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PRINCIPAL INVESTIGATOR: S. Ward Casscells, M.D.

CONTRACTING ORGANIZATION: University of Texas Health Science Center at Houston
Houston TX 77030-3900

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S. Ward Casscells, M.D.

E-Mail: S.Ward.Casscells@uth.tmc.edu

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14. ABSTRACT
The primary objectives of the DREAMS project for FY 2006 are threefold: 1) further advance our studies focused on delineating the mechanisms underlying cell protection under stress conditions in wounds, 2) continue development of a new axial flow pump designed to maintain cardiac output in cardiogenic and hemorrhagic shock, and 3) initiate a study designed to optimize the biocompatibility of carbon-based nanomaterials suitable for military applications. These three objectives are in accord with the Science, Triage, and Treatment (STAT) component of DREAMS that aims to develop new ways of diagnosing and treating tissue injuries and infection in combat situations.

15. SUBJECT TERMS
None provided.

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TABLE OF CONTENTS

PROJECT I.C.1

Molecular Regulation of Apoptosis in Wound Healing

Principal Investigator:

Yong-Jian Geng, MD, PhD

INTRODUCTION

For the continued work involving cell protection under stress conditions (Project I.C.1), we will continue to focus on regulating vascular cell apoptosis during wound healing. Thus far, we have identified several stress-responding factors that are potentially important for protecting vascular cells against apoptotic stimulation by oxidized cholesterol or oxysterols. We have found that gelsolin-deficient smooth muscle cells become less sensitive to stress factors such as gentle heating. In the presence of proinflammatory cytokines, many cells in the wound are generating nitric oxide at high levels as the consequence of expression of a cytokine-inducible nitric oxide synthase. Studies performed during DREAMS FY 2004 showed that heat shock reduces expression of the nitric oxide-synthesizing enzyme in smooth muscle cells. Interestingly, we observed that smooth muscle cells deficient in expression of the actin-binding protein gelsolin become resistant to the heat shock response, suggesting that heat shock-induced change in nitric oxide production may be mediated by gelsolin.

In order to continue this study, we are collaborating with colleagues at Harvard Medical School in order to gain access to gelsolin-knockout mice, which we intend to use for our in vivo studies. Difficulties in the original research design prevented our use of these animals in FY 2004 and thus, we aim to validate our promising in vitro results in the gelsolin-knockout mouse model. We anticipate that in FY 2005, we will be able to identify stress factor-associated regulatory proteins in controlling cell death or proliferation.

RESULTS & DISCUSSION

Superoxide is one of the main pro-oxidants in the cell and as such, SOD serves a key antioxidant role. The physiological importance of SODs is illustrated by the severe pathologies evident in mice genetically engineered to lack these enzymes. Mice lacking SOD2 die several days after birth, amidst massive oxidative stress3. Mice lacking SOD1 develop a wide range of pathologies, including hepatocellular carcinoma4, an acceleration of age-related muscle mass loss5, an earlier incidence of cataracts and a reduced lifespan. Mice lacking SOD3 do not show any obvious defects and exhibit a normal lifespan.

For this portion of the study, murine embryonic stem cells (ESC) were infected with adenoviral vectors carrying SOD cDNA. After creation of mouse skin wound lesions, SOD-ESCs were implanted within the lesion, which were measured for size pre- and post-implantation over a two-week period. Shown below is a mouse with a lesion treated with SOD-ESCs after seven days and compated with a lesion treated with PBS buffer alone. Each of the lesions were of comparable size prior to cell implantation.

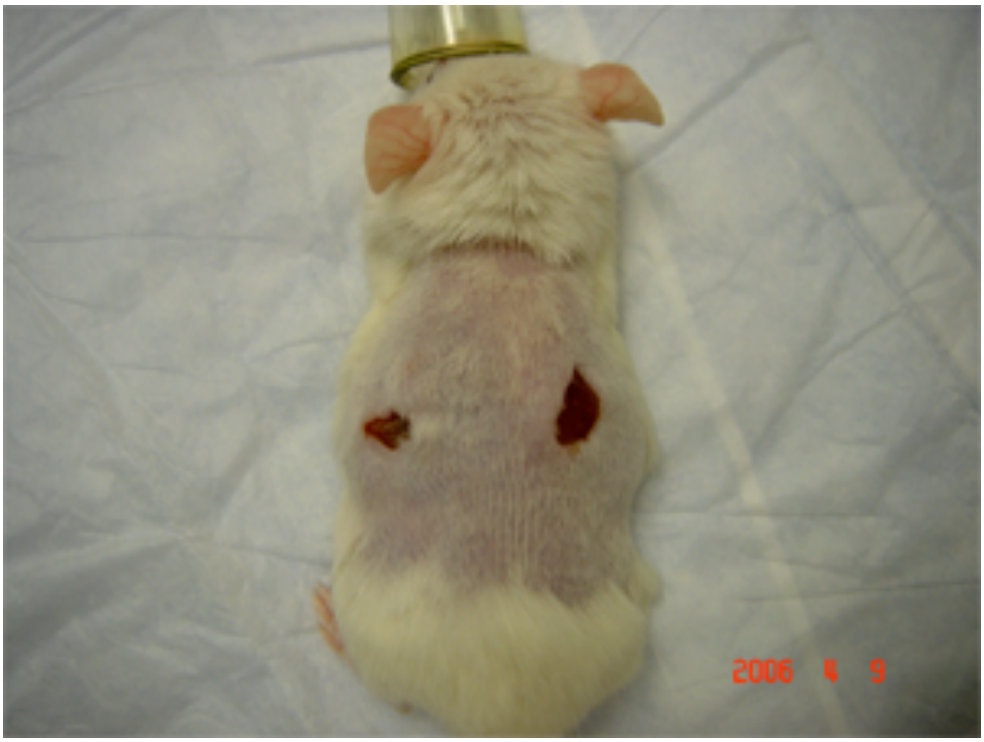

FIGURE 1.
Lesion treated with SOD-ESCs (shown at left) compared to PBS-treated wound (shown at right) after seven days. Note the diminished overall size of the lesion after stem cell treatment.

