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Characterization and Application of Dynamic in vitro Models of Human Airway

Hemangkumar J. Patel
Utah State University

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CHARACTERIZATION AND APPLICATION OF DYNAMIC IN VITRO MODELS

OF HUMAN AIRWAY

by

Hemangkumar J. Patel

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

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2011
ABSTRACT

Characterization and Application of Dynamic in vitro Models of Human Airway

by

Hemangkumar J. Patel, Doctor of Philosophy
Utah State University, 2011

Major Professor: Dr. Soonjo Kwon
Department: Biological Engineering

In recent years, respiratory diseases have emerged as a leading cause of mortality across the globe. In the United States alone respiratory diseases are the fourth leading cause of deaths annually. Moreover, with the rapid increase of industrialization and urbanization, the occurrences of respiratory diseases are expected to remain high with strong chances of increasing in the future. To ameliorate the epidemic of respiratory disease, it is first important to understand its underlying mechanisms.

Respiratory research studies in animals have elucidated the chronological order of the pathological events and systemic responses inside the lung, but understanding the response of individual cell types inside the lung is necessary to outline the initiators and mediators of the pathological events. Many research studies have aimed to understand the behavior of individual cell types, from the lung, under different pathological conditions specific to the respiratory system. However, the cell culture systems used in most of these studies were limited by the absence of the dynamic cell growth environment present in actual lung tissues. The lung exists in a mechanically active environment, where different
amounts of circumferential and longitudinal expansion and contraction occur during breathing movements. Thus, simulating the biomechanical environment in \textit{in vitro} cell culture models may improve the cellular functionality and the outcome of the research studies. Moreover, the stimulation of biomechanical forces in \textit{in vitro} cell cultures provides the advantage of mimicking the mechanical environment, related to different pathological conditions.

In our study we used a dynamic \textit{in vitro} cell culture system capable of implementing cyclic equibiaxial deformation in cell monolayers to stimulate different biomechanical environments similar to conditions inside the lung. The dynamic cell growth condition was used to determine the effects of ventilator-induced lung injury and nano-material/pollutant exposure in A549 cell cultures. Examples of such pollutants are diesel particulate matter, multi-walled carbon nanotubes, and single-walled carbon nanotubes. Our results indicated that the dynamic cell growth condition specific to ventilator induced lung injury facilitated an increase in inflammatory and tissue remodeling activities in A549 cells. Under the nano-material/pollutant exposure assessment studies, the dynamic cell growth condition induced changes in inflammation and oxidative stress level which closely resembled those in \textit{in vivo} studies.
To my parents

Jayantibhai Patel and Minaxiben Patel

And my loving wife

Nisha Patel
I would like to express my deepest gratitude to my professor and mentor, Dr. Soonjo Kwon, for extending me the opportunity to dream, create, and discover in his laboratory during my doctoral studies. I would also like to thank other professors on my graduate advisory committee, including Dr. Timothy Doyle, Dr. Kamal Rashid, Dr. David Britt, and Dr. Anhong Zhou, for their valuable guidance, support, and advice. Special thanks to Dr. Kytai Nguyen for laying down a strong foundation in my development as a scientist. I would like to thank Dr. Ronald Sims and all Biological Engineering department staff members, including Anne Martin, Paul Veridian, and Jed Moss, for their support and help with all graduate student formalities and activities.

I am also thankful to all members (Aaron Winder and Joseph Camire) and alumni (Rena Baktur, Erik Ostler, Ross Booth, Emily Stoker, Forest Purser, Sterling Fife, Jaewook Chung) of the Integrated Tissue Engineering Lab for providing a supportive work environment and extending their friendship. I am also thankful to all my friends at Utah State University (Sitaram Harihar, Tripti Bhaskaran, Kripa Nidhan Chauhan, Mukta Sharma Chauhan) for creating a friendly environment during the course of my studies. Special thanks to my friends (Nilesh Prajapati, Vishal Patel, Naresh Solanki, Sunil Desai, Tarak Patel, Nikunj Patel, Vikas Deshpande, Suraaj Vyas, and Venkat Krishnaraj) for encouraging me to join the doctoral studies and providing me with friendly support and advice during my time at Utah State University.

