Journal of Cellular Biochemistry 83:574–584 (2001)

Effect of Culture in a Rotating Wall Bioreactor on the Physiology of Differentiated Neuron-Like PC12 and SH-SY5Y Cells

Steven S.-S. Wang and Theresa A. Good*

Department of Chemical Engineering, Texas A&M University, College Station, TX 77843-3122

Abstract A variety of evidence suggests that nervous system function is altered during microgravity, however, assessing changes in neuronal physiology during space flight is a non-trivial task. We have used a rotating wall bioreactor with a high aspect ratio vessel (HARV), which simulates the microgravity environment, to investigate the how the viability, neurite extension, and signaling of differentiated neuron-like cells changes in different culture environments. We show that culture of differentiated PC12 and SH-SY5Y cells in the simulated microgravity HARV bioreactor resulted in high cell viability, moderate neurite extension, and cell aggregation accompanied by NO production. Neurite extension was less than that seen in static cultures, suggesting that less than optimal differentiation occurs in simulated microgravity relative to normal gravity. Cells grown in a mixed vessel under normal gravity (a spinner flask) had low viability, low neurite extension, and high glutamate release. This work demonstrates the feasibility of using a rotating wall bioreactor to explore the effects of simulated microgravity on differentiation and physiology of neuron-like cells. J. Cell. Biochem. 83: 574–584, 2001.

Key words: simulated microgravity; neurite; nitric oxide; glutamate

The structure and function of the human nervous system, compared with the earth environment, is believed to be altered in space. Microgravity is known to produce a number of neurological disturbances during space flight [Fujii and Patten, 1992]; however, the underlying mechanism of these disturbances is yet to be elucidated. A variety of studies have been performed to examine the effect of space flight and microgravity on the nervous system. These studies have predominantly been carried out using whole animals flown in space, and then examined after sacrifice upon return.

Morphological and structural evidence from neurons of the central and peripheral nervous system indicate that neurons experience hypoactivity and hyperactivity during microgravity and increased gravity, respectively [Krasnov, 1994]. Biochemical evidence points to neuron hypoactivity during microgravity. Cellular oxidative potential decreases in dorsal root ganglia [Ishihara et al., 1997], acetylcholinesterase activity and RNA content decrease in cervical ganglia [Roy et al., 1996], and total catecholamine excretion decreases in humans [Robertson et al., 1994]. Significant changes in the neuromuscular junction occur during space flight including a decrease in the number of synaptic vesicles and the degeneration of axonal terminals [Riley et al., 1990; Roy et al., 1996]. Neurotransmitter receptor changes have also been reported under microgravity conditions. Spacelab studies examined the number and affinity of different receptors in rat cortices, including seritonin, dopamine, noradrenergic, and GABA receptors and found that the alteration is neurotransmitter-specific [Miller et al., 1985, 1989]. In addition, the neuronal cytoskeleton appears to be responsive to the gravitational environment, as evidenced by alterations in axonal neurofilaments and microtubules during space flight [Riley et al., 1990].

Little attention has been directed toward the earth-bound cell culture experiments to

Grant sponsor: National Aeronautics and Space Agency; Grant number: NCC8-169.

*Correspondence to: Theresa A. Good, Department of Chemical Engineering, Texas A&M University, TAMU 3122, College Station, TX 77843-3122.

E-mail: tgood@tamu.edu

Received 24 January 2001; Accepted 2 July 2001

DOI 10.1002/jcb.1252

examine the effect of simulated microgravity on neurons, however, Gruener and Hoeger [Gruener and Hoeger, 1990, 1991] reported disruptions in synapse formation, alterations in cytoskeletal morphology, and acetylcholine receptor aggregation in neurons cultured in a vector-free gravity clinostat environment. Simulated microgravity reactors provide a valuable tool with which to examine the effects of the microgravity environment on neuronal physiology.

Simulated microgravity rotating wall bioreactors are based on a previous clinostat design. The culture vessel is completely filled with fluid, leaving zero -headspace. Oxygen is delivered to the reactor via diffusion through a large membrane which makes up one of the surfaces of the culture vessel. The culture vessel is horizontally rotated such that within the simulated microgravity bioreactors, the gravitational vector is effectively canceled by continuously averaging. In addition, fluid shear stresses are minimized through synchronous movement. Cells and small particles can be suspended in a sustained free fall condition, thus simulating a microgravity environment for cultured cells [Becker et al., 1993; Jessup et al., 1993; Spaulding et al., 1993; Goodwin et al., 1993a,b].

We report here the establishment of differentiated neuron-like cell cultures, PC12 and SH-SY5Y, on microcarriers within a simulated microgravity rotating wall bioreactor high aspect ratio vessel (HARV, Synthecon, Houston, TX) system. We examined the effects of culture in the simulated microgravity bioreactor on the viability, morphology, and release of small molecule neurotransmitters/ retrograde messengers of the differentiated PC12 and SH-SY5Y as compared to cell physiology in static culture controls (cells grown on microcarriers in petri dishes) and in high-shear spinner flasks. These results provide insight into the role of culture environment on they physiology of differentiated neuron-like cells and may shed light on the effects of microgravity on cells of the nervous system.

METHODS

Materials

Eagle's Minimum Essential Media (MEM, RPMI 1640 Media fetal bovine serum, penicillin, streptomycin, 2.5S Nerve Growth Factor (NGF), fungizone and trypsin-EDTA were purchased from GibcoBRL (Grand Island, NY). Collagen-coated Cytodex-3 microcarriers were obtained from Amersham Pharmacia (Piscataway, NJ). Ethanol was purchased from Fisher Scientific (Houston, TX). $N^{(G)}$-nitro-L-arginine methyl ester (L-NAME), human recombinant nerve growth factor-β (β-NGF), crystal violet, lactate dehydrogenase (LDH) diagnostic kit and glutamine/glutamate determination kit were obtained from Sigma (St. Louis, MO). Dulbecco phosphate-buffered saline (PBS) was obtained from Pierce Chemical Co. (Rockford, IL).

Cell Culture

The rat pheochromocytoma PC12 cells (ATCC, Rockville, MD) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum, 3 mM L-glutamine, 100 U/ml penicillin, and 100 μg/mL streptomycin. The human neuroblastoma SH-SY5Y cells (a kind gift from Dr. Evelyn Tiffany-Castiglioni, College of Veterinary Medicine, Texas A&M University, College Station, Texas) were cultured in MEM medium (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum, 3 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/mL fungizone. PC12 and SH-SY5Y cells were induced to differentiate into neuron-like cells by the addition of nerve growth factor-β (β-NGF). Both cell cultures were grown in a humidified 5% (v/v) CO_2-air environment at 37°C.

