Neonatal Rat Heart Cells Cultured in Simulated Microgravity

Robert E. Akins[1], Nancy A. Schroedl[1], Steve R. Gonda[2], and Charles R. Hartzell[1]

[1] Department of Medical Cell Biology, Nemours Research Programs, Alfred I. duPont Institute, Wilmington, DE 19899
and
[2] Medical Sciences Division, Biotechnology Program, Johnson Space Center, NASA, Houston, TX 77068

Corresponding Author:

Robert E. Akins, Jr., Ph.D.
Department of Medical Cell Biology
Nemours Research Programs
P.O. Box 269
Wilmington, DE 19899

Phone: (302) 651-6815
FAX: (302) 651-6897
Internet: akins@helios.medsci.udel.edu

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SUMMARY

In vitro characteristics of cardiac cells cultured in simulated microgravity are reported. Tissue culture methods performed at unit gravity constrain cells to propagate, differentiate, and interact in a two dimensional (2D) plane. Neonatal rat cardiac cells in 2D culture organize predominantly as bundles of cardiomyocytes with the intervening areas filled by non-myocyte cell types. Such cardiac cell cultures respond predictably to the addition of exogenous compounds, and in many ways they represent an excellent in vitro model system. The gravity-induced 2D organization of the cells, however, does not accurately reflect the distribution of cells in the intact tissue. We have begun characterizations of a three-dimensional (3D) culturing system designed to mimic microgravity. The NASA designed High-Aspect-Ratio-Vessel (HARV) bioreactors provide a low shear environment which allows cells to be cultured in static suspension. HARV-3D cultures were prepared on microcarrier beads and compared to control-2D cultures using a combination of microscopic and biochemical techniques. Both systems were uniformly inoculated and medium exchanged at standard intervals. Cells in control cultures adhered to the polystyrene surface of the tissue culture dishes and exhibited typical 2D organization. Cells in cultured in HARVs adhered to microcarrier beads, the beads aggregated into defined clusters containing 8 to 15 beads per cluster, and the clusters exhibited distinct 3D layers: myocytes and fibroblasts appeared attached to the surfaces of beads and were overlaid by an outer cell type. In addition, cultures prepared in HARVs using alternative support matrices also displayed morphological formations not seen in control cultures. Generally, the cells prepared in HARV and control cultures were similar; however, the dramatic alterations in 3D organization recommend the HARV as an ideal vessel for the generation of tissue-like organizations of cardiac cells in simulated microgravity.

Key words: heart cells, microgravity, culture method, HARV bioreactor, tissue generation

INTRODUCTION

BACKGROUND

It is not known at what level gravity interacts with cellular processes; however, several mechanisms have been postulated to account for gravitational effects (14, 21). These mechanisms include calcium signaling, mechanical force transmission along fibrous cytoskeletal networks, cell-cell communication, and disruption of ligand-receptor binding. Within cells, effects such as subtle changes in micro-convection currents, or in the distribution of tension along the cytoskeletal elements which resist organelle sedimentation may be involved in gravity responses. Gravity-imposed restrictions at the cellular level may specify particular interactions among groups of cells, or between cells and the surrounding substrate, while inhibiting others. In addition, gravity may affect extra-cellular factors such as fluid shear at the cell surface, changes in fluid mixing, or mass transfer of solutes. At each level, within cells, cellular, and extra-cellular, gravitational forces may determine cellular morphology and/or responsiveness.

Experiments using cells cultured in suspension have shown significant changes in prokaryotic and eukaryotic cell functions in spaceflight-induced microgravity (14, 4). These effects include increased growth rates in Bacilli; increased antibiotic resistance in E. coli; increased substrate attachment in cultured human kidney cells; increased interferon secretion and decreased blastogenic response in cultured lymphocytes; increased interleukin-1 and tumor necrosis factor-α secretion in a macrophage cell line after lipopolysaccharide stimulation, to mention a few. Even in the absence of direct evidence for any specific mechanisms, it has become increasingly clear that spaceflight-induced microgravity has profound effects on cells in vitro.

Spaceflight-induced microgravity has also been shown to significantly alter cardiac morphology and function, and several studies concerning the reduction of myocardial contractility have been performed. Light and electron microscopic examinations of cardiac tissue dissected from rats flown on orbital space missions have indicated that a significant degree of cardiac atrophy occurred after as little as 14 days (7). Increased lipid and glycogen storage, decreased microtubule

number, and alterations in mitochondrial density and mitochondria-to-myofibril ratios have also been reported in heart cells in flights as short as 7 and 12.5 days (15, 16). In addition, the ratio of β-myosin-heavy-chain RNA to ribosomal RNA in rat heart decreased during 14 days of spaceflight, indicating that the transcriptional regulation of sarcomere components was affected by the absence of gravity (22). The mechanisms responsible for the in vivo changes in cardiac function in microgravity are not known, but they certainly involve multiple aspects including alterations in peripheral blood flow, blood pressure, and hormonal status as well as any direct effects of weightlessness on the constituent cells.

Cardiac cells represent an excellent system to study the interaction of gravity with cellular processes. The exquisite three dimensional organization, coordinated contractile activity, and regulation of the heart are associated with many of the aspects related to gravitational responsiveness: calcium signaling, force transmission along fibrous cytoskeletal networks, cell-cell communication, etc. Furthermore, methods designed to generate 3D structures from heart cells in vitro must take into account the high degree of shear sensitivity associated with cardiac cells. Heart cell cultures in 3D, therefore, would actually benefit from exposure to microgravity (real or simulated) as a result of the low shear environment. Using NASA-designed High-Aspect-Ratio-Vessel (HARV) rotating-wall bioreactors, we have begun to examine cultured heart cells in our ground-based laboratory under conditions which simulate microgravity and induce three-dimensional tissue formation.

