures that the fiber is always held at precisely the center of rotation. A tachometer for monitoring the speed of rotation is outside the photograph.

Preliminary investigations of chicken skeletal muscle cells in the Clinostat have been carried out over the past few months, and while we have made major progress in developing the appropriate conditions, it should be made very clear that we are in the early stages of analyzing muscle cells and that all conclusions are based on a limited amount of information. Additionally, we attempted to grow several different cell types to determine the best ones for future experiments, and to see if one cell type grew better than the others. Chicken skeletal muscle cells and chick cardiac muscle cells grew best, whereas fetal bovine skeletal muscle cells grew very slowly.

Light micrographs of skeletal muscle cells stained with hematoxylin and eosin after 7 days in culture are shown in Figures 3

and 4. Figure 3 is a longitudinal section (i.e., parallel to the long axis of the hollow fiber) through the monolayer of chicken skeletal muscle cells, and Figure 4 is a cross section through the monolayer of cardiac muscle cells. Figure 5 shows that bovine skeletal muscle cells did not grow as well. In these light micrographs, the cells are confluent, and although fusion can be observed occasionally from these sectioning planes, the extent of fusion and myofibril formation is far more apparent in electron micrographs (Figure 6 and Figure 7). These micrographs in Figure 6 and Figure 7 were taken from a representative control multinucleated myotube after 6 days in a hollow fiber in the absence of rotation. Cross striations are readily apparent in most sections, and the myofibrils appear to be aligned appropriately. Moreover, portions of myotube nuclei are visible.

The micrographs shown in Figure 8 and Figure 9 were taken from myotubes that had been rotating horizontally in the Clinostat at 100 rpm for 6 days. Although the following conclusions are very tentative and are based on a limited amount of information, at least two differences between the Clinostat cells and the controls have been observed so far. First, it was more difficult to find myotubes in the Clinostat cultures than in the controls (i.e., more sections from different blocks had to be examined to find myotubes, even though the same number of muscle cells was originally placed into both sets of fibers). Second, while most of the sections from the Clinostat myotubes do have some cross striations (c.f., Figure 8), we observed that the Clinostat myofibrils seem to be more poorly organized. The sections contain abundant amounts of filamentous-appearing structures that resemble myofibrillar material; however, the extent of organization and alignment seems not to be as precise as in the controls. One possible explanation that we have not yet had time to evaluate is that a higher percentage of the sections from the Clinostat cultures are at oblique angles; however, it also easy to find this poorly organized material in sections that also contain readily identifiable sections of intact myofibrils. Thus, we can not rule out that the possibility that Clinostat cultures contain as many myofibrils, but that they are more randomly oriented in the myotubes. These are questions that can only be answered by a detailed continuation of the present research and by expanding it to include the additional parameters we have proposed to measure. Again, while these results are interesting, their preliminary nature cannot be over-emphasized.

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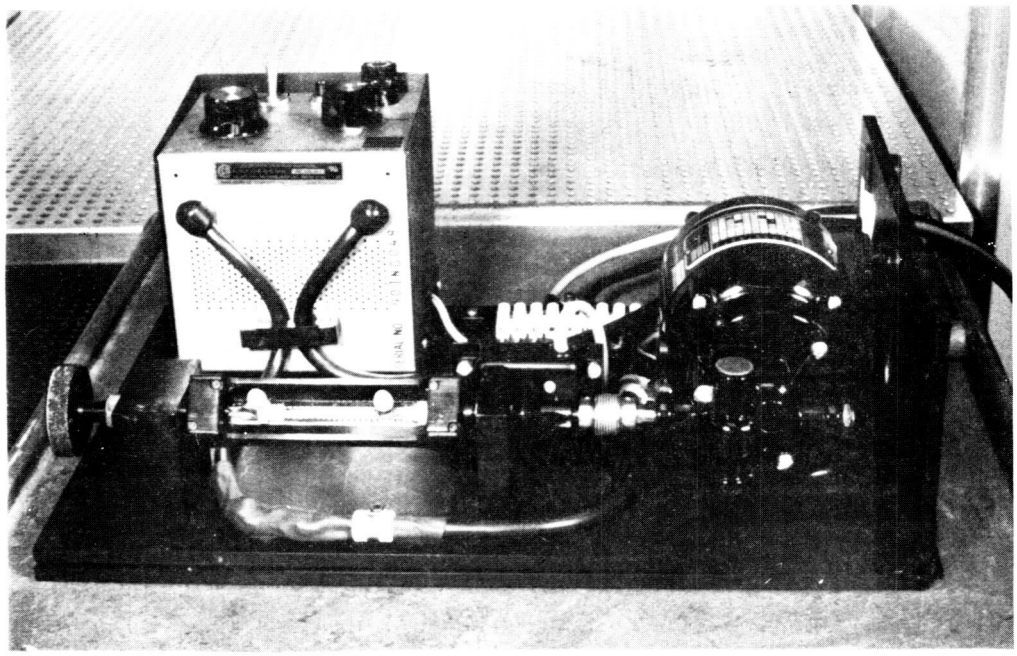