Culture Systems

Two cell lines were grown on the collagen-coated Cytodex 3 microcarriers (Amersham Pharmacia, Piscataway, NJ). Cytodex-3 microcarriers were pretreated according to the manufacture's instructions before use. Cells and microcarriers were cultured in three culture systems, petri dishes which served as a static culture condition (control), 100 ml spinner flasks (Bellco Glass, Vineland, NJ) which served as a high shear normal gravity culture environment, and high aspect rotating-wall vessel (HARV) (Synthecon, Houston, TX) which simulated a low-shear microgravity environment ($10^{-2} \times$ g). The cell density in the bioreactors was approximately 1×10^6 cells/ml. The spinner flask was operated at 18∼20 rpm, which was just sufficient to keep the microcarriers suspended in the culture medium. The HARV

system was operated according to the manufacture's instructions. The culture vessel was rotated at 16 rpm, which was sufficient to keep the microcarriers suspended and in continuous free fall. In each culture system cells were differentiated for 8 days via the addition of 10 ng/ml of β-NGF. During the course of each experiment, cell samples were removed from the spinner flask, the static petri dish and the HARV once every other day after differentiation. Liquid samples taken from three culture systems were stored in −20°C for further analyses. Cell number was assessed using the crystal violet staining method and cell injury was assessed using an LDH assay, performed using a prepackaged kit as per the manufacturer's directions.

Light Microscopy

The morphology of cell samples removed from the cultures was observed with the Zeiss Axiovert 100 Transmitted Light Microscope (Thornwood, NJ). The Images were captured with the Zeiss continuous image capture 128 system. Typically, for each observation (i.e., number of cells with neurites) approximately 80–100 cells were examined from between three to five experimental runs for each culture environment.

Nitric Oxide Production and Inhibition

Nitric oxide (NO), formed by the cells, is rapidly converted to nitrite in the cell culture medium. Nitrite was measured chemiluminescently from a 100 μl sample of culture medium using a Sievers Nitric Oxide Analyzer (NOA) 270B (Boulder, CO). The Nitric Oxide Analyzer signal was calibrated against nitrite standards prepared with sodium nitrite.

NO was inhibited by $N^{(G)}$-nitro-L-arginine methyl ester (L-NAME). After cell attachment to microcarriers, cells were transferred into the spinner flask or the HARV bioreactor. NGF-β was added to both reactors, and to one set of reactors, L-NAME (final concentration: 2 mM) was added. After 3 h culture medium was replaced to remove the L-NAME, and new NGF-β was added. Neurite development in all reactors was followed via light microscopy before and after L-NAME addition.

Glutamate Release

Glutamate is one of the most widely used excitatory neurotransmitters within the central nervous system. Changes in extracellular glutamate concentration may be a useful indicator of excitability or injury in neuronal cells. In the spectrophotometric assay, glutamate undergoes oxidative deamination by the catalysis of glutamate dehydrogenase. Glutamate concentration was measured enzymatically by following its oxidative deamination in the presence of glutamate dehydrogenase. The assay was performed using a glutamine/glutamate determination kit according to the manufacturer's instructions.

Shear Stress Application

SH-SY5Y cells were plated at a density of 300,000 cells in a 35 mm polystyrene culture dish. After 5∼6 days differentiation in the culture dish, with NGF-β, cells were then placed in a modified cone and plate viscometer [Triyoso and Good, 1999], where cells were exposed to shear stress via rotation of a cone above the cells. Shear stress was transmitted via the movement of the culture medium above the cells. Stress was proportional to the speed of rotation of the cone.

Data Analysis

Data are presented as the mean ± SD of n independent determinations, where n is indicated in each figure legend. To determine whether a given data set was significantly different from a control data set, the Student's *t* test with unequal variance was used.

RESULTS

We established differentiated PC12 and SH-SY5Y cultures on microcarriers in the simulated microgravity HARV bioreactor and characterized structural changes in the cultured cells within the HARV. Comparisons were made with cells grown on microcarriers a static petri dish and in a spinner flask, which served as static and well mixed normal-gravity controls, respectively. The morphological observations of neuron-like PC12 and SH-SY5Y cells grown in three different culture systems are depicted in Figure 1. The top panel (Fig. 1A, B, C) and the bottom panel (Fig. 1D, E, F), show the differentiated SH-SY5Y and PC12 cells on cytodex-3 microcarriers in the spinner flask (A, D), the simulated microgravity HARV bioreactor (B, E), and the petri dish (C, F). Throughout the experiments, morphological changes, such as