NEONATAL RAT HEART CELLS IN CULTURE

Approximately 70% of the cells isolated by enzymatic digestion of neonatal rat hearts are cardiomyocytes. Cardiomyocytes isolated from 2-3 day-old rats are fully differentiated and non-proliferative (see 2); therefore, they represent a valuable model system for the study of physiological adaptations in the absence of cell proliferative activity. Additionally, cardiac cell cultures sustain spontaneous, rhythmic, and synchronous contractile activity from the first day after seeding onto fibronectin coated culture dishes. These cells adapt to changes in their physical

or mechanical environment through alterations in gene expression or through structural reorganization. (18, 23). In our methodology, cardiac cell cultures are typically carried out in the absence of serum, and the effects of pharmacological agents can be easily determined in vitro by the direct addition of compounds to the medium. Beating cardiac cell cultures respond in a predictable manner to inotropic and chronotropic agents, such as isoproterenol and carbachol (1), and changes in contractile activity can be easily induced and observed in vitro. Cultured neonatal rat cardiac cells, then, represent an excellent system to address physiological adaptations to diverse influences including microgravity.

One of the principle interests of our research group is to evaluate the specific effects of exposure to a low gravity or simulated-microgravity environment on excitable cell types. This work exists as part of a long term goal directed toward the potential use of microgravity as an aid to in vitro formation of physiologically important 3D structures. It is anticipated that the ability to culture cardiac cells under conditions of microgravity will allow cell:cell and/or cell:substrate interactions that are not possible when using traditional 2D culture methods or high shear 3D culture methods. Indeed, it is hoped that this research will lead to an improved understanding of the requirements for 3D tissue formation and that researchers will eventually be able to use in vitro culture methods for the generation of functional tissue replacements.

THREE DIMENSIONAL CELL CULTURE IN THE NASA HARV BIOREACTOR

Advantages of Culturing in the HARV

The NASA designed HARV bioreactor (SYNTHECON, INC., Houston, TX) is a rotating wall culture vessel which allows cells to be maintained in perpetual suspension within a low shear environment with such that the net gravity vector is zero (i.e., in simulated microgravity). The HARV consists of a base, a controller, and the culture vessel. The base holds a small motor linked by a belt drive mechanism to the shaft of the culture vessel mounting. Since the HARV culture vessel is a closed system, the base also contains a small air pump which provides a sterile filtered air and CO_2 mixture. The controller supplies the variable voltages to drive the motor and air pump.

The culture vessel is a machined polycarbonate (Lexan) culture dish operated in the upright position. A vessel is comprised of two portions: the first screws onto the base and contains several small pores that are covered by a silicone membrane (which serves as a gas exchange interface); the second has a circular recessed area and two luer-locking ports through which cells and media can be accessed. The two halves are bolted together and sealed by means of a compressible O-ring. Once assembled and filled, the HARV can be mounted onto the motorized base and rotated.

The key to the utility of the HARV system is that the culture vessel and the fluid contained therein approximate a solid body during rotation (10). When cells, microcarrier beads, or other materials are added to the HARV, they sink to the bottom of the vessel at a constant rate (except for a nearly instantaneous initial acceleration due to gravity) related to the gravitational field, the difference in density between the particles and the medium, the size of the particles, medium viscosity, and other factors. Rotation of the HARV results in a path determined by the combination of sedimentation and movement along with the medium. Increasing the rotation rate can prevent the particles from reaching the bottom of the vessel: they will appear to describe an elliptical path through the medium relative to the observer. Further increases in the rotation rate results in the dimunition of the elliptical path until the particles become essentially motionless relative to the medium. At this point the vessel, medium, and contents approximate a single solid body: Particles (e.g. cells or microcarrier beads) maintain three dimensional orientations relative to each other and the surrounding medium in an apparently low shear environment (<0.52 dyne/cm^2; ref. 10). These conditions are similar to those expected to prevail in true microgravity.

In addition, the rotation of the HARV, like a clinostat, normalizes the gravity vector to zero. In practice the 10 ml vessels used in our studies are rotated at approximately 30 RPM, and only gravitational responses which occur over a 2 to 3 second time frame may be expected to be affected. However, structures would be stressed equally in each direction during a single rotation. In theory, then, the HARV system offers a reasonable approximation of a microgravitational environment in a ground-based laboratory.

Criteria for Evaluating Cells in 3-D Culture

The information presented here has been acquired during the course of detailed studies of excitable cells in a simulated microgravity environment. To evaluate the potential changes induced by the culture conditions of the HARV, neonatal rat heart cells are currently being characterized by a combination of biochemical and cell biological techniques. Since, at a preliminary level, the co-ordinated activity of the heart depends on specific, sequential events (including cytosolic Ca^{2+} elevation, conformational changes and interactions among the contractile proteins, energy availability, and the alignment of myocytes and organization of cellular structures), we are in the process of examining: 1) Contractile Function: cardiac cell beat frequency and strength; 2) Ca^{2+} Handling: Ca^{2+} distribution, mobilization, and sequestration; 3) Protein Composition: the expression of essential proteins; 4) Energy Metabolism: shifts in metabolism or ATP availability; 5) Cellular Morphology: the spatial distribution of cardiac cell types; 6) Ultrastructural Morphology: the organization of cellular components and organelles.

Culture of Neonatal Rat Cardiac Cells.