Figure 1. Clinostat: Horizontal Position

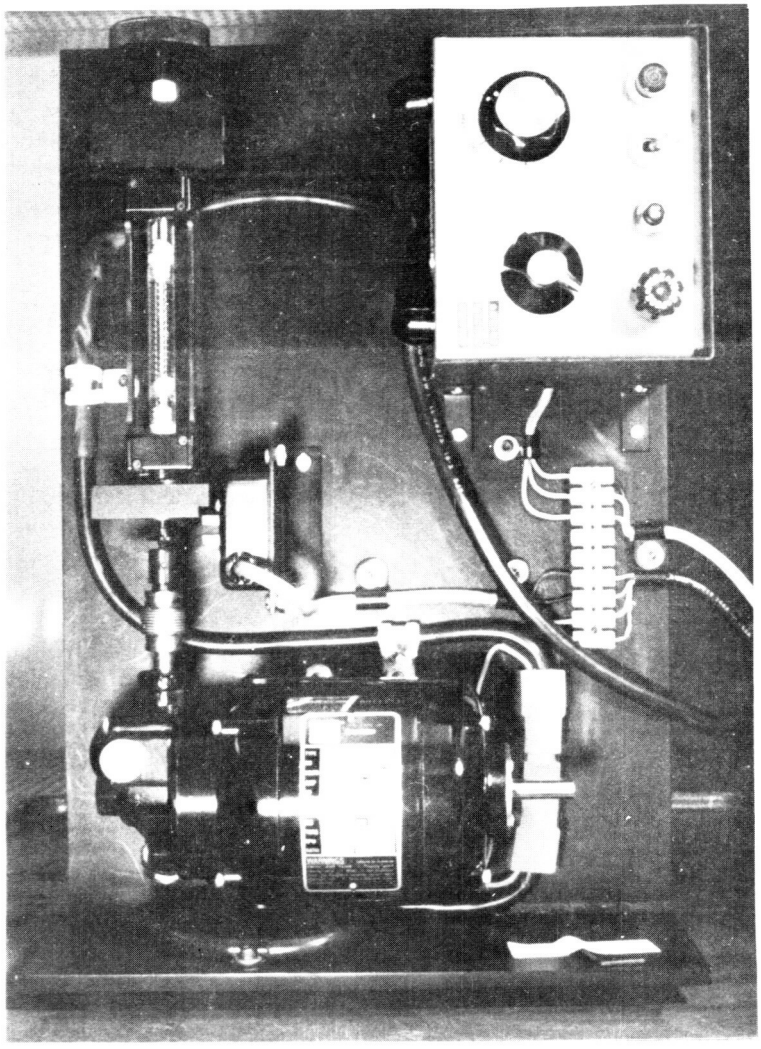

Figure 2. Clinostat: Vertical Position

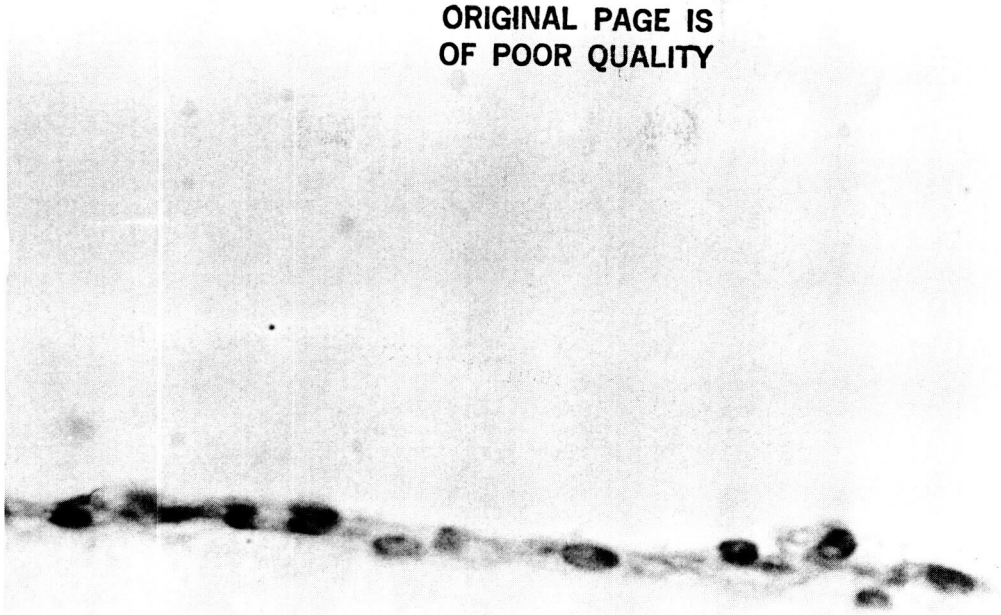