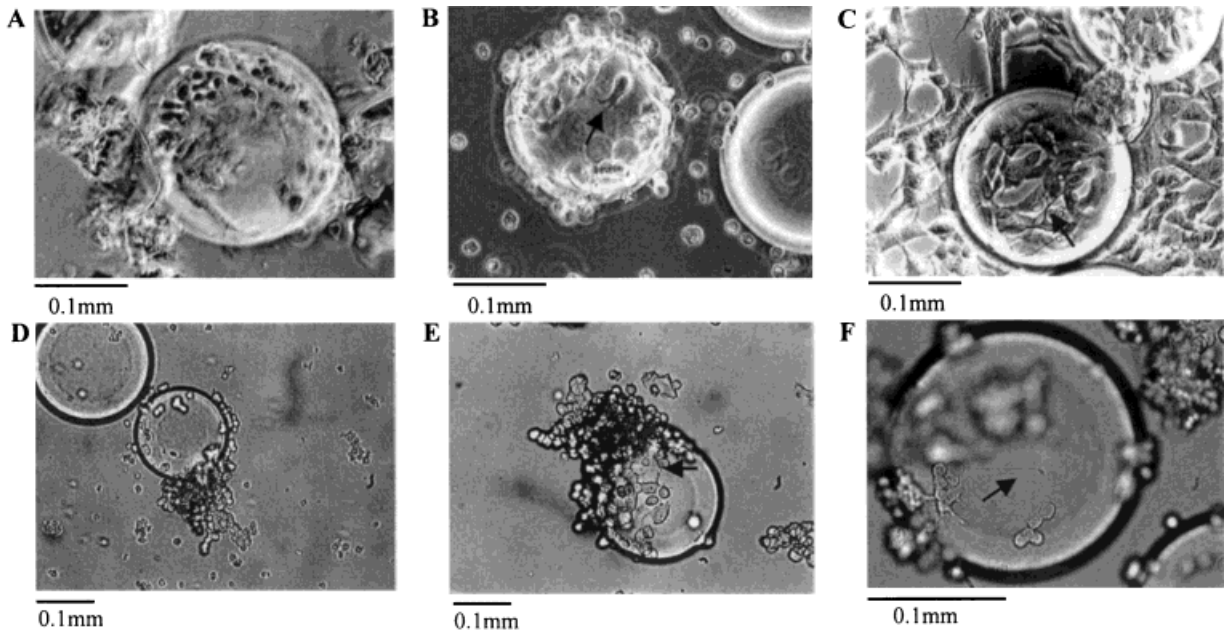

Fig. 1. Representative micrographs of differentiated neuron-like SH-SY5Y (**A**, **B**, **C**) and PC12 cells (**D**, **E**, **F**) grown in three different culture systems: spinner flasks (A, D); HARV (B, E); petri dishes (C, F). Arrows indicate the neurites extended from the cell body.

cell aggregation, neurite length, and number of cells bearing neurites per bead were considered. More cell aggregates were found in the spinner flask and the HARV cultures than the static control. Neurite length was the longest in static petri dish environment, intermediate in the simulated microgravity HARV, and shortest in the spinner flask. Cells with neurites were more numerous in the static petri dish than either the spinner flask or HARV. The fewest number of cells with neurites were found in the spinner flask. In addition, microcarrier disruption was severe in the spinner flask, while relatively little microcarrier disruption was observed in either the static control or the HARV. All morphological findings are summarized in Table I.

We examined the measures of cell number and cell injury for the PC12 and SH-SY5Y cells in the three different culture systems. We used crystal violet staining to count the total number of cells (both alive and dead) on microcarriers during the course of an experiment and used LDH activity released from cells into culture medium to give a measure of cell injury or loss of cell viability. For all three culture environments, cell number per microcarrier bead, as measured from crystal violet staining, increased slightly during the course of an 8 day experiment, which is typical of nerve

TABLE I. Morphological Observations in Three Culture Environments*

Cell culture system	Length of neurites	Percentage of cells with neurites (%)	Aggregation	Microcarrier disruption
Spinner flask	Short	34 ± 2	Present	Severe
HARV-simulated microgravity	Medium	53 ± 3	Present	Mild
Static petri dish	Long	88 ± 1	Absent	Absent

*Three to five experiments were performed in each culture environment with each cell type. For a given experiment, 80–100 cells were examined for the presence of neurites and neurite length. The percentage of cells with neurites during each experiment was determined. The average ± SD of the percentage of cells with neurites from each experiment are reported for each of the three culture environments.

growth factor differentiated PC12 and SH-SY5Y cells, however, the changes in cell number per microcarrier observed was not significantly different in the three different culture systems ($P > 0.5$).

The accumulated LDH activity in the culture medium per cell as a function of cultivation time after differentiation is shown in Figure 2. At all times after 2 days in culture, the differentiated cells in the spinner flask released significantly more LDH into the culture medium as compared to the static petri dish and HARV cultures ($P < 0.01$). For the SH-SY5Y cells, the ratio of the LDH activities in the spinner flask over the static cultures ranged from 22.1–25.8, while the same ratio was only 2.3–4.3 for cells grown in the HARV relative to the static cultures. Similar trends were also observed with the PC12 cultures, with LDH release relative to static cultures ranging from 6.5–14.3 for the spinner flask and 0.8–2.2 for the HARV. This is probably due to more cell damage in the spinner flask than the other two culture systems.

We measured NO and glutamate released from cells in each of the culture environments as both are used as neurotransmitters and could indicate changes in cell excitability or other changes in cell physiology. In addition, at high concentrations, both are toxic to neuron-like cells. For both NO and glutamate, we measured the concentration of the analyte in the culture medium of each environment on a daily basis and also calculated the total amount of analyte accumulated in the reactor over the course of the experiment. As shown in Figure 3A and C, the measured glutamate concentrations in the spinner flask and the HARV environments for both cell types were significantly higher than that measured in the static condition on the average ($P < 0.05$ and 0.1 for spinner flask and HARV, respectively). The total accumulated glutamate released by both cell types in the spinner flask was significantly greater than both the accumulated glutamate released from cells in the HARV and from cells in the static petri dish (Fig. 3B, D, $P < 0.005$). At all times in culture after Day 2, accumulated glutamate was not significantly different in the HARV than in the static condition.

As shown in Figure 4A, the concentration of NO released by the SH-SY5Y cells was slightly higher in the HARV than in the spinner flask and the static control with the exception of Day 2. A similar trend was observed for PC12 cell culture (Fig. 4C). In addition, the calculated accumulated NO produced in the HARV was slightly higher than that in the other two culture conditions after Day 4 with the SH-SY5Y cells (Fig. 4B) and at all times with the PC12 cells (Fig. 4D). The differences in accumulated NO between the HARV and the spinner flask were not significant, however,