Cardiac cells were prepared from two or three day neonatal rats as previously described (6, 20). Briefly, cells were liberated using a mixture of purified enzymes, inoculated onto fibronectin-coated tissue culture plastic, and maintained in a defined serum free heart medium (SFHM), based on that previously (6, 12) with high glucose medium substituted for the standard low glucose medium. The specific ratio of cells to SFHM to fibronectin coated surface area was maintained in HARV and control cultures (1×10^6 cells per milliliter SFHM per 4.8 cm^2 of surface area). HARV cultures were typically carried out using Biosilon microcarrier beads (Nunc, Inc., Naperville, IL). Biosilon beads are comprised of the same plastic (Nunc Δ-Si polystyrene) as the two dimensional culture control dishes. A complete discussion of microcarrier bead culture systems is included in the accompanying paper (13).

Biosilon beads were chosen in an effort to normalize some of the cell attachment conditions between HARV and control cultures, and they were used in a majority of the cultures described herein. In some instances, other support matrices were utilized for 3-D cultures. These included

thin collagen fibers (Organogenesis, Inc., Canton, MA), absorbable and non-absorbable suture materials (Davis and Geck Inc., Manati, P.R.), and gelatin Cultispheres (Hyclone Laboratories, Inc., Logan, Utah). Recently, cultures involving 3D conformations of polyglycolic acid fibers have also been succesfully attempted (5). Cells, fibronectin-coated-support-matrix, and SFHM were added to the HARVs or to control plates immediately after cell isolation. HARV rotations were determined empirically to provide suspension of the visible beads without apparent movement relative to the medium or walls of the HARVs.

Cultures were fed with complete changes of medium 24 hours after initial plating and then at 48 hour intervals until termination. Control cells and HARVs were observed daily, and, after six days, the cultures were sacrificed by one of several methods: freezing in liquid nitrogen for subsequent biochemical analyses; fixation in 2 % glutaraldehyde (with post-fixation in 1% osmium tetra-oxide) for electron microscopy; fixation with either -20 °C methanol, HISTOCHOICE (Amresco Inc.), or 2 % paraformaldehyde in PBS (pH 7.3) for subsequent light microscopic and fluorescent assays.

RESULTS OF CULTURING CARDIAC CELLS IN THE HARV BIOREACTOR

ENVIRONMENTAL CONDITIONS

Growth media from the HARV and parallel control dishes were sampled, and pH, pCO_2, and pO_2 were compared. **Table I** summarizes the results obtained. Statistical difference were not seen between media from heart cell cultures grown at unit gravity and those grown in HARV-simulated microgravity; however, values for media from the closed HARV systems consistently indicated elevations in dissolved gas levels compared to control. In addition, control and HARV cultured cells utilized glucose at similar rates. In **Figure 1** glucose depletion rates are compared for cells from a representative experiment: no statistical difference was seen. During the 48 hour time period between culture feedings, medium glucose levels were depleted by 20 to 30 % over initial levels in both HARV and control cultures (**not shown**) indicating that ample nutrient sources were continuously available.

HEART CELLS GROWN IN THE HARV

As a preliminary assessment of the HARV culture method, the degree to which viable cardiac cells attached to the available substrate was quantified by cell counting. **Figure 2** shows the plating efficiency of HARV-based cultures versus controls. Cells attached with the same efficiency in both HARV and control cultures.

We have used an assortment of microscopic techniques to visually assess the condition of HARV and control cultures. Phase contrast (PHACO) microscopic observations revealed spontaneous, rhythmic contractions in control cultures within 20 hours after plating; thereafter, entire culture dishes beat in synchrony. Cells in control cultures were anchorage-dependent and contact-inhibited such that a monolayer of mixed cell types was formed without obvious 3D structure. Cells cultured in HARVs adhered to microcarrier beads, and beads aggregated into defined clusters containing approximatly 8 to 15 beads per cluster. These clusters appeared generally non-contractile. **Figure 3** shows PHACO and corresponding fluorescence micrographs

of control and HARV cultures which have been immunofluorescently stained for anti-sarcomeric actin to label myocytes. Control cultures were populated with a high proportion of cardiomyocytes which were arranged in discrete, radiating groups. In the gaps between cardiomyocyte bundles, cell types representing the non-myocyte portion of the heart were evident. HARV cultures, on the other hand, exhibited a significantly different organization. Cells accumulated in layers both along and between beads. The combination of 3D cell distribution and the rounded surface of the plastic beads made light microscopy difficult; however, scanning electron microscopic observations clearly revealed the divergent cell distributions. **Figure 4A** shows a scanning electron microscope image of a control culture. Cells were observed in typical formations with non-myocyte cells organized into a thin mononlayer. These cells were presumably also found on microcarrier beads from HARV cultures. A distinct layer of covering cells appeared in a "cobblestone-like" pattern along the surface of the cell/bead aggregate (**Figure 4B**).

Intriguing results were obtained when non-plastic support matrices were used in the HARVs. Collagen threads, prepared so that the protein bundles run parallel to the long axis of the thread, provided a substructure which allowed cells to grow in a well defined orientation: parallel to the long axis of the collagen. As shown in **Figure 5**, neonatal cardiac cells grown on such fibers in HARVs formed elegant networks of oriented cells. Prevalent among these cells were formations reminiscent of the intercalated discs found in vivo.

METABOLIC ACTIVITY IN HARV CULTURES

A key indication of contractile activity in cultured cardiac cells is altered energy metabolism (6, 19, 20). Accordingly, we measured specific enzymatic activities in HARV and control cultures. **Figure 6** shows the combined results of assays for five different enzyme specific activities. Although CK and NCR levels were altered in the HARV cultured cells, the metabolic activities of the two culture systems were quite similar overall; the differences in CK and NCR may indicate a shift toward oxidative metabolism in the HARV cultures.