Further studies compared the efficacy of SOD-ESCs with lacZ-ESCs in wound healing in vivo. After injection of 0.5×10^6 cells and measuring wound size via assumption of a geometrical area determined by length vs width (in mm^2), wound healing was assessed daily for a two week period. As can be seen below, SOD-ESCs were better suited for wound healing, where less than ~10% of the wound remained after two weeks, as compared to the lacZ-ESCs, where ~20% of the wound remained after two weeks.

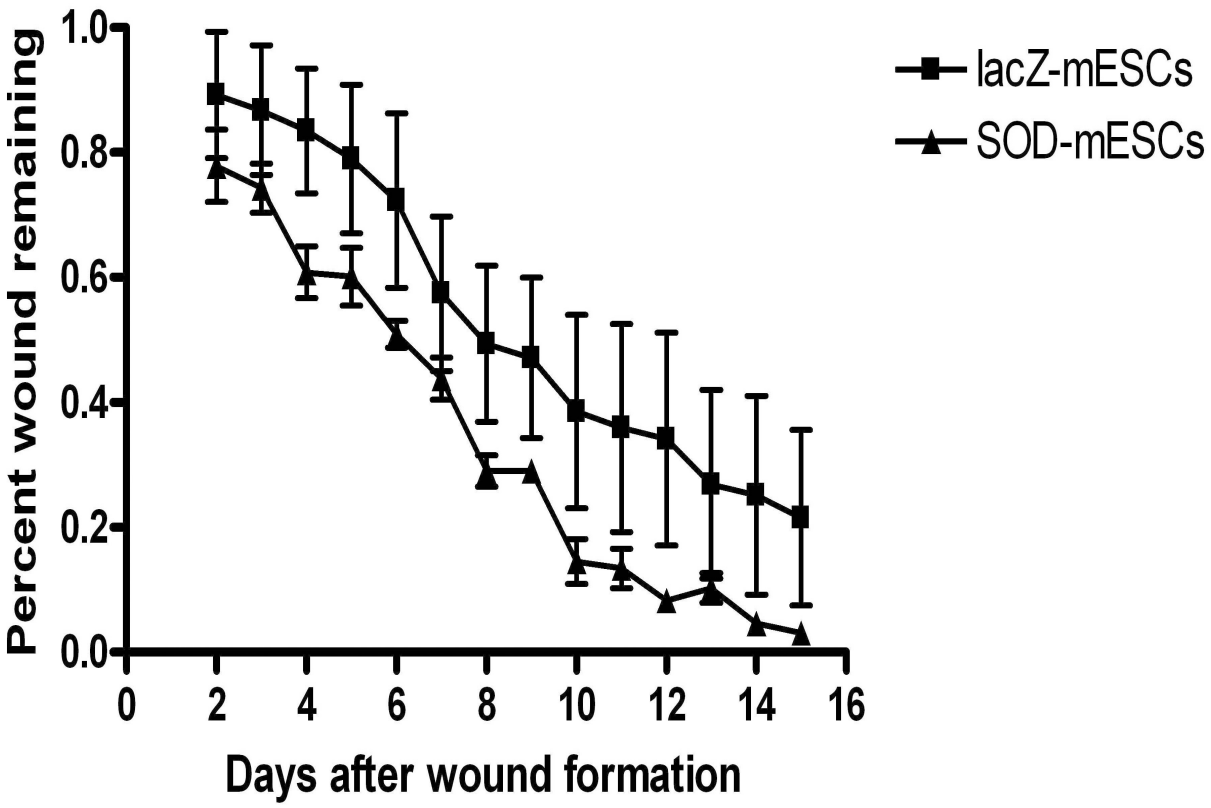

FIGURE 2.
Kinetics of sink wound healing in lacZ-mESC and SOD-ESC treated SCID mice. Wound area is determined by measuring length and width. Each value represents mean ± SEM (n = 4 and $P < 0.05$ at days 3-7.

KEY RESEARCH ACCOMPLISHMENTS

This project made considerable progress this year, which can be summarized by four main accomplishments, below.

- Successfully conducted high-throughput cDNA microarray experimentation using vascular tissue damaged by atherosclerosis via oxidized lipids and inflammation.
- Identified at least twelve (12) genes involved in the tissue response to wound and oxidative injury.
- Characterized in vitro several candidate genes involved in wound healing, such as apoj and SOD.
- Performed the first attempt at using SOD-gene transfected stem cells for wound healing in an apoE k/o mouse model.

CONCLUSIONS

The following conclusions were drawn from the present study.