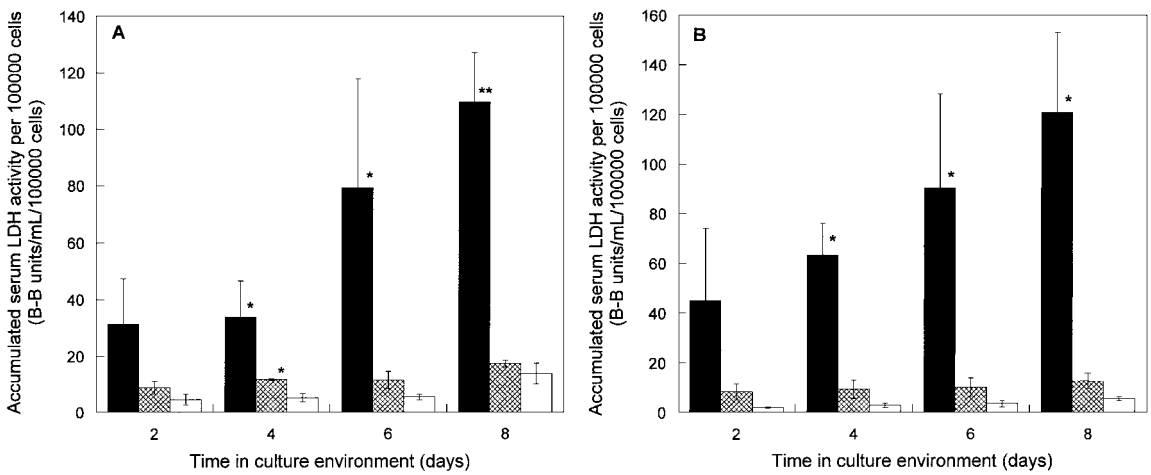

Fig. 2. The accumulated serum LDH activity per cell as a function of cultivation time after differentiation in three culturing environments: spinner flask (solid bar), HARV (cross hatched bar), and petri dish (open bar). **A**: SH-SY5Y cells (**B**) PC12 cells. Accumulated LDH was calculated by taking into account the amount of LDH that was removed from the reactor in each previous cell medium exchange plus the amount of LDH that was currently in the reactor. * and ** indicate that the increase in the accumulated serum LDH in HARV or spinner flask relative to the static control system was significant at $P < 0.01$ and $P < 0.005$, respectively. (n = 3∼5)

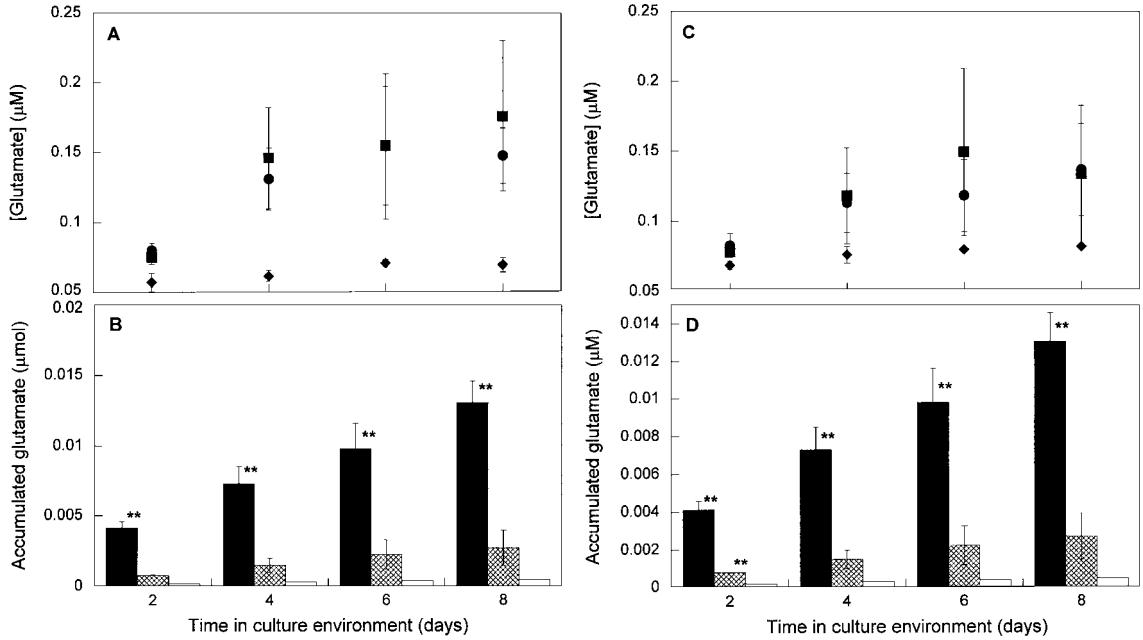

Fig. 3. **A**, **C**: The concentration of glutamate in culture medium and (**B**, **D**) the total accumulated glutamate in three culture systems: spinner flask (circle, solid bar), HARV (square, cross hatched bar), and petri dish (diamond, open bar). (A, B) SH-SY5Y cells (C, D) PC12 cells. Accumulated glutamate was calculated analogously to accumulated LDH. * and ** indicate that the increase in the concentration or the accumulation of glutamate in HARV or spinner flask relative to the static control was significant at $P < 0.01$ and $P < 0.005$, respectively. (n = 3∼5)

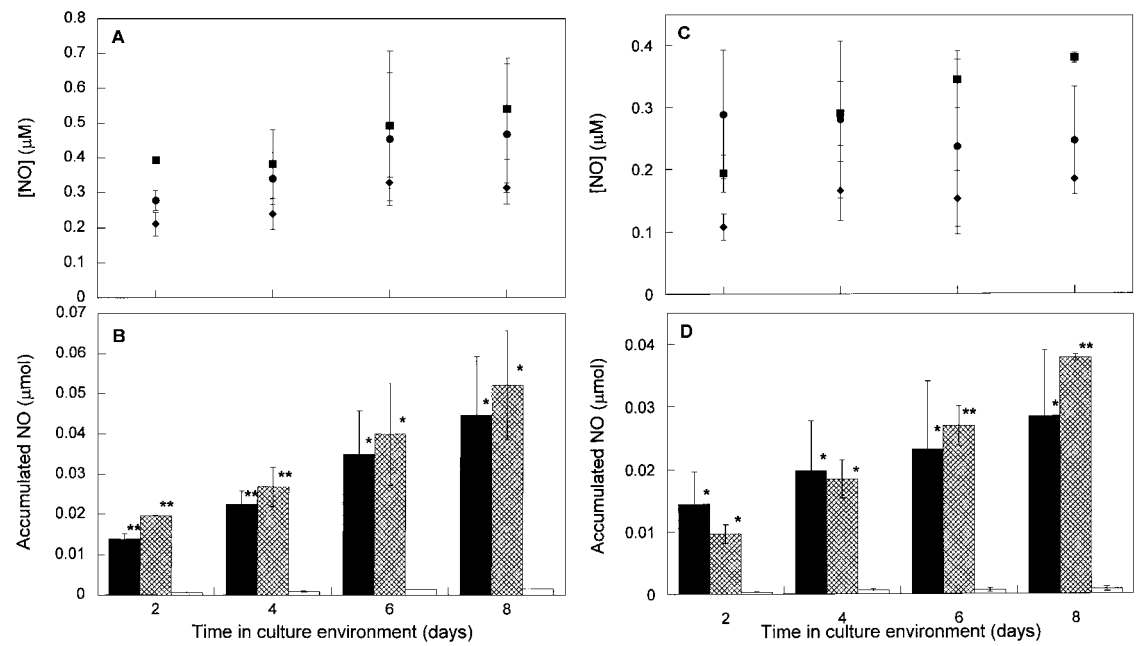

Fig. 4. The concentration and the accumulation of NO in three culture systems: spinner flask (circle, solid bar), HARV (square, cross hatched bar), and petri dish (diamond, open bar). **A**, **B:** SH-SY5Y cells (**C**, **D**) PC12 cells. * and ** indicate that the increase in the concentration or the accumulation of NO in HARV or spinner flask relative to the static control was significant at $P < 0.01$ and $P < 0.005$, respectively. (n = 3∼5)