Figure 3. Chick Skeletal Muscle Cells

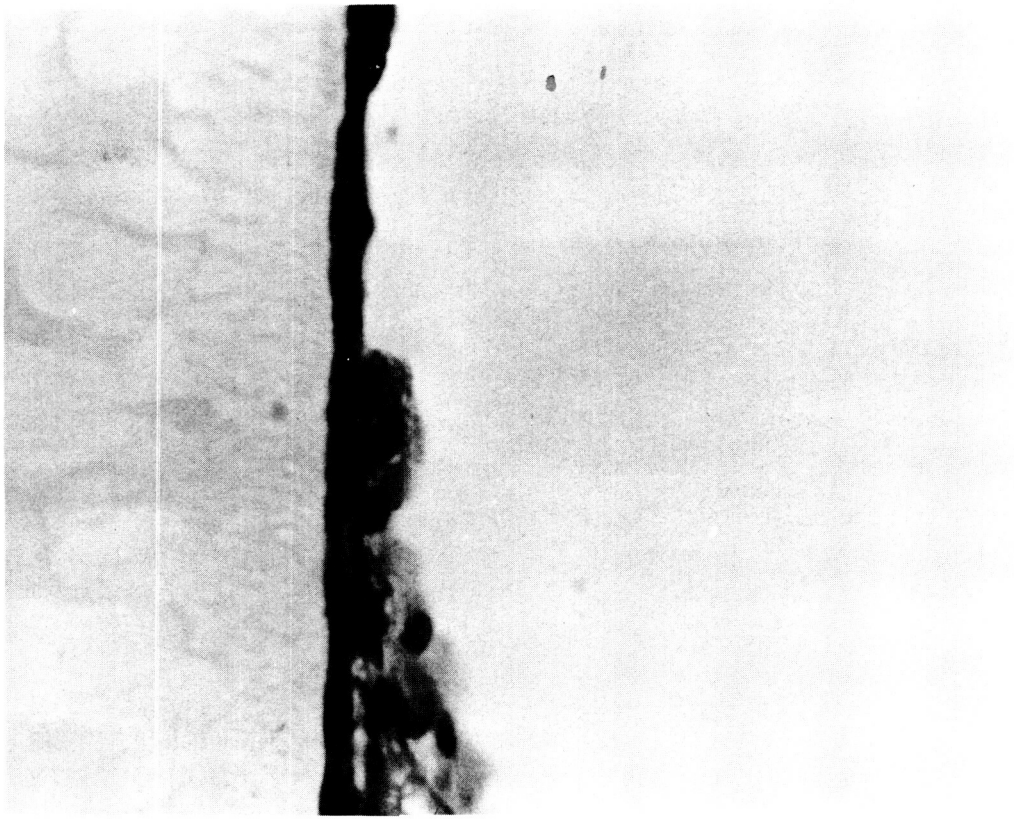

Figure 4. Chick Cardiac Muscle Cells

Figure 5. Bovine Skeltal Muscle Cells