DISCUSSION AND FUTURE PROSPECTS

We have been studying the effects of simulated microgravity on in vitro characteristics of cultured cardiac cells. In this regard, we have utilized NASA-HARV bioreactors and adapted our standard cardiomyocyte cell system for 3D culture. Comparisons between standard-2D cultures and HARV-3D cultures have indicated that the systems are remarkably similar in terms of general culture characteristics; however, the establishment of a HARV-based culture system has allowed the generation of elegant 3D structures. We are continuing to evaluate the HARV-based culture method both as a means to further understand the effects of microgravity on cultured cells and as a means to generate 3D cellular architectures for experimental study and potential tissue substitutes.

Neonatal rat cardiac cells are anchorage dependent and, in suspension culture, solid matrices are typically provided for cell attachment. In the HARV culture system, cardiac cells were allowed to attach to support matrices for 24 hours; unattached cells were removed during the first medium exchange. The attachment of cells to substrate was similar in the HARVs and in control cultures. Cell attachment efficiencies ranged from 70 to 80 percent of viable cells present. This figure is not surprising in the standard 2D cultures where cells necessarily make contact with the substrate under the force of gravity; however, such a result in the HARVs must be interpreted in light of the presumed solid-body HARV rotation and the resulting static arrangement of cells and microcarrier beads. Clearly, it is difficult to specify solid body rotation based on empirical observation alone, and it is possible that the beads in the HARV are describing "orbits" of great enough dimension to allow for substantial interactions with the surrounding beads and cells. It may also be possible that the number of beads and cells loaded into a HARV allows for a high number of cells to be in contact with at least one bead. It is clear, however, that both HARV and control culture methods resulted in similar cell attachments.

The degree to which HARV and control cultures altered the media in which they were grown was also quite similar. The pH of SFHM remained relatively constant during the 48 hours between feedings, as did pCO_2 and pO_2 levels. Control and HARV cultures both utilized medium glucose at similar rates. It should be noted that the determination of pCO_2 and pO_2 values was

carried out in a manner that is internally consistent but that does not result in values for comparison to determinations done using alternative methods. In the absence of O_2-buffering capacity in SFHM, this caveat is especially true for pO_2 measurements. Nonetheless, our results indicated a fairly good agreement between control and HARV values; differences were likely due to the modulation of gas exchange between the medium and the incubator environment in the two systems. Finally, it should be pointed out that these values represent estimations of the overall medium condition and may have little direct bearing on the immediate cellular micro-environment, which would be strongly dependent on overall metabolic activity and the mass transfer of both nutrients and cellular waste materials.

Morphological observations have suggested that control and HARV cultures differ in terms of the arrangement of cell types. These observations were not unexpected considering the 3D nature of the HARV culture system and the presence of multiple cell types in the cardiac isolates. The heart is comprised of an endocardium, which is contiguous with the endothelial surface of the vasculature; the endocardium is surrounded by the myocardium, which comprises most of the mass of the organ; the heart is encased in an epicardial layer. It is heavily vascularized, contains collagenous extra-cellular matrix, and is subdivided into functionally and morphologically distinct regions. The organization of the various cell types in control cultures does not accurately reflect the in vivo situation. The arrangement of cells in the HARV cultures, however, suggests a more physiologically accurate situation where cells are differentially distributed by cell type relative to the support matrix. In the case of collagen supports, cells were also dramatically aligned and finely associated, as in the intact tissue.

In terms of functional characterizations, both contractile activity and metabolic enzyme activities were assessed. Control cardiac cells beat visibly in vitro in the range of 1 to 4 times per second. The HARV cultures appeared generally non-contractile. This observation may be accounted for by the apparent masking of contractile cells under a layer of non-contractile covering cells, or by a reduction in actual contractility. Based on metabolic enzyme specific activities, the HARVs were again similar to controls. All the enzyme activities tested resulted in specific

activities that were indistinguishable from controls except for a decrease in levels of CK and an increase in levels of NCR. This shift toward a more aerobic phenotype may be related to the minor differences in pH, pCO_2, and pO_2 levels noted in the two culture systems.

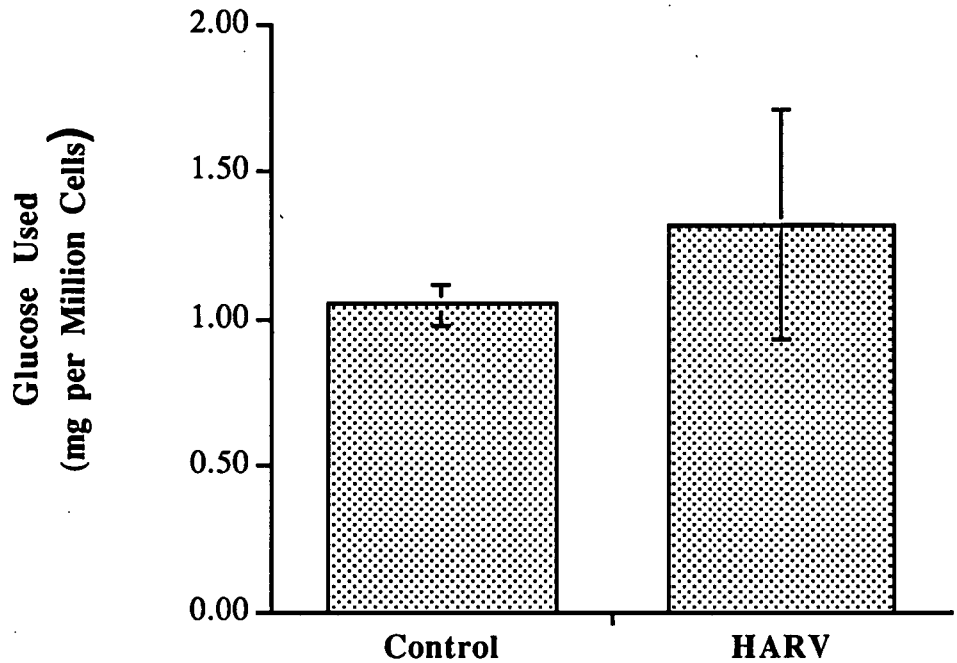
Standard tissue culture systems confine cells to proliferate and differentiate in two dimensions along a defined surface, usually a tissue culture plate, or propel cells into suspension under conditions of high shear. HARV bioreactors, on the other hand, allow cells to form 3D aggregates under conditions similar to those found in microgravity (8, 9, 10). The relatively quiescent environment of the HARV in combination with the rotation-induced simulation of microgravity allows specific cell:cell and cell:matrix interactions to occur without gravitational constraints. In our case, such three dimensional formations, in some cases, closely resembled tissue structures seen in vivo: tissue structures which would be difficult or impossible to produce using other culture methods. By virtue of its approximation of microgravity, the HARV system allows direct observation, manipulation, and preparation of complex 3D systems and architectures. On the basis of a thorough understanding of cell culture in microgravity, we hope to establish reliable methods for the formation of 3D tissue arrangements, and, as outlined above, we are moving ahead with more detailed characterizations of HARV-based cardiac cell cultures.