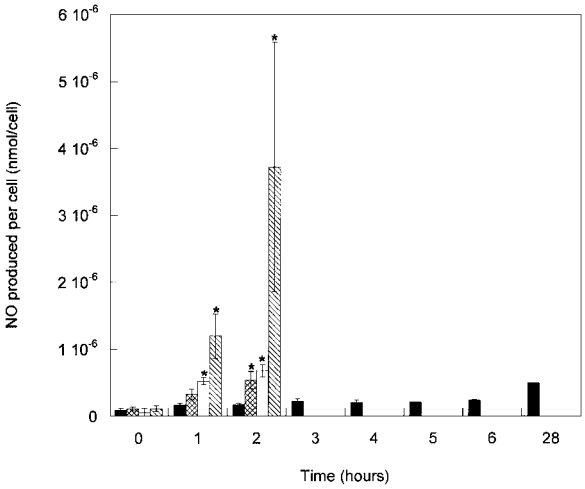

Fig. 5. Amount of NO released into culture medium per cell in the SH-SY5Y cultures grown in the simulated microgravity HARV (solid bar), or exposed to shear stress at 0.08 dyn·cm^{-2} (cross hatched bar),0.24 dyn·cm^{-2} (open bar), and 0.36 dyn·cm^{-2} (diagonally striped bar). (n = 3∼5)

accumulated NO in both the HARV and the spinner flask were significantly greater than that produced in the static culture for both cell types ($P < 0.01$).

In order to try to understand why NO accumulation was elevated in both the spinner flask and in the HARV, we examined how NO production per cell changed with shear stress, and compared that to NO production observed in the HARV. Figure 5 shows the short-term change in the amount of NO production per cell by differentiated SH-SY5Y cultures either exposed to shear stress in a modified cone and plate viscometer or exposed to the HARV culture environment. Cells subjected to shear stress produced significantly more NO after two hours than cells in the HARV ($P < 0.01$), even at the lowest shear stresses utilized (0.08 dyn·cm^{-2}). At the highest shear stress examined, 0.36 dyn·cm^{-2}, NO levels measured in the culture medium after 2 h were 22.1 times that measured in the HARV.

In order to address if the enhanced neurite outgrowth observed in the HARV relative to that observed in the spinner flask was at all related to the slightly higher levels of NO seen in the HARV, we examined the effect of inhibiting NO synthesis on neurite extension in both culture systems. We used PC12 cells for these experiments, as PC12 cell neurites are more numerous and easier to observe than SH-SY5Y cell neurites.

The NO synthase inhibitor, L-NAME, was added to the PC12 cells cultured within the spinner flask and the HARV. As shown in Figure 6, no obvious neurite extension was observed in either three-dimensional culture environment 3–10 h after the addition of the NO synthase inhibitor.

DISCUSSION

A variety of neurological disturbances have been reported for whole organisms exposed to microgravity. These disturbances include space motion sickness, space adaptation syndrome, postural illusions, postural imbalance, visual

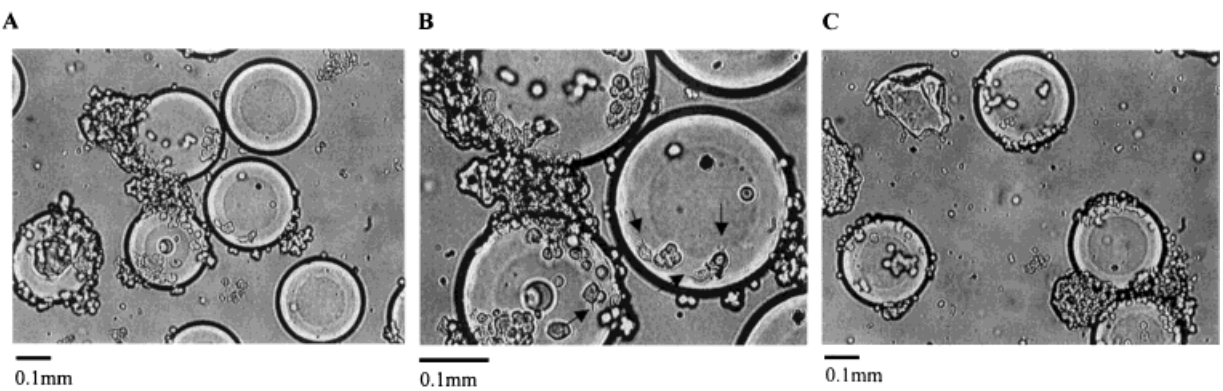

Fig. 6. Effect of nitric oxide synthase inhibition on neurite extension in three-dimensional culture systems. **A**: Initial micrograph (**B**) 8–10 h after NGF addition in the absence of L-NAME (**C**) 8–10 h after NGF addition in the presence of L-NAME. Representative micrographs taken from cells in a spinner flask are shown. Arrows indicate neurites. Approximately 80 cells were examined prior in each 3D culture environment after NGF addition in the presence and absence of L-NAME. In the absence of L-NAME, the percentage of cells with neurites were comparable to that reported in Table I. In the presence of L-NAME, no neurites were detected in either HARV or spinner flask cell out of more than 150 cells observed.

disturbances, neuromuscular weakness and fatigue [Fujii and Patten, 1992]. With advancements in aerospace technology and prolonged space flights, both organism and cellular level understanding of the effects of microgravity on cells of the nervous system will become increasing important in order to ensure the safety of prolonged space travel.