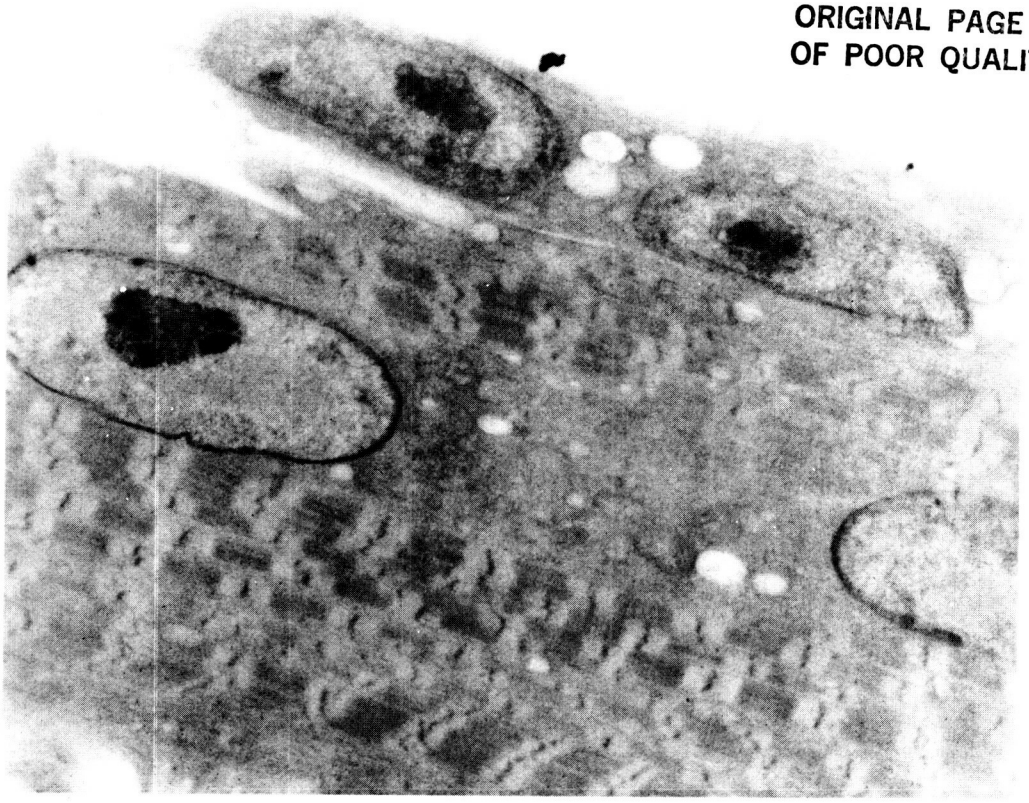

Figure 6. Electron Micrograph of Control Chick Skeletal Muscle

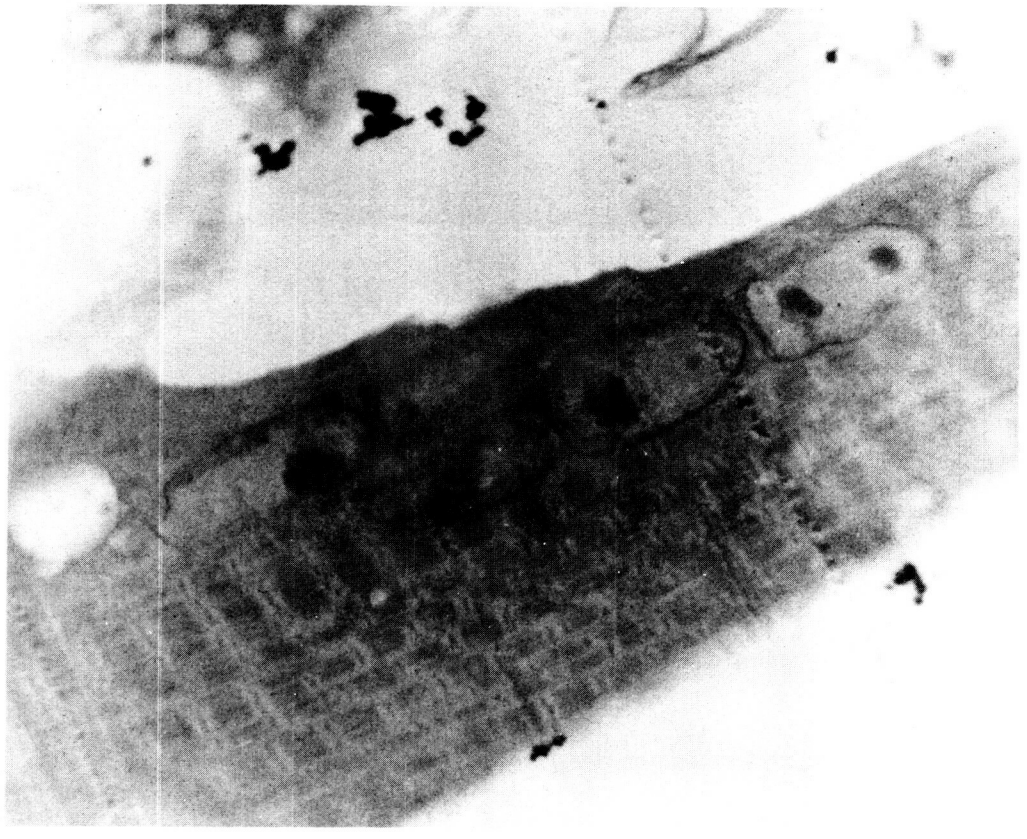