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for their enormous encouragement, support, and love without which this journey would not have been possible for me. I am really grateful for their patience and love during my studies.

Hemangkumar J. Patel
CONTENTS

ABSTRACT ................................................................................................................ iii

ACKNOWLEDGEMENTS ........................................................................................ vi

LIST OF TABLES ..................................................................................................... xii

LIST OF FIGURES .................................................................................................. xiii

LIST OF SYMBOLS, NOTATIONS, AND DEFINITIONS ................................... xix

CHAPTER

1 INTRODUCTION ................................................................................................... 1

1.1 Background ...................................................................................................... 1

1.2 Respiratory Research Using Whole-Animal Research Models ....................... 2

1.3 Use of in vitro Cell Culture Models for Respiratory Research ...................... 3

1.4 Experimental Model Selection ......................................................................... 6

1.5 Ventilation-Induced Lung Injury ..................................................................... 7

1.6 Evaluation of DPM Exposure in Respiratory System ...................................... 9

1.7 Evaluation of Carbon Nanotube Exposure in Respiratory System .......... 13

1.8 Hypothesis and Objectives ............................................................................. 15

1.9 Dissertation Outline ....................................................................................... 16

1.10 References ...................................................................................................... 17

2 INTERPLAY BETWEEN CYTOKINE-INDUCED AND CYCLIC EQUIBIAXIAL DEFORMATION-INDUCED NITRIC OXIDE PRODUCTION AND METALLOPROTEASES EXPRESSION IN HUMAN ALVEOLAR EPITHELIAL CELLS ........................................................................................... 27

2.1 Abstract .......................................................................................................... 27

2.2 Introduction .................................................................................................... 28

2.3 Materials and Methods ................................................................................... 31

2.3.1 Cell Culture ............................................................................................ 31

2.3.2 Exposure of A549 Monolayers to TNF-α .............................................. 31

2.3.3 Cyclic Equibiaxial Deformation ............................................................ 31

2.3.4 Nitrite Measurement .............................................................................. 33

2.3.5 Total MMP-2 Expression .................................................................... 33
4 ALTERNATIVE ESTIMATION OF HUMAN EXPOSURE TO MULTI-WALLED CARBON NANOTUBES USING DYNAMIC CULTURE OF AIRWAY EPITHELIAL CELLS

4.1 Abstract

4.2 Introduction

4.3 Materials and Methods

4.3.1 Cell Culture

4.3.2 MWCNT Solution Preparation

4.3.3 MWCNT Exposure Study

4.3.4 IL-8 Measurement

4.3.5 Reactive Oxygen Species (ROS) Measurement

4.3.6 GSH Measurement

4.3.7 Total Protein Measurement

4.3.8 Statistical Analysis

4.4 Results

4.4.1 Effect of MWCNT Exposure on A549 Cell Growth

4.4.2 Effect of MWCNT Exposure on Cellular Inflammation

4.4.3 Characterization of Oxidative Stress Through ROS Measurement

4.4.4 Characterization of Oxidative Stress Through GSH Measurement

4.5 Discussion

4.7 References

5 EFFECT OF DYNAMIC ENVIRONMENT ON THE INTERACTION BETWEEN SINGLE-WALLED CARBON NANOTUBE AND HUMAN AIRWAY EPITHELIAL CELL MONOLAYER