In order to begin to address the effects of microgravity on cells of the nervous system through the use of *in vitro* ground-based experiments, we used two differentiated neuron-like cell lines, human neuroblastoma SH-SY5Y cells and rat pheochromocytoma PC12 cells, cultured in a HARV-rotating wall bioreactor. The HARV-rotating wall bioreactor simulates microgravity via the motion of the cells within the reactor which effectively cancels the average gravity vector. The SH-SY5Y cell line is of human and neuronal origin, consists of a homogeneous population of neuroblast-like cells, can be differentiated via nerve growth factor to take on a neuron-like phenotype, forms neurites, and expresses ion channels. SH-SY5Y cells are dopaminergic but also express function glutamate receptors and are susceptible to glutamate toxicity [Amano et al., 1994; Yoshioka et al., 1996]. The PC12 cell line is phenotypically endocrine resembling the normal adrenal chromaffin cells. They contain numerous secretory granules like those found in adrenal chromaffin cells and sympathetic nerve cells and upon NGF-induced differentiation, PC12 cells develop a sympathetic neuron-like phenotype [Tischler and Greene, 1978; Fujita et al., 1989]. Given that some of the abnormalities observed in long-term space flight and on return to Earth have been attributed to reduced sympathetic activity [Robertson et al., 1994], both PC12 and SH-SY5Y cell lines provide convenient model systems with which to explore neurophysiological responses to microgravity.

Our results show that differentiated PC12 cells and SH-SY5Y cells extend neurites and aggregate when cultured in simulated microgravity, while in static culture, cells extend neurites without aggregation, and in a spinner flask, cells aggregate without extending neurites (Fig. 1; Table I). It appears that certain aspects of tissue development, such as cell aggregation, are enhanced in both 3 dimensional culture environments (HARV and spinner flask), while cell differentiation, as indicated by neurite extension, is enhanced in both static culture and the HARV, but not in the spinner flask. While there have been few if any reports of the effects of culture in simulated microgravity on neuron or neuron-like cells, non-differentiated PC12 cells have been previously cultured in the NASA rotating wall vessel (RWV) bioreactor [Lelkes et al., 1998]. In the simulated microgravity environment, PC12 cells formed macroscopic, tissue-like organoids several millimeters in diameter and displayed evidence or neuroendocrine differentiation [Lelkes et al., 1998]. Like the PC12 cells, other cell lines of non-neuronal origin, such as BHK-21 [Goodwin et al., 1993a], DU 145 human prostate carcinoma [Clejan et al., 1996], neonatal rat heart cells [Akins et al., 1997], and bovine chondrocytes [Baker and Goodwin, 1997] displayed dramatic changes in three dimensional organization upon culture in a simulated microgravity environment. In addition, enhanced differentiation was seen in a number of the same cell lines [Goodwin et al., 1993b].

We saw no significant loss of viability or cell injury, as indicated by the absence of significant LDH release, by either the differentiated PC12 or SH-SY5Y cells in the simulated microgravity environment (Fig. 2). This is in dramatic contrast to the LDH release seen in the spinner flask which we take to indicate that significant cellular injury occurred in this culture environment. We believe that the high cell injury observed in the spinner flask, which is also accompanied by disruption of the microcarrier beads, is due to the high shear stresses present in the spinner flask [Croughan and Wang, 1991; Venkat et al., 1996; Gregoriades et al., 2000]. While the stirring speeds used in our experiments, less than 20 rpm, are well below those considered damaging to most cells, a variety of evidence indicates that neuron-like cells are injured by mild shear stress levels [LaPlaca and Thibault, 1997; Laplaca et al., 1997; Triyoso and Good, 1999; Edwards et al., 2001]. The shear stresses expected to be present in the HARV (< 0.5 dyn·cm^{-2}, [Becker et al., 1993; Prewett et al., 1993]), are a factor of two or more lower than those which have been observed to cause damage to neuron-like cells [Triyoso and Good, 1999; Edwards et al., 2001].

While large increases in LDH release were observed in the spinner flask, no similar changes in cell number on beads were observed as measured using crystal violet staining. Two

possibilities for this apparent discrepancy in results are put forward. Since crystal violet will stain living and dead cells, we could have observed no change in total cell number and had increasing numbers of dead cells in the spinner flask over time, which gave rise to the observed LDH release. While this is plausible, it is doubtful that dead cells would have remained attached to the microcarrier beads over a period of 8 days. More probably, the high shear stress present in the spinner flask induced an increase in membrane permeability and the observed increase in LDH release into the spinner flask came from "leaky" or injured cells instead of "dead" cells.

We examined glutamate release and NO production from the differentiated neuron-like cells in the 3 different culture environments. Glutamate is the most widely used neurotransmitter in the central nervous system but is also neurotoxic at high levels [Otori et al., 1998; Ye and Sontheimer, 1998; Kawasaki et al., 2000]. PC12 and SH-SY5Y cells, when differentiated, are both vulnerable to glutamate excitotoxicity [Amano et al., 1994; Calderon et al., 1999; Lee et al., 1999]. Nitric oxide is an important signaling molecule that is involved in neuronal development, neurite extension, and long term potentiation [Gally et al., 1990; Schuman and Madison, 1991; Bruhwyler et al., 1993; Zhuo et al., 1993]. However, NO, like glutamate, is neurotoxic at high levels [Chao et al., 1992]. As seen in Figure 3, glutamate concentrations in culture medium taken from spinner flask cultures were higher than those in the HARV or static cultures. Elevated glutamate could signal increased excitability of cells in the spinner flask, or, more likely, could be associated with the increased cellular injury seen in the spinner flask relative to the other culture conditions.

The increased accumulated NO production seen in both the spinner flask and the simulated microgravity cultures (Fig. 4) indicate that increased cellular signaling and changes in cell physiology occur in the two culture environments relative to static cultures. The increased NO production, because it is seen associated both with un-injured cells in the HARV and with injured cells in the spinner flask, is probably not directly associated with cellular injury in our experiments.

It should be noted that both glutamate and NO are released locally, and that the concentrations at the site of release may be much higher than those measured in the bulk culture medium. The shear field in the spinner flask is predicted to be much more inhomogeneous than that in the HARV [Venkat et al., 1996; Begley and Kleis, 2000], which could lead to regions of high local shear stress induced NO production [Triyoso and Good, 1999] or shear stress induced cell membrane damage [Laplaca et al., 1997] and glutamate release. In addition, NO, because of its short half life, acts locally. Thus, especially for NO, while there were no significant differences in the average concentrations measured in the spinner flask and the HARV, there may have been significant differences in local concentrations in the two culture environments which may be related to the observed differences in neurite extension and cell injury in the two systems.