- Stem cell implantation promoted wound healing, especially those with genetically manipulated cells with SOD gene delivery via an adenoviral vector
- Vascular inflammation characterizes unstable atherosclerotic plaques
- Caspase expression and activation may occur in rupturing or ruptured plaques
- Degradation of extracellular matric releases biologically active peptides
- HDL-associated apolipoprotein-J may regular vascular inflammation and apoptosis

NEXT YEAR'S RESEARCH GOALS

The funding for the DREAMS program, which supported the work performed under this project in fiscal year 2006, has expired and therefore will not continue the work originally described in the DREAMS 2006 scope of work. However, the investigator will seek additional funds through TATRC for additional support of this work to further advance the in vivo work in Apo-E k/o mice.

PUBLICATIONS & PRESENTATIONS

1. Madonna, R; Di Napoli, P; Massaro, M; Grilli, A; Felaco, A; De Caterina, A; Tang, D; Geng, YJ. *Simvastatin attenuated expression of cytokine-inducible nitric oxide synthase in embryonic cardiac myoblasts,* J. Biol. Chem., 8, 280(14), 13503, 2005.

2. Willerson, JT; Yeh, ET; Geng, YJ; Perin, EC. *Blood-derived progenitor cells after recanalization of chronic coronary artery occlusions in humans.* Circulation Res. 14, 97(8), 735, 2005.

3. He, R; Guo, DC; Estrera, AL; Safi, HJ; Huynh, TT; Yin, Z; Cao, SN; Lin, J; Kurian, T; Buja, LM; Geng, YJ; Miliwicz, DM. *Characterization odf the inflammatory response and apoptotic cells in the aortas of patients with ascending thoracic aortic aneurysm and dissections.* J. Thorac. Cardiovascl. Surg., Machr, 131(3), 671, 2006.

4. Madonna, R; Rinaldo, L; Rossi, C; Geng, YJ; De Caterina, R. *Protracyclin improves transcoronary myocardial delivery of adipose tissue-derived stromal cells.* Eur. Heart. J., 2006 (in press)

PROJECT II.E

Initial Evaluation of a New Axial Flow Pump to Maintain Cardiac Output

Principal Investigator:

O.H. Frazier, MD

INTRODUCTION
The Cardiovascular Research Laboratory (CVRL), under the direction of Dr. O.H. Frazier at the Texas Heart Institute, discontinued this project in fiscal year 2005 and did not carry out experiments during the performance period of this reporting cycle. No funds were spent on this project as a result.

RESULTS & DISCUSSION
None Available.

KEY RESEARCH ACCOMPLISHMENTS
None Available.

CONCLUSIONS
None Available.

NEXT YEAR'S RESEARCH GOALS
This project has been completed and will not receive any future funding.

PUBLICATIONS & PRESENTATIONS
No publications and/or presentations for fiscal year 2006

PROJECT III.D

Biocompatibility of Carbon-Based Nanomaterials

Principal Investigator:

Jodie L. Conyers, PhD

INTRODUCTION

The focus on project III.D in fiscal year 2006 was to characterize the toxicity and biocompatibility of a number of water-soluble fullerene and carbon nanotube constructs in a variety of cell cultures. Currently, our laboratory is focused on better understanding the interaction of fullerenes and nanotubes with living cells. We are developing new synthetic strategies to render various fullerenes and nanotubes more water soluble and more biocompatible. By doing so, we hope to develop a new class of carbon-based nanomaterials that can exist within the human body and provide some predetermined therapeutic and/or diagnostic advantage currently unachievable with current materials.

We anticipate that these unique materials can be designed to target specific sites of disease, infection, or injury within the body once introduced into the blood stream, whereby they may deliver a payload of drugs or remain localized within the diseased area for diagnosis (e.g. using CT/MRI imaging). Certain fullerene derivatives may also prove useful in combating the deleterious effects of chemical and/or biological warfare agents and radiation exposure. Before one can evaluate these applications in animal and/or human subjects, we must first fully understand how these nanomaterials impact living cells.

RESULTS & DISCUSSION

A total of six (6) cell types were used for this work for assessing nanomaterial cytotoxicity. Each of the cell lines is described below.

- HepG2 human liver cells, an epithelial hepatocellular carcinoma line (AATC)
- Human coronary artery (HCA) smooth muscle cells (Cascade Biologics, Inc.), positive for a-actin expression and negative for von Willebrand factor (vWf)
- Mouse macrophage (Cambrex, Inc.), *Mus. Musculus* strain BALB/c, expresses interleukin I, lysozyme, and complement C3, active in antibody-dependant phagocytosis

- Human renal, renal cortical, and renal proximal tubule epithelial cells (Cambrex, Inc.), positive stain for cytokeratin
- Cardiac myocytes (canine derived)
- Human coronary artery (HCA) endothelial cells (Cambrex, Inc.), positive for acetylated LDL and vWf, negative for smooth muscle a-actin

Fullerenes used for this study included two amphiphilic fullerenes, AF1 and AF3, C3, and DF1, whereas nanotube samples included pluronic-wrapped single-walled carbon nanotubes (SWNTs), phthalic acid covalently modified, and sulfonic acid covalently modified.

Cytotoxicity was assessed via a cell proliferation assay (MTT), the cyto-tox ONE homogeneous membrane integrity assay (LDH), an Annexin-V-FLUOS flow cytometric assay, and a cellular DNA fragmentation ELISA.

Figure 1 below shows the results of the MTT assay in mouse macrophage with a number of carbon nanomaterials at 10- and 100-fold dilution. Slight toxicity was observed for concentrated sulfonated nanotubes and AF3 but little toxicity when diluted. In liver cells, however, all but the amphi-fullerene AF1 showed significant cytotoxicity, especially the pluronic wrapped and sulfonic acid-functionalized nanotubes (Figure 2).