TABLE I:

	pH	pCO_2	pO_2
CONTROL	7.51 ± 0.04	35 ± 4	90 ± 8
HARV	7.43 ± 0.04	37 ± 4	96 ± 8

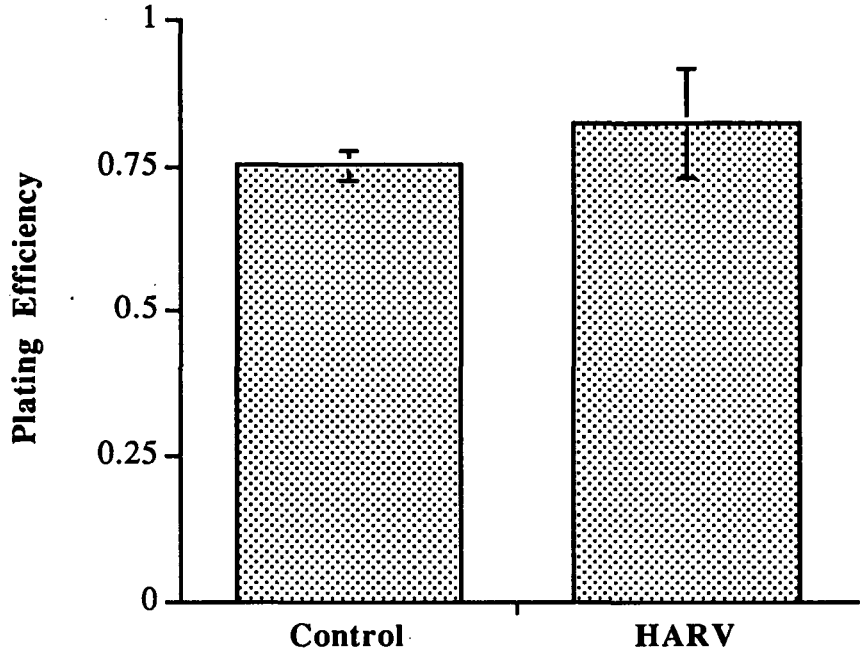
Comparison of control and HARV culture media. Samples were taken from duplicate experiments after 48 hours of culture and kept on ice. Values were determined using a Corning Blood Gas Analyzer without compensation for the absence of hemoglobin. Mean values ± S.E. (n=5) are presented; pCO2 and pO2 are reported as equivalent mmHg.

FIGURE 1:



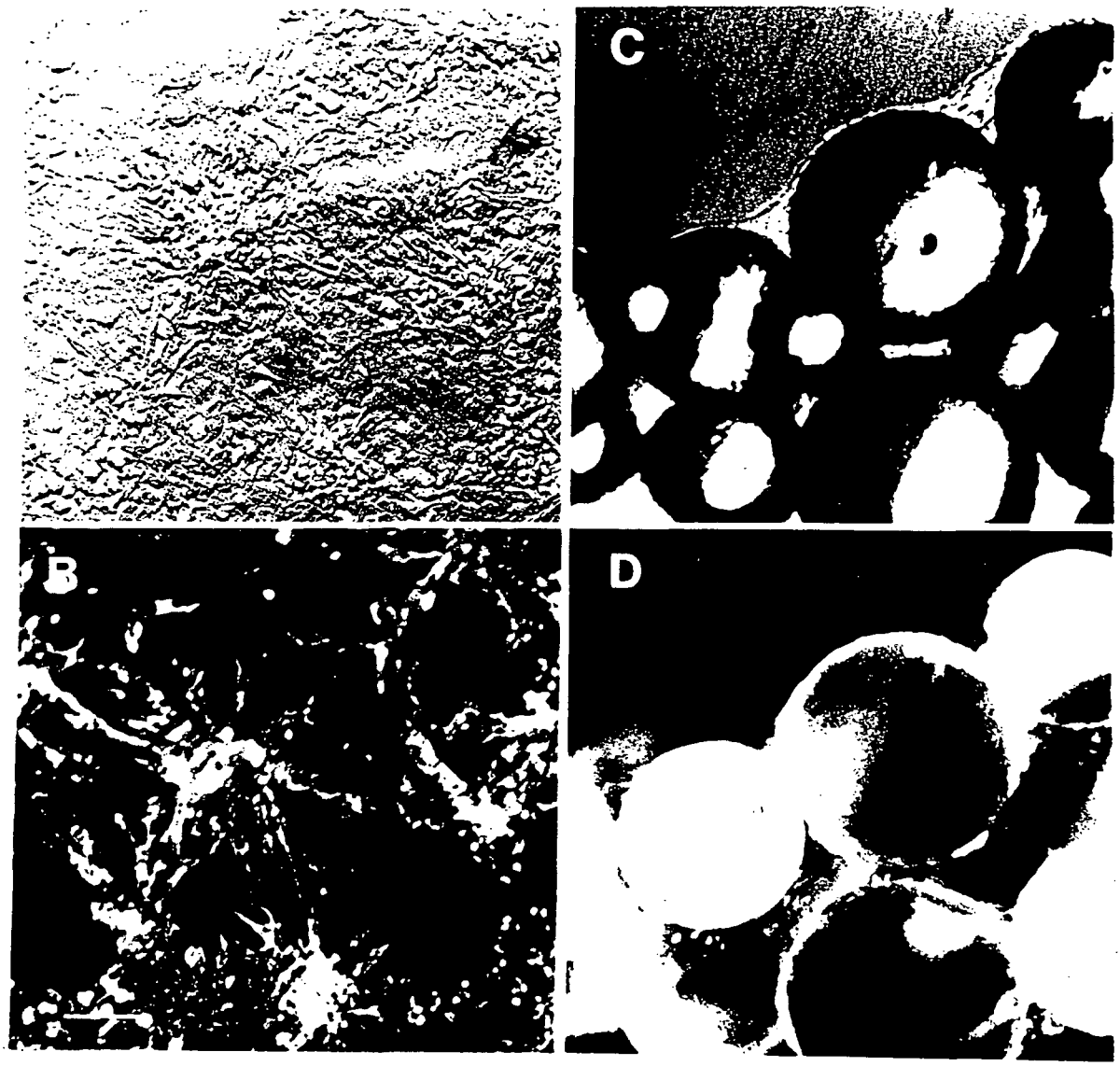
Comparison of glucose utilization. Medium was removed from HARV and control cultures 48 hours after the previous feeding. Samples were stored frozen at -20°C until assayed. Glucose was measured using a colorimetric, enzymatic (Trinder) assay kit (Sigma, St. Louis, MO). Values were normalized to the number of cells in each culture and are presented are means ± SE, n=9 for control and n=3 for HARV. No significant difference was found.

FIGURE 2:



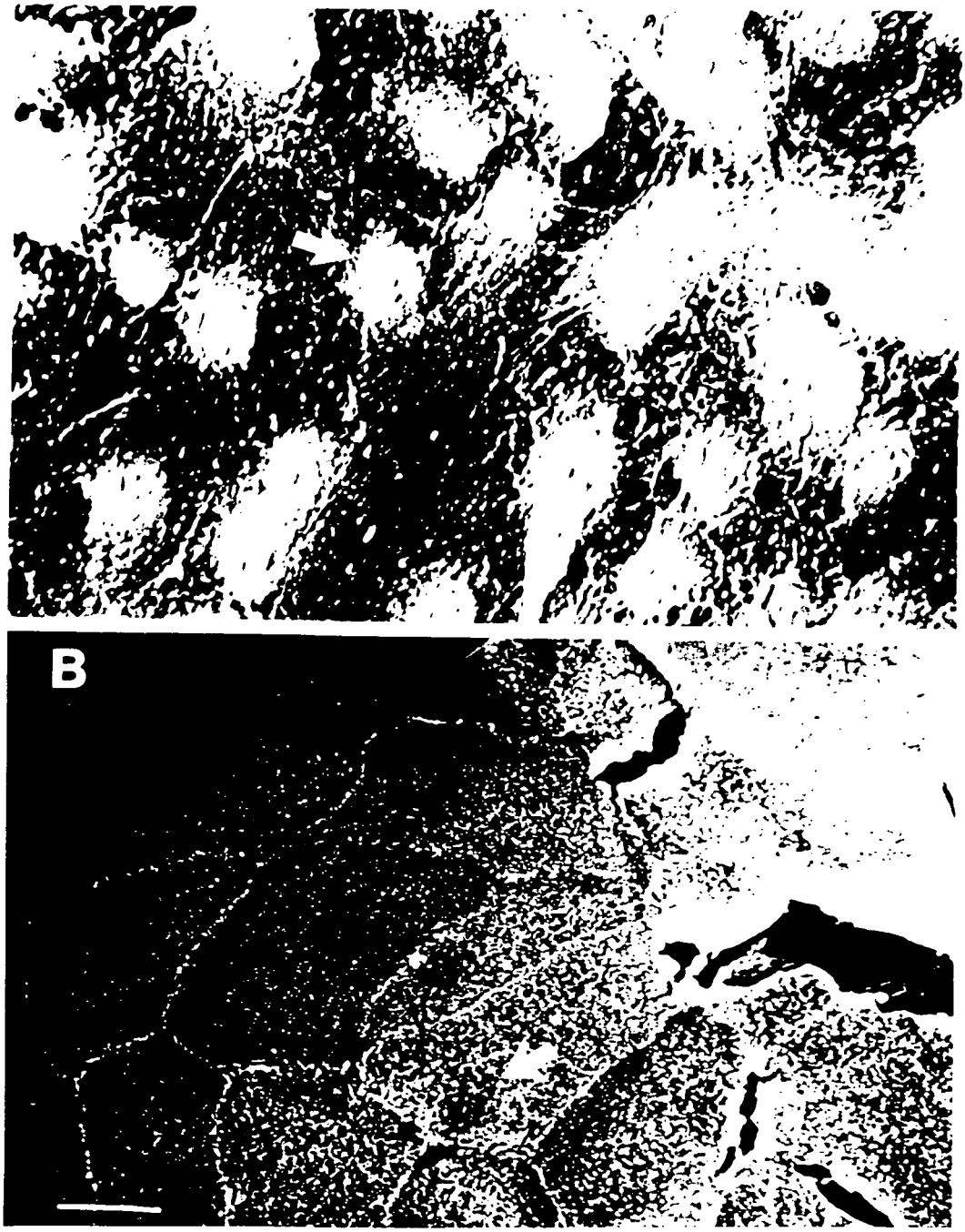
Comparison of cell attachment. The fraction of cells attached during the initial phase of culture (i.e. plating efficiency) was determined as the fraction of cells attached to the plastic substrate divided by the total number of viable cells. Cell counts were determined using a hemacytometer and trypan blue (0.2% final; Sigma, St Louis, MO) exclusion. Values reported are the mean ratios ± SE. n=21 for control and n=3 for HARV. No significant difference was found.