In order to assess if NO levels seen in the HARV could be a result of shear stress within the bioreactor, we compared directly the NO production by differentiated SH-SY5Y cells exposed to a controlled shear stress generated by a cone and plate viscometer and the NO production by cells in the simulated microgravity environment (Fig. 5). Even at the lowest shear stress examined, 0.08 dyn cm^{-2}, NO production was significantly greater than that observed in the HARV, suggesting that NO production in the HARV is not related to shear stress. NO production associated with high shear stresses (3–10 times higher than those used in our study) has previously been reported [Triyoso and Good, 1999]. We speculate that NO production observed in the HARV is due to some cellular signaling unique to simulated microgravity.

We suspect that the NO levels seen in the simulated microgravity environment may have supported neurite outgrowth. Outgrowth of neurites is a fundamental morphological change associated with differentiation and neural development [Fujita et al., 1989] and may also play a pivotal role in the formation of collateral circuits and functional recovery after damage to the central or peripheral nervous systems [Gage et al., 1990; Van der Zee et al., 1992]. It has been shown that NGF differentiated PC12 cells require NO and NO synthase for neurite outgrowth and differentiation [Phung et al., 1999]. In addition, hippocampal neurons and NGF differentiated PC12 cells,

when treated with NO donors, had longer neurites and many more neurite-bearing cells compared to cultured untreated with NO [Hindley et al., 1997]. While our results show no difference in NO levels in HARV and spinner flask, we do show that prevention of NO release via treatment with the NO synthase inhibitor L-NAME completely inhibits neurite extension in both culture systems (Fig. 6). Other factors associated with neurite extension, such as intracellular calcium levels, GTPase activity, integrin receptor expression, tau and microtubule associated protein expression, and cytoskeletal tension [Valtorta and Leoni, 1999], might be associated with the moderate neurite extension seen in simulated microgravity and/or the absence of neurite extension in the spinner flask.

During our experiments, we made no attempt to examine differences in cell response to the three reactor environments at different stages of NGF induced differentiation. As most of our data were collected during differentiation, our results may have more implications for the developing nervous system than the adult nervous system. However, our results may be relevant to processes in an adult astronaut such as nervous system remodeling, regeneration, or repair.

In conclusion, we show that culture of differentiated PC12 and SH-SY5Y cells in the simulated microgravity HARV bioreactor resulted in high cell viability, moderate neurite extension, and cell aggregation accompanied by NO production. Neurite extension was less than that seen in static cultures, suggesting that less than optimal differentiation occurs in simulated microgravity relative to normal gravity. The shear stress associated with the rotating culture vessel was probably not the cause of the changes in cell physiology, as cells grown in a mixed vessel under normal gravity (a spinner flask) had low viability, low neurite extension, and high glutamate release. This work demonstrates the feasibility of using a rotating wall bioreactor to explore the effects of simulated microgravity on differentiation and physiology of neuron-like cells. In addition, we show that there are significant differences between cells cultured in normal gravity and simulated microgravity that cannot be accounted for by the increased rotation or mixing in the simulated microgravity reactor, suggesting that microgravity associated changes in neuronal physiology, at the cellular level, can be studied in *in vitro* ground-based experiments.

REFERENCES

Akins RE, Schroedl NA, Gonda SR, Hartzell CR. 1997. Neonatal rat heart cells cultured in simulated microgravity. In Vitro Cell Dev Biol Anim 33:337–343.

Amano S, Ohashi M, Ishiko T, Hazama F. 1994. Humoral factors derived from glial cells protect cultured neuroblastoma cells against glutamate toxicity. Brain Res 645:347–350.

Baker TL, Goodwin TJ. 1997. Three-dimensional culture of bovine chondrocytes in rotating-wall vessels. In Vitro Cell Dev Biol Anim 33:358–365.

Becker JL, Prewett TL, Spaulding GF, Goodwin TJ. 1993. Three-dimensional growth and differentiation of ovarian tumor cell line in high aspect rotating-wall vessel: morphologic and embryologic considerations. J Cell Biochem 51:283–289.

Begley CM, Kleis SJ. 2000. The fluid dynamic and shear environment in the NASA/JSC rotating-wall perfused-vessel bioreactor. [In Process Citation]. Biotechnol Bioeng 70:32–40.

Bruhwyler J, Chleide E, Liegeois JF, Carreer F. 1993. Nitric oxide: a new messenger in the brain. Neurosci Biobehav Rev 17:373–384.

Calderon FH, Bonnefont A, Munoz FJ, Fernandez V, Videla LA, Inestrosa NC. 1999. PC12 and neuro 2a cells have different susceptibilities to acetylcholinesterase-amyloid complexes, amyloid25-35 fragment, glutamate, and hydrogen peroxide. J Neurosci Res 56:620–631.

Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK. 1992. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. J Immunol 149:2736–2741.

Clejan SC OCK, Cowger NL, Cheles MK, Haque S, Primavera AC. 1996. Effects of simulated microgravity on DU 145 human prostate carcinoma cells. Biotechnol Bioeng 50:587–597.

Croughan MS, Wang DI. 1991. Hydrodynamic effects on animal cells in microcarrier bioreactors 17.

Edwards ME, Wang SS, Good TA 2001. Role of viscoelastic properties of differentiated SH-SY5Y human neuroblastoma cells in cyclic shear stress injury. Biotechnol Prog (in press).

Fujii MD, Patten BM. 1992. Neurology of microgravity and space travel. Neurol Clin 10:999–1013.

Fujita K, Lazarovici P, Guroff G. 1989. Regulation of the differentiation of PC12 pheochromocytoma cells. Environ Health Perspect 80:127–142.

Gage FH, Buzsaki G, Armstrong DM. 1990. NGF-dependent sprouting and regeneration in the hippocampus. Prog Brain Res 83:357–370.

Gally JA, Montague PR, Reeke GN Jr, Edelman GM. 1990. The NO hypothesis: possible effects of a short-lived, rapidly diffusible signal in the development and function of the nervous system. Proc Natl Acad Sci U S A 87:3547–3551.

Goodwin TJ, Prewett TL, Wolf DA, Spaulding GF. 1993a. Reduced shear stress: a major component in the ability of mammalian tissues to form three-dimensional assemblies in simulated microgravity. J Cell Biochem 51:301–311.