Figure 7. Electron Micrograph of Control Chick Skeletal Muscle

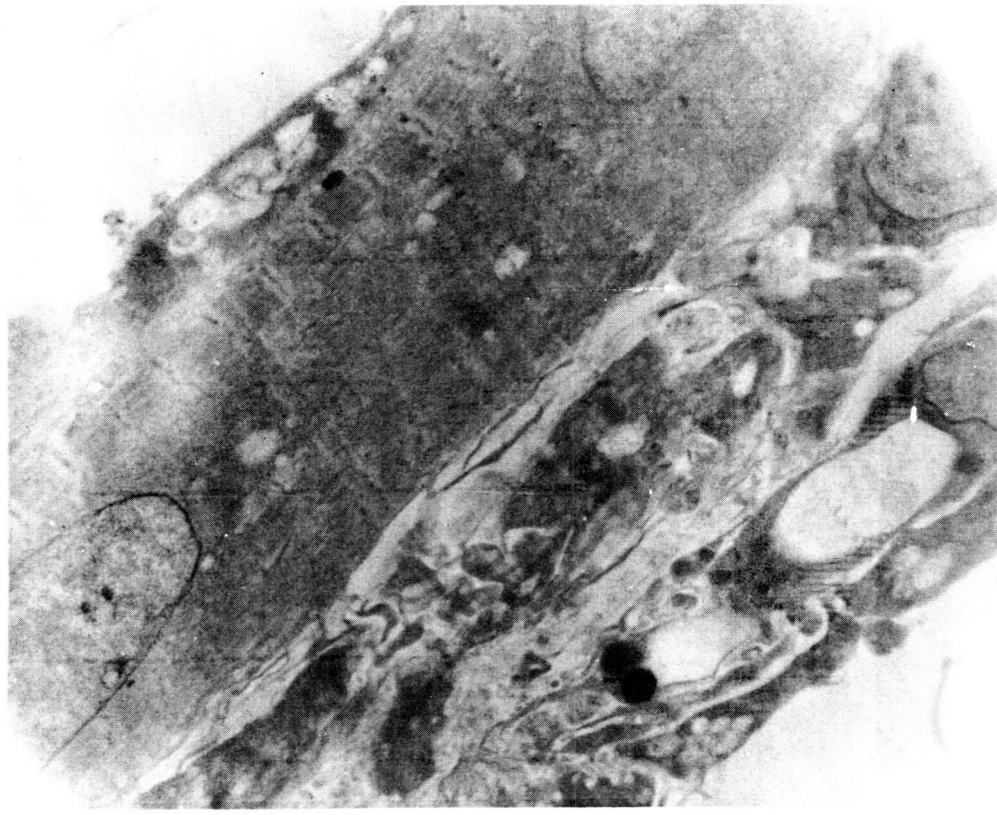

Figure 8. Electron Micrograph of Clinostat