FIGURE 3:



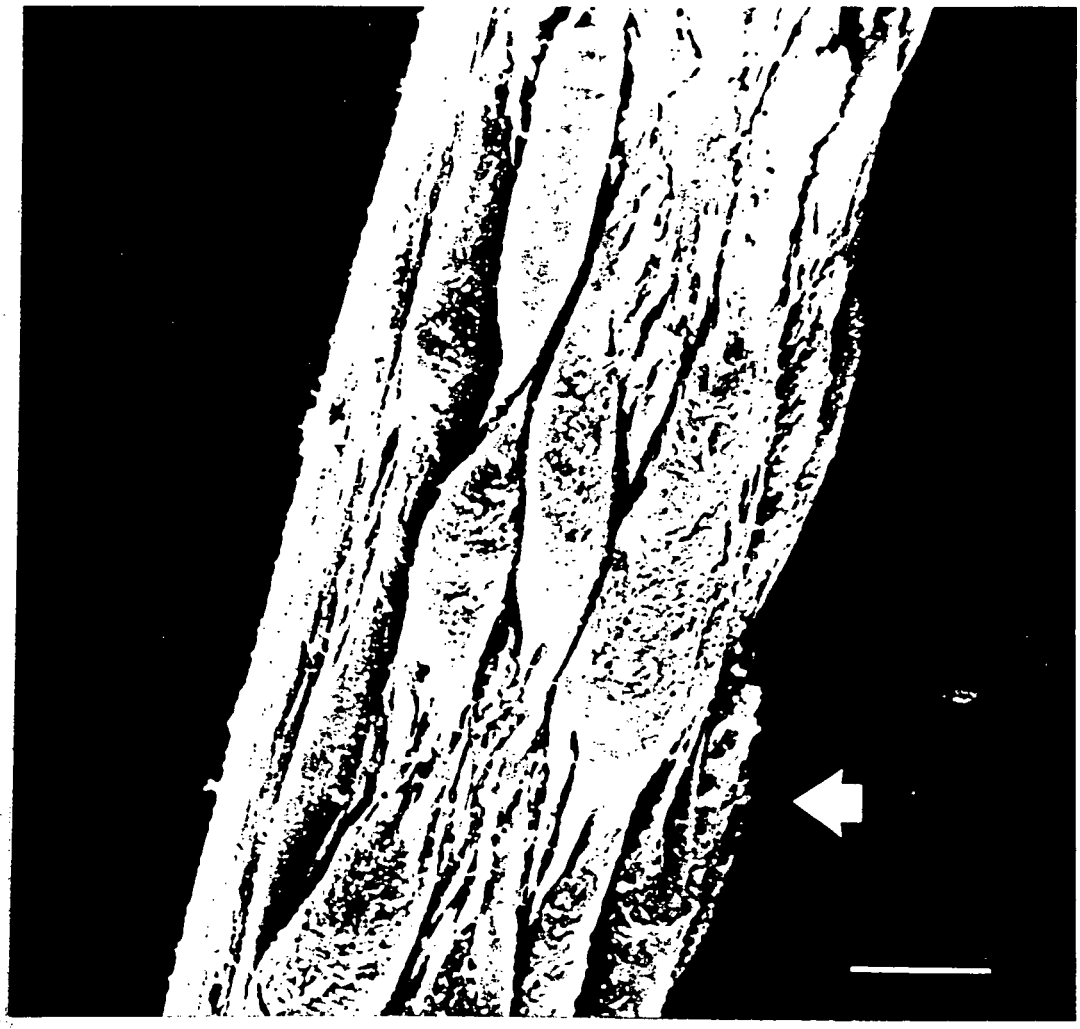
Light microscopy of control and HARV cultures. The distribution of cells in control and HARV cultures can be seen. Myocytes were abundant in both culture types. Myocytes were typically associated with other myocytes in radiating groups. In HARV cultures, 3D aggregates of beads and cells formed. **Panel A:** Hoffman Modulation Contrast (HMC) image of a control culture after 6 days. Cells were confluent. **Panel B:** Immunofluorescent (IF) image of Panel A stained using a sarcomeric actin monoclonal antibody (Sigma); myocytes were positive. **Panel C:** HMC image of a HARV culture after 6 days. Cells were present in multiple layers along and between beads. **Panel D:** IF image of Panel C. Myocytes were positive. Resolution was poor due to the out-of-focus cells and the poor optical properties of polystyrene beads. Bar = 100 μm.