5.1 Abstract

5.2 Introduction

5.3 Materials and Methods

5.3.1 Cell Culture

5.3.2 Cyclic Equibiaxial Deformation

5.3.3 Short/Long-SWCNT Solution Preparation

5.3.4 Exposure of Short/Long-SWCNT to A549 cells

5.3.5 Total Protein Measurement

5.3.6 IL-8 Measurement

5.3.7 Reactive Oxygen Species (ROS) Measurement
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Relationship between equibiaxial elongations level and percentage total lung capacity</td>
<td>6</td>
</tr>
<tr>
<td>2.1</td>
<td>Qualitative analysis results from zymography of media samples from A549 cells exposed to cyclic equibiaxial deformation in the absence of TNF-α (A – Active, I – Inactive)</td>
<td>41</td>
</tr>
<tr>
<td>2.2</td>
<td>Qualitative analysis results from zymography of media samples from A549 cells exposed to cyclic equibiaxial deformation in the presence of TNF-α (A – Active, I – Inactive).</td>
<td>42</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>a) Flexcell® 4000T Plus™ System, b) Working diagram of Flexcell® 4000T Plus™ System. Figure adapted from Flexcell® 4000T Plus™ System user manual.</td>
</tr>
<tr>
<td>2.1</td>
<td>(a) Experimental Scheme for cyclic equibiaxial deformation of A549 cells. Following confluency, A549 cell monolayers were incubated in serum-free media for 24 hours. The cell monolayers were then either exposed to the different levels of cyclic equibiaxial deformation for 24 hours and grown in static condition for another 48 hours or growth in static condition. Time 0 hour marks the end of serum free media incubation and refers to the starting point of cyclic equibiaxial deformation or static condition growth (for controls). (b) Experimental Scheme for cyclic equibiaxial deformation of A549 cells in presence of TNF-α. Following confluency, A549 cell monolayers were incubated in serum-free media for 24 hours before TNF-α exposure. Cell layers were exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour marks the end of TNF-α exposure and refers to the starting point of cyclic equibiaxial deformation.</td>
</tr>
<tr>
<td>2.2</td>
<td>(a) Effect of cyclic equibiaxial deformation on inflammatory response of A549 cells. Nitrite concentration was measured from media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour refers to the starting point of cyclic equibiaxial deformation. *significantly higher than the control (p &lt; 0.05). (b) Effect of cyclic equibiaxial deformation on A549 cell proliferation. Total protein concentration was measured in cell lysate of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour refers to starting point of cyclic equibiaxial deformation. *significantly higher than the control (p &lt; 0.05).</td>
</tr>
</tbody>
</table>
2.3 a) Effect of cyclic equibiaxial deformation on inflammatory response of A549 cells in the presence of TNF-α. Nitrite concentration was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α (10 ng/ml). Cell monolayers were exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. * significantly higher than the control (p < 0.05). # significantly higher than other time points under the same condition (p < 0.05). (b) Effect of cyclic equibiaxial deformation on A549 cell proliferation in the presence of TNF-α. Total protein concentration was measured in cell lysate of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α. Cell monolayers were exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. * significantly higher than the control (p < 0.05).

2.4 (a) Effect of cyclic equibiaxial deformation on MMP-2 expression in A549 cell monolayers. Total MMP-2 expression was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour refers to starting point of cyclic equibiaxial deformation. *significantly higher than the control (p < 0.05). (b) Effect of cyclic equibiaxial deformation on MMP-2 expression in A549 cell monolayers in the presence of TNF-α. Total MMP expression was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α. Cell monolayers were exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to the different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. *significantly higher than the control (p < 0.05).
2.5 (a) Effect of cyclic equibiaxial deformation on MMP-2 activation in A549 cell monolayers. Active and pro-active forms of MMP-2 were analyzed by zymography following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). Inverted view of zymography gels were presented for better visualization of the gelatinase activity. Inactive and active forms of MMP-2 were detected at 72 and 62 kD region, respectively. One of three gel pictures was presented in this figure. All results were presented in Table 2.1. (b) Effect of cyclic equibiaxial deformation on MMP-2 activation in A549 cell monolayers in the presence of TNF-α. Active or pro-active forms of MMP-2 were analyzed by zymography following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α. Inverted view of zymography gels was presented for better visualization of the gelatinase activity. One of three gel pictures was presented in this figure. All results were presented in Table 2.2.

2.6 (a) Effect of cyclic equibiaxial deformation on TIMP-2 expression in A549 cell monolayers in the absence of TNF-α. Total TIMP-2 expression was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). The cell monolayers were exposed to the different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour refers to starting point of cyclic equibiaxial deformation. *significantly higher than the control (p < 0.05). (b) Effect of cyclic equibiaxial deformation on TIMP-2 expression in A549 cell monolayers in the presence of TNF-α. Total TIMP-2 expression was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α. Cell monolayers are exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to the different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. *significantly higher than the control (p < 0.05).