Figure 3 shows the results of cellular toxicity as determined by the LDH membrane integrity assay in macrophage cells. Compared to control, all nanomaterials exhibited little to no toxicity, a result that was consistent to those collected in liver (Figure 4) and kidney (Figure 5) cells.

Figure 6 reports the results of the DNA ELISA assay used for toxicity assessment in smooth muscle cells. Here, we observed toxicity when concentrated phthalic acid and sulfonated tubes were used, but not when the samples were diluted up to 100-fold. Similar results were observed in macrophage (Figure 7) and Liver (Figure 8) cells.

Figure 9 summarizes the toxicity assays and nanomaterial samples used for this study, where grey boxes are those experiments that have been completed. Overall, we are consistently seeing little cytotoxicity with various carbon nanomaterials, especially the water-soluble fullerene constructs. While it is known that SWNTs have a propensity to aggregate and “bundle” in solution, limited toxicity of nanotube samples could be attributed to chemical functionalities that allow individual nanotubes to overcome the van derWalls attraction between tubes. This behavior could potentially be overcome by increasing the overall size of the chemical functional groups.

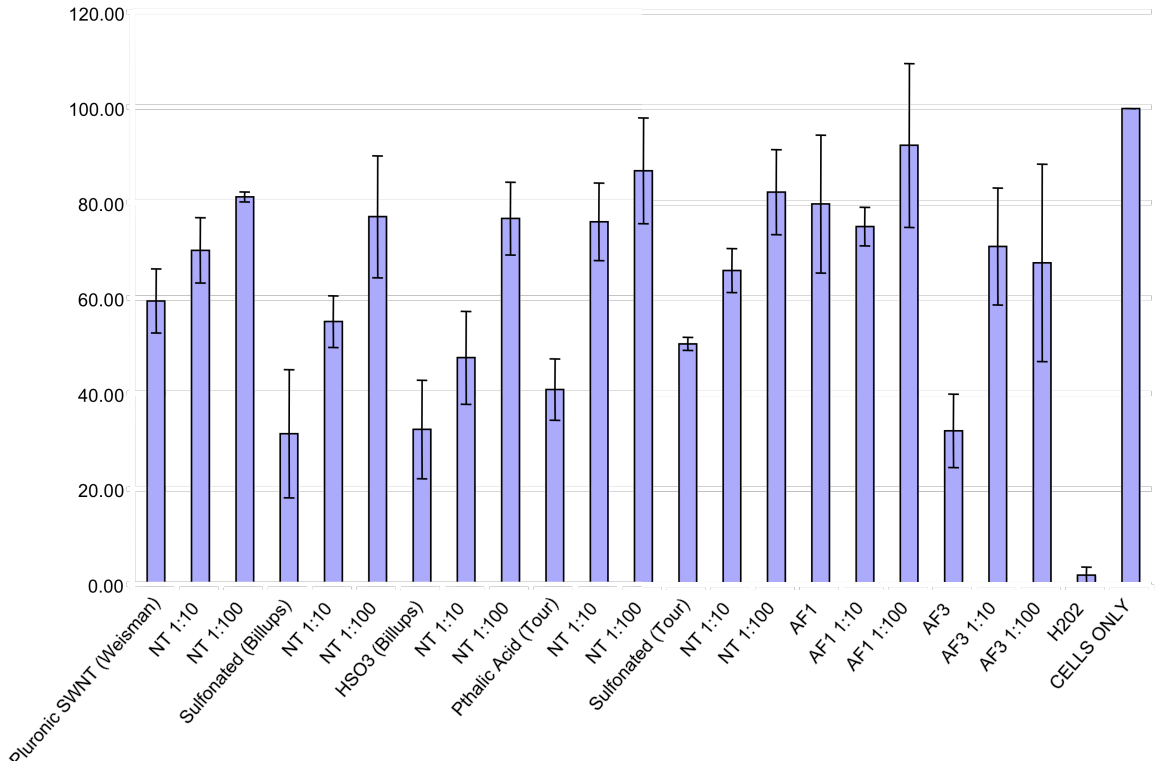

FIGURE 1. MTT in Macrophage Cells

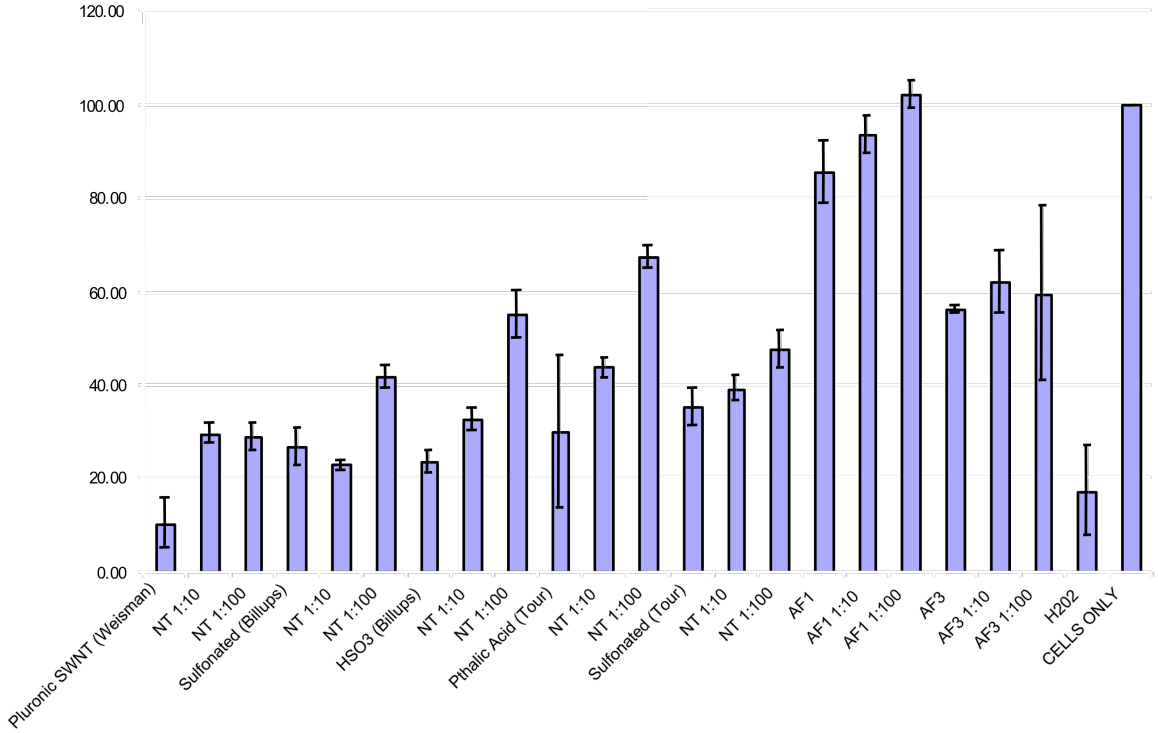