FIGURE 4:



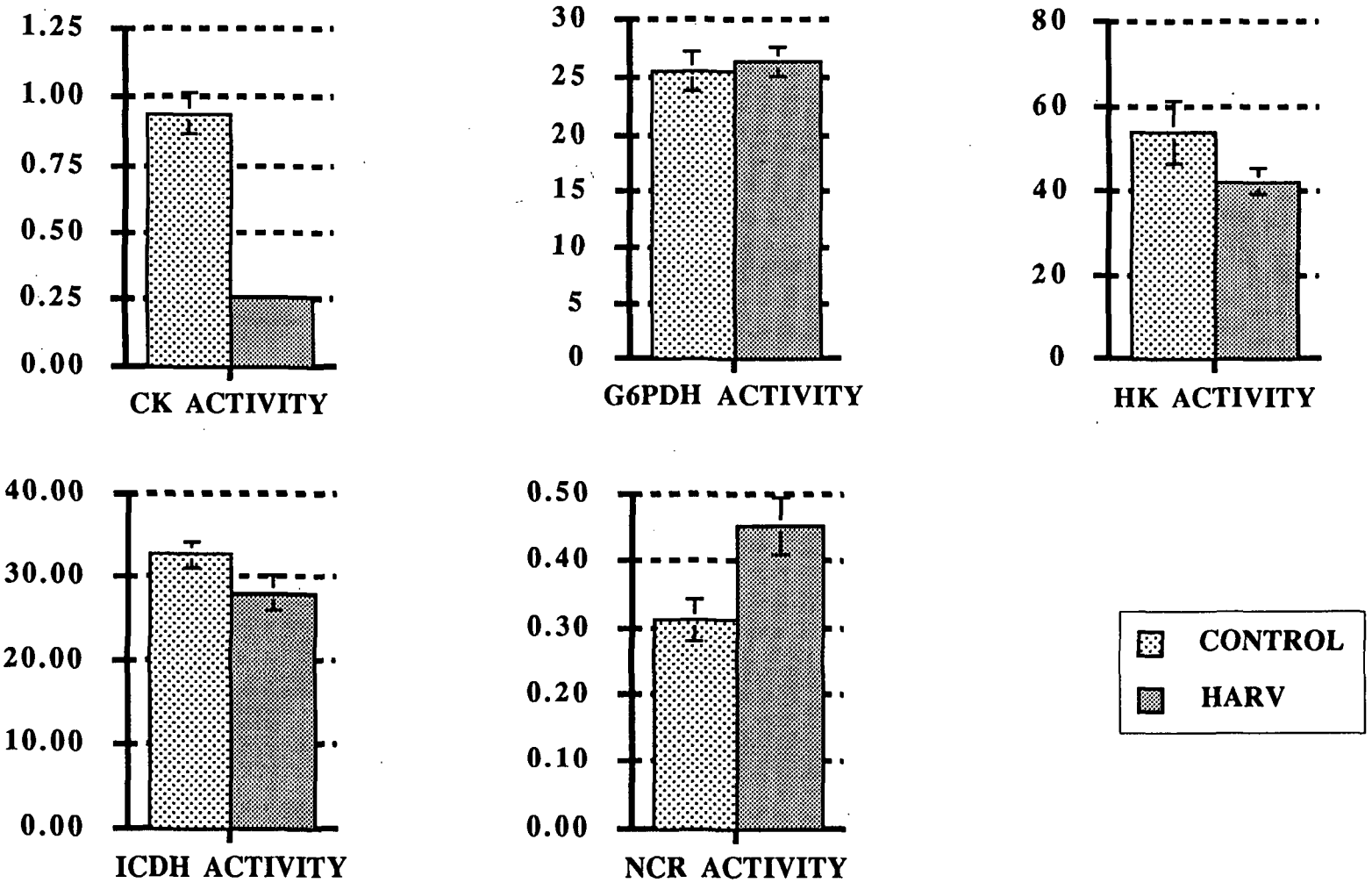
Surface characteristics of cells on polystyrene. Scanning electron microscope (SEM) observations were carried out after 6 days in culture. **Panel A:** Control culture showing a region of confluent cells (primarily non-myocytes). The sample was tilted approximately 40 degrees in the SEM to allow visualization. Samples were remarkably flat; nuclei were seen with distinct nucleoli (arrow). Little 3D structure was evident. **Panel B:** HARV culture showing a region along the surface of a bead aggregate. Thin cell layers were evident which encased entire clusters. These cells presumably correspond to the population of non-myoytes seen in Panel A. Nuclei could be seen (arrow); however, they were less distinct than in controls. Surface microvilli and cell junctions were clearly evident. In areas where the outer cell layer was broken, other cell types were clearly evident in a 3D arrangement along and between the beads (not shown). Bar = 20 μm.

FIGURE 5:



Surface characteristics of cells on oriented collagen. Scanning electron microscope (SEM) observations were carried out after 6 days of HARV culture. Presumptive cardiomyocytes were seen in distinct groupings along the length of collagen threads. The cells were almost exclusively oriented along the long axis of the threads, and other cell types were visible in alternative organizations. The cells formed distinct junctional complexes (arrow) reminiscent of intercalated discs. Bar = 10 μm.

FIGURE 6:



Comparison of enzyme specific activities. Control and HARV samples were collected and stored frozen at -80 °C as previously described (5). Values presented are means of specific activities (U/mg) ± calculated errors (pooled SE for activity + protein determinations) n=3 for each. Significant differences between HARV and control values were found in both CK and NCR ($p < 0.05$; Scheffe's Multiple Comparison). Data are from a representative cell isolation. Individual values varied from experiment to experiment; however, CK activities were consistently reduced and NCR activities were consistently elevated in HARV cultures (n=5 cell isolations).

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