Goodwin TJ, Schroeder WF, Wolf DA, Moyer MP. 1993b. Rotating-wall vessel coculture of small intestine as a prelude to tissue modeling: aspects of simulated microgravity. Proc Soc Exp Biol Med 202:181–192.

Gregoriades N, Clay J, Ma N, Koelling K, Chalmers JJ. 2000. Cell damage of microcarrier cultures as a function of local energy dissipation created by a rapid extensional flow. Biotechnol Bioeng 69:171–182.

Gruener R, Hoeger G. 1990. Vector-free gravity disrupts synapse formation in cell culture. Am J Physiol 258:C489–C494.

Gruener R, Hoeger G. 1991. Vector-averaged gravity alters myocyte and neuron properties in cell culture. Aviat Space Environ Med 62:1159–1165.

Hindley S, Juurlink BH, Gysbers JW, Middlemiss PJ, Herman MA, Rathbone MP. 1997. Nitric oxide donors enhance neurotrophin-induced neurite outgrowth through a cGMP-dependent mechanism. J Neurosci Res 47:427–439.

Ishihara A, Ohira Y, Roy RR, Nagaoka S, Sekiguchi C, Hinds WE, Edgerton VR. 1997. Effects of 14 days of spaceflight and nine days of recovery on cell body size and succinate dehydrogenase activity of rat dorsal root ganglion neurons. Neuroscience 81:275–279.

Jessup JM, Goodwin TJ, Spaulding G. 1993. Prospects for use of microgravity-based bioreactors to study three-dimensional host-tumor interactions in human neoplasia. J Cell Biochem 51:290–300.

Kawasaki A, Otori Y, Barnstable CJ. 2000. Muller cell protection of rat retinal ganglion cells from glutamate and nitric oxide neurotoxicity. Invest Ophthalmol Vis Sci 41:3444–3450.

Krasnov IB. 1994. Gravitational neuromorphology. Adv Space Biol Med 4:85–110.

LaPlaca MC, Thibault LE. 1997. An in vitro traumatic injury model to examine the response of neurons to a hydrodynamically-induced deformation. Ann Biomed Eng 25:665–677.

Laplaca MC, Lee VM, Thibault LE. 1997. An in vitro model of traumatic neuronal injury: loading rate-dependent changes in acute cytosolic calcium and lactate dehydrogenase release. J Neurotrauma 14:355–368.

Lee YS, Ha JH, Yong CS, Lee DU, Huh K, Kang YS, Lee SH, Jung MW, Kim JA. 1999. Inhibitory effects of constituents of Gastrodia elata Bl. on glutamate- induced apoptosis in IMR-32 human neuroblastoma cells. Arch Pharm Res 22:404–409.

Lelkes PI, Galvan DL, Hayman GT, Goodwin TJ, Chatman DY, Cherian S, Garcia RM, Unsworth BR. 1998. Simulated microgravity conditions enhance differentiation of cultured PC12 cells towards the neuroendocrine phenotype. In Vitro Cell Dev Biol Anim 34:316–325.

Miller JD, McMillen BA, Murakami DM, McConnaughey MM, Williams HL, Fuller CA. 1985. Effects of weightlessness on neurotransmitter receptors in selected brain areas. Physiologist 28:S203–S204.

Miller JD, McMillen BA, McConnaughey MM, Williams HL, Fuller CA. 1989. Effects of microgravity on brain neurotransmitter receptors. Eur J Pharmacol 161:165–171.

Otori Y, Wei JY, Barnstable CJ. 1998. Neurotoxic effects of low doses of glutamate on purified rat retinal ganglion cells. Invest Ophthalmol Vis Sci 39:972–981.

Phung YT, Bekker JM, Hallmark OG, Black SM. 1999. Both neuronal NO synthase and nitric oxide are required for PC12 cell differentiation: a cGMP independent pathway. Brain Res Mol Brain Res 64:165–178.

Prewett TL, Goodwin TJ, Spaulding GF. 1993. Three-dimensional modeling of T-24 human bladder carcinoma cell line: a new simulated microgravity culture vessel. J Tissue Cult Meth 15.

Riley DA, Ilyina-Kakueva EI, Ellis S, Bain JL, Slocum GR, Sedlak FR. 1990. Skeletal muscle fiber, nerve, and blood vessel breakdown in space-flown rats. Faseb J 4:84–91.

Robertson D, Convertino VA, Vernikos J. 1994. The sympathetic nervous system and the physiologic consequences of spaceflight: a hypothesis. Am J Med Sci 308:126–132.

Roy RR, Baldwin KM, Edgerton VR. 1996. Response of the neuromuscular unit to spaceflight: what has been learned from the rat model. Exerc Sport Sci Rev 24:399–425.

Schuman EM, Madison DV. 1991. A requirement for the intercellular messenger nitric oxide in long-term potentiation. Science 254:1503–1506.

Spaulding GF, Jessup JM, Goodwin TJ. 1993. Advances in cellular construction. J Cell Biochem 51:249–251.

Tischler AS, Greene LA. 1978. Morphologic and cytochemical properties of a clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Lab Invest 39:77–89.

Triyoso DH, Good TA. 1999. Pulsatile shear stress leads to DNA fragmentation in human SH-SY5Y neuroblastoma cell line. J Physiol (Lond) 515:355–365.

Valtorta F, Leoni C. 1999. Molecular mechanisms of neurite extension. Philos Trans R Soc Lond B Biol Sci 354:387–394.

Van der Zee CE, Fawcett J, Diamond J. 1992. Antibody to NGF inhibits collateral sprouting of septohippocampal fibers following entorhinal cortex lesion in adult rats. J Comp Neurol 326:91–100.

Venkat RV, Stock LR, Chalmers JJ. 1996. Study of hydrodynamics in microcarrier culture spinner vessels: A particle tracking velocimetry approach. Biotechnol Bioeng 49:456–466.

Ye ZC, Sontheimer H. 1998. Astrocytes protect neurons from neurotoxic injury by serum glutamate. Glia 22:237–248.

Yoshioka A, Ikegaki N, Williams M, Pleasure D. 1996. Expression of N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptor genes in neuroblastoma, medulloblastoma, and other cells lines. J Neurosci Res 46:164–178.

Zhuo M, Small SA, Kandel ER, Hawkins RD. 1993. Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. Science 260:1946–1950.