FIGURE 2. MTT in Liver Cells

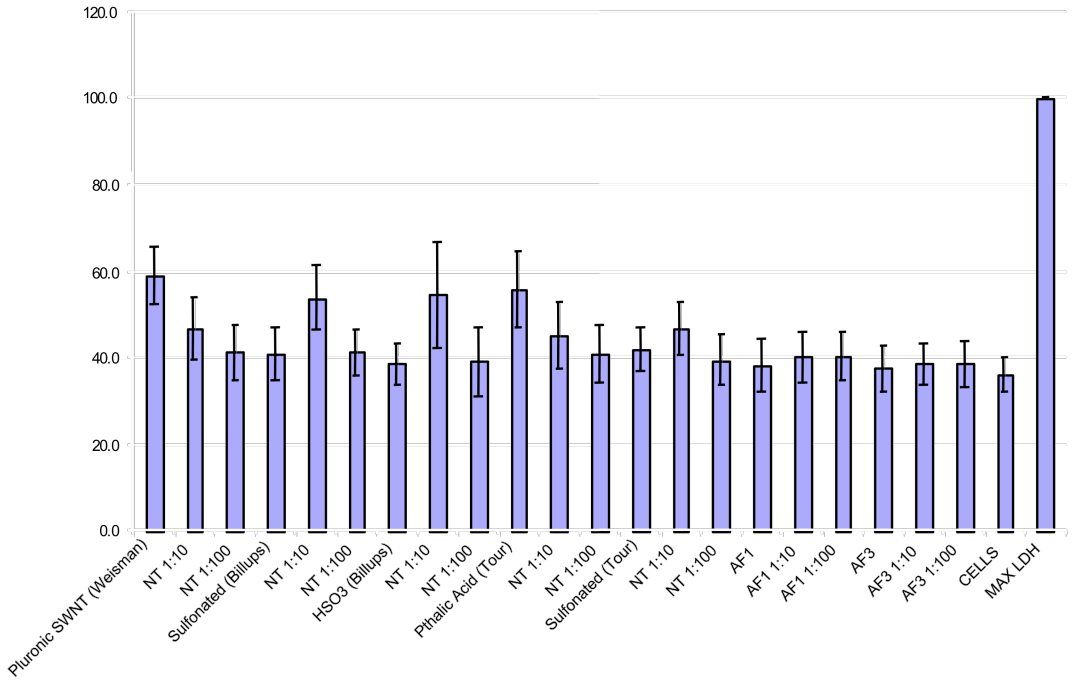

FIGURE 3. LDH in Macrophage Cells

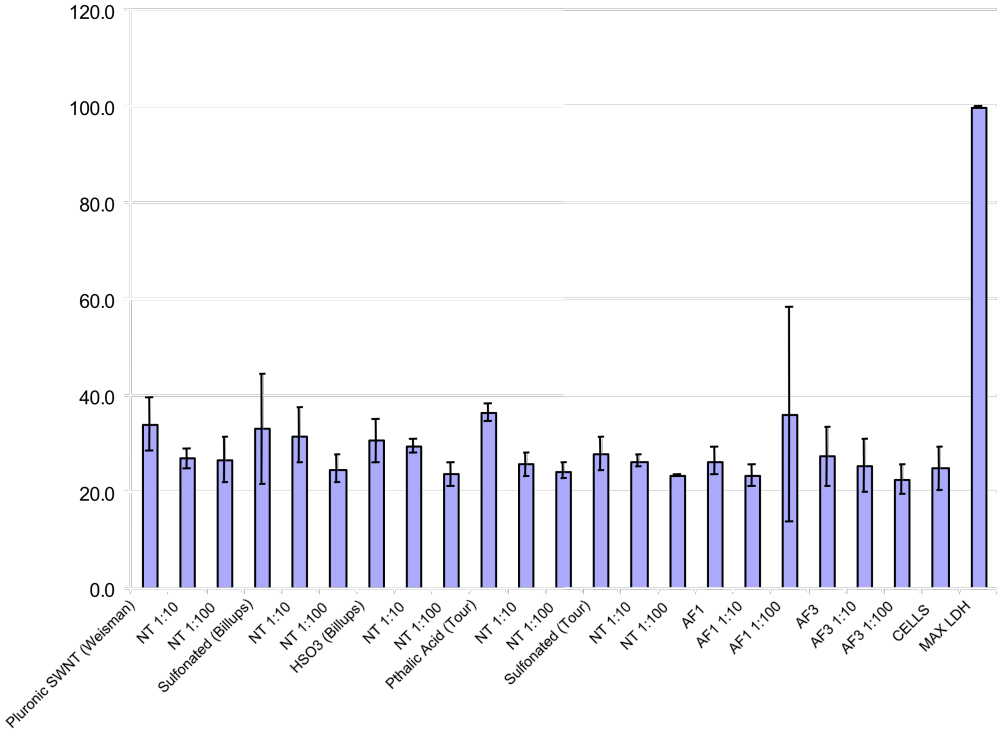

FIGURE 4. LDH in Liver Cells

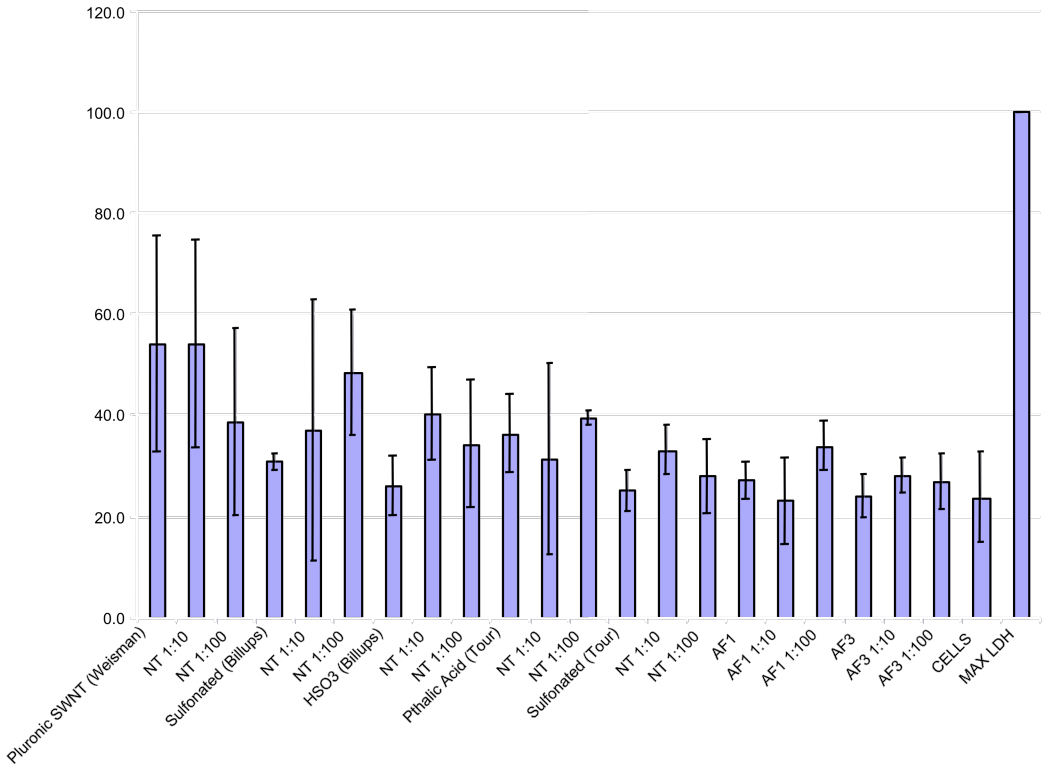

FIGURE 5. LDH in Kidney Cells

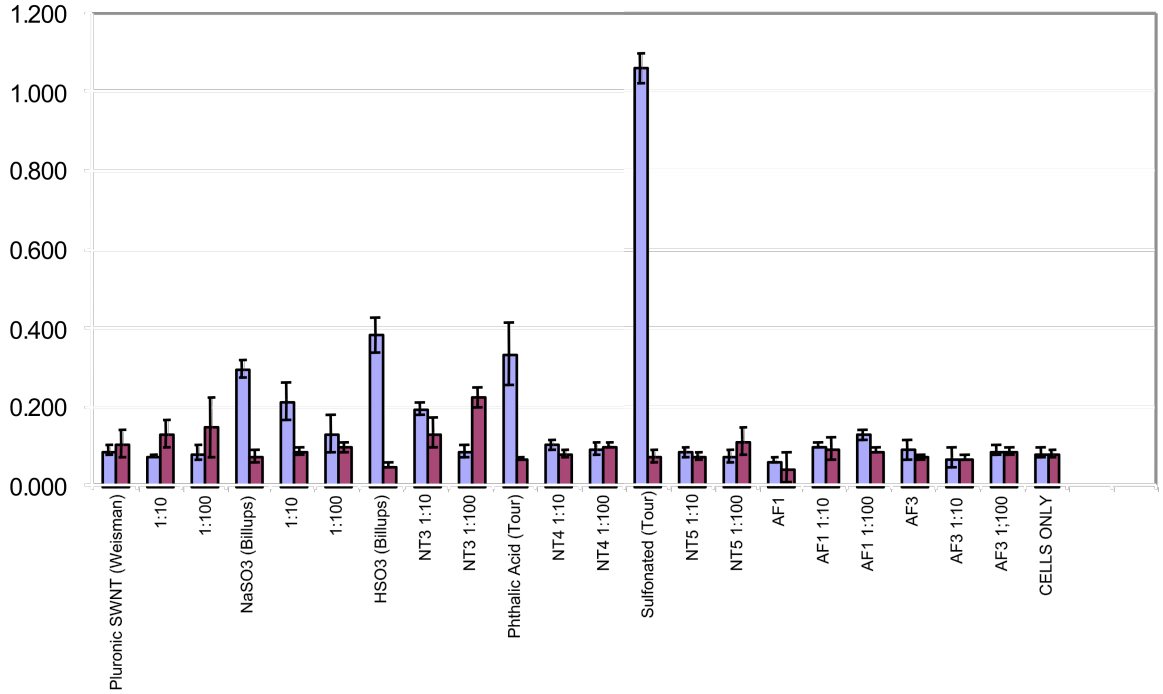

FIGURE 6. CDF ELISA in Smooth Muscle Cells

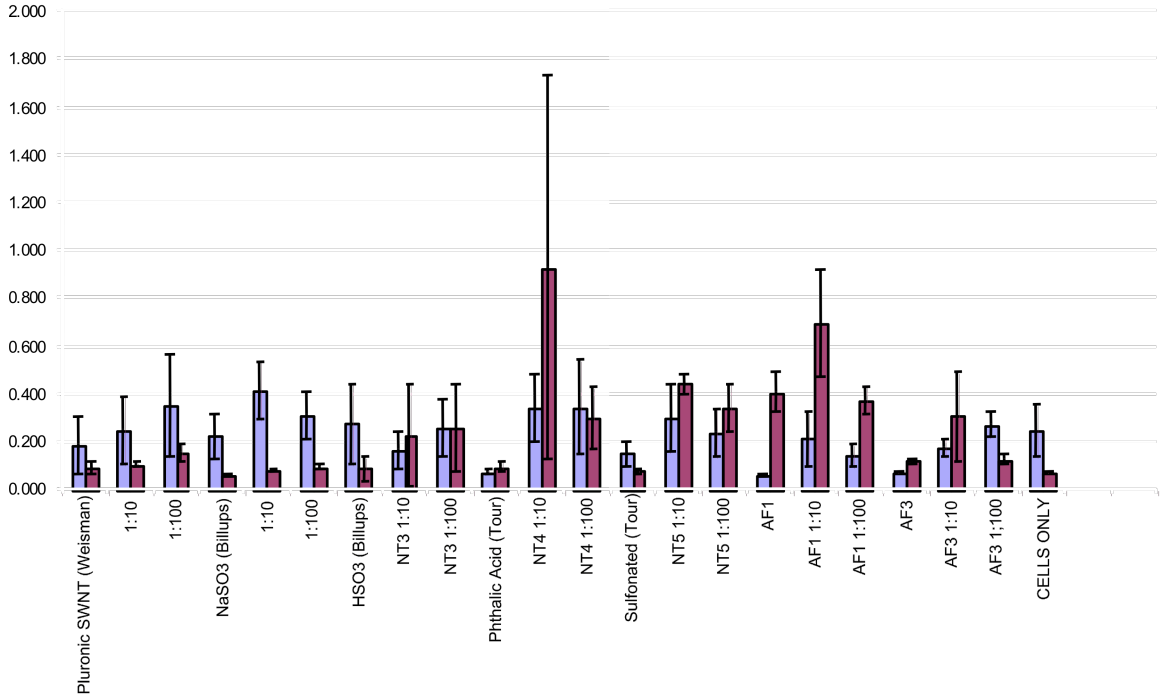

FIGURE 7. CDF ELISA in Macrophage Cells

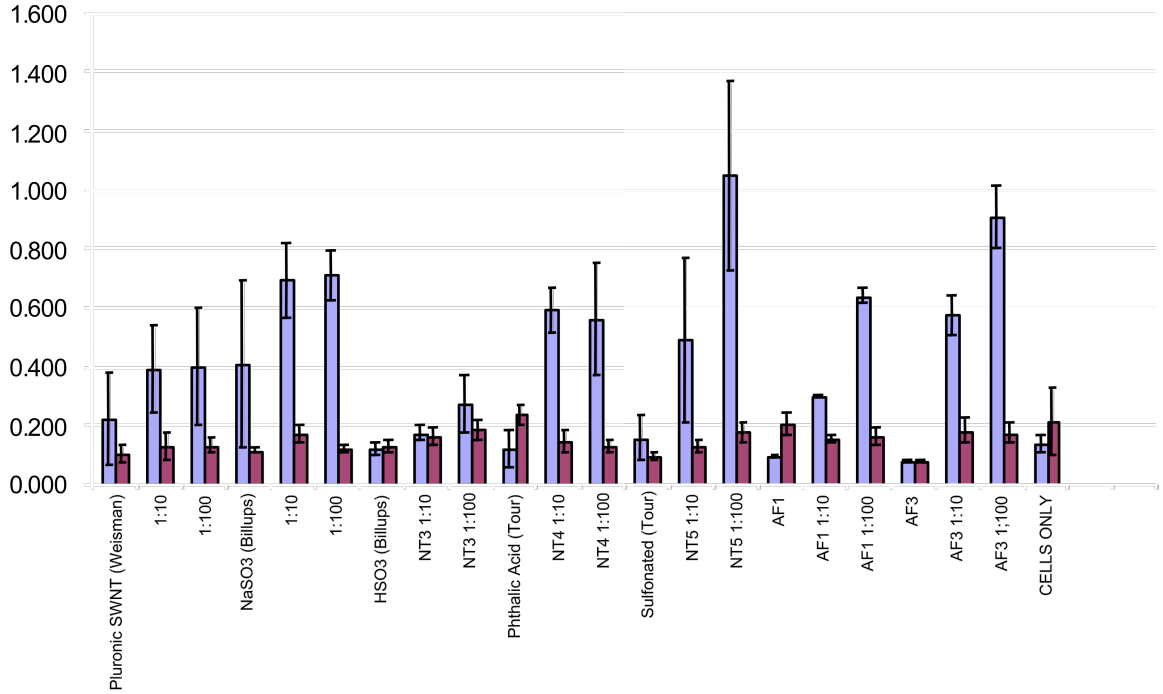

FIGURE 8. CDF ELISA in Liver Cells

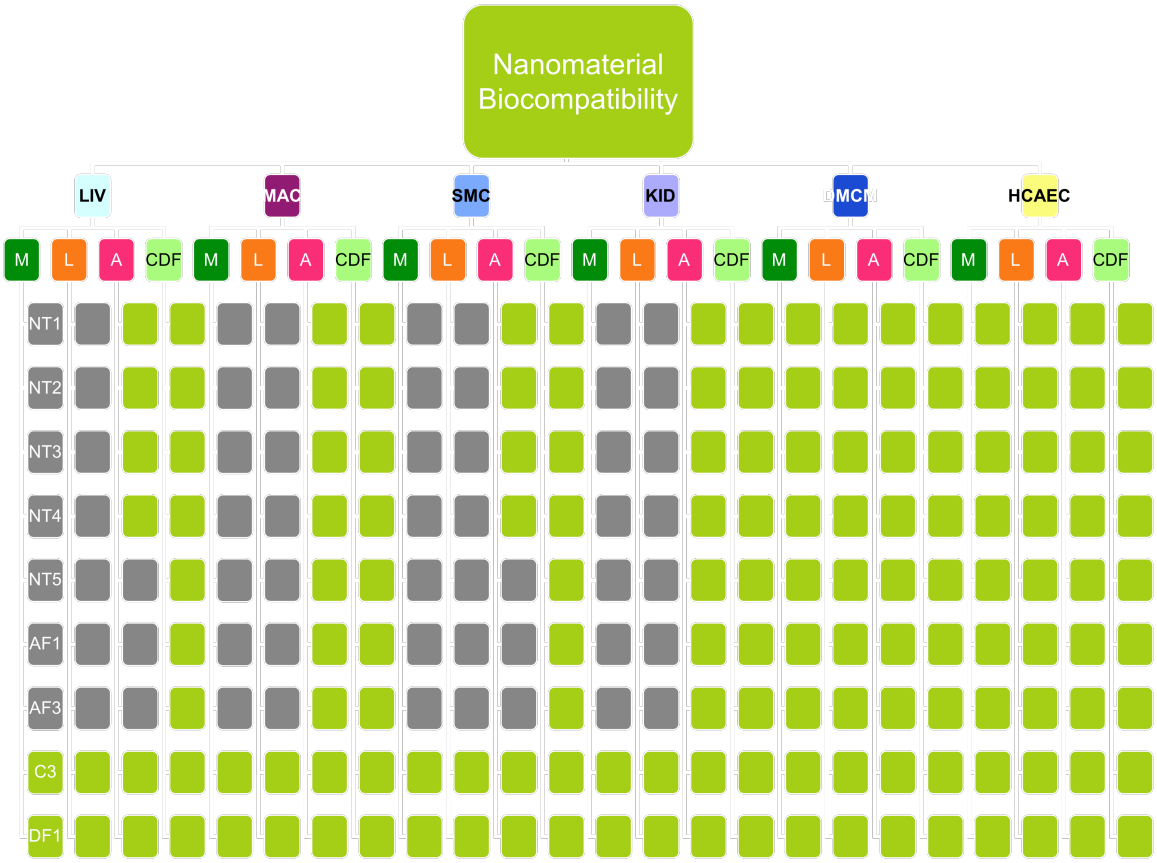

FIGURE 9.
Experiments completed (grey) versus those remaining (green) for the MTT (dark green), LDH (orange), annexin-V (pink), and CDF DNA (light green) assays in each of the cell types (LIV-liver, MAC-macrophage, SMC-smooth muscle, KID-kidney, DMCM-cardiac myocytes, HCAEC-endothelial cells).

KEY RESEARCH ACCOMPLISHMENTS

This work was an initial attempt at assessing the biocompatibility of carbon-based nanomaterials that are being designed for specific biological applications, such as drug delivery, diagnostic imaging, and protection from oxidative injury. While little has been known about the fate of biological systems in the presence of fullerenes and nanotubes, suitable functionalization with water solubilizing ligands should be able to overcome toxicities associated with aggregation, oxidation, and insolubility.

CONCLUSIONS

With adequate water solubility designed within carbon-based nanomaterial constructs, cellular toxicity can be overcome. However, significant variability in the data is observed in a number of cell types and therefore cannot be relied

upon. A better understanding of the cellular process of nanomaterial internalization is required before we can make assumptions about what molecular pathways the nanomaterials affect, if any.

NEXT YEAR'S RESEARCH GOALS

Funding for this project ended in September 2006 and therefore will not continue under this funding source.

PUBLICATIONS & PRESENTATIONS

This work was basic research and has yet to be presented or published for peer review.