Treated Chick Skeletal Muscle Cells.

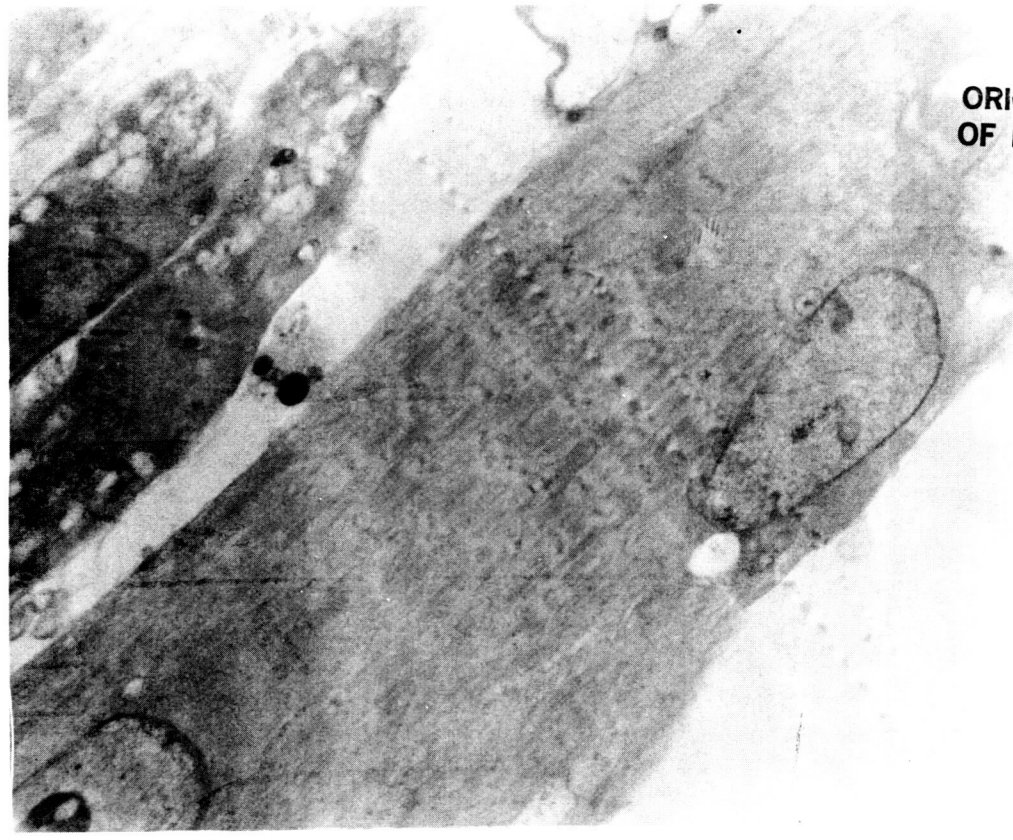

Figure 9. Electron Micrograph of Clinostat Treated Chick Skeletal Muscle Cells.