3.1 Schematic Diagram of Flexcell® Tension Plus™ 4000T system. A computer driven system controls the pressure of vacuum, which pulls down the silastic membrane around loading post and implements the circumferential strain in cell culture growing on top of the loading post.

3.2 Cell proliferation in A549 cultures grown under static or dynamic cell growth condition exposed at 0 (control), 0.01, 0.1, 1, 3, 5, 10, and 20 ppm of DPM. Total protein level was measured from cell lysate to characterize
the cell proliferation during the exposure to different concentrations of DPM under static and dynamic cell growth conditions. a) 24 hours exposure of DPM, b) 48 hours exposure of DPM, and c) 72 hours exposure of DPM. *Significantly different from the static condition at each concentration of DPM (p<0.05). †Significantly different from the control for static cell growth condition (static condition at 0 ppm of DPM) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 ppm of DPM) (p<0.05). ....... 61

3.3 Cellular Inflammation in A549 cultures grown under static or dynamic cell growth condition exposed at 0 (control), 0.01, 0.1, 1, 3, 5, 10, and 20 ppm of DPM. IL-8 was measured from culture media to characterize the inflammatory response of cells exposed to different concentrations of DPM under static and dynamic cell growth conditions. Total amount of protein obtained from cell lysate of each sample was used to normalize the IL-8 measurement of same sample, to compensate the variation of IL-8 measurement resulted from different number of cells at different cell growth conditions. a) 24 hours exposure of DPM, b) 48 hours exposure of DPM, and c) 72 hours exposure of DPM. *Significantly different from the static condition at each concentration of DPM (p<0.04). †Significantly different from the control for static cell growth condition (static condition at 0 ppm of DPM) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 ppm of DPM) (p<0.05). ......................................................................................................... 63

3.4 CRP level in A549 cells grown under static and dynamic cell growth condition exposed at 0 (control), 0.01, 0.1, 1, 3, 5, 10, and 20 ppm of DPM. CRP was measured from culture media to characterize the inflammatory response of cells exposed to different concentrations of DPM under static and dynamic cell growth conditions. Total amount of protein obtained from cell lysate of each sample was used to normalize the CRP measurement of same sample, to compensate the variation of CRP measurement resulted from different number of cells at different cell growth conditions. a) 24 hours exposure of DPM, b) 48 hours exposure of DPM, and c) 72 hours exposure of DPM. *Significantly different from the static condition at each concentration of DPM (p<0.05). †Significantly different from the control for static cell growth condition (static condition at 0 ppm of DPM) (p<0.05). ......................................................................................................... 63

3.5 ROS production in A549 cells grown under static and dynamic cell growth condition exposed at 0 (control), 0.01, 0.1, 1, 3, 5, 10, and 20 ppm of DPM. ROS was measured from culture media to characterize the inflammatory response of cells exposed to different concentrations of DPM under static and dynamic cell growth conditions. Total amount of protein obtained from cell lysate of each sample was used to normalize the ROS measurement of same sample, to compensate the variation of
ROS measurement resulted from different number of cells at different cell growth conditions. a) 24 hours exposure of DPM, b) 48 hours exposure of DPM, and c) 72 hours exposure of DPM. *Significantly different from the static condition at each concentration of DPM (p<0.05). +Significantly different from the control for static cell growth condition (static condition at 0 ppm of DPM) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 ppm of DPM) (p<0.05). .......................................................... 67

4.1 Schematic Diagram of Flexcell® Tension Plus™ 4000T system. A computer driven system controls the pressure of vacuum, which pulls down the silastic membrane around loading post and implements the circumferential strain in cell culture growing on top of the loading post...... 85

4.2 Cell growth of A549 under static or dynamic cell growth conditions during 0 (control), 5, 10, and 20 µg/ml MWCNT exposure. Total protein concentration was measured in each cell lysate of the A549 culture following the exposure to MWCNT at different levels (0, 5, 10, and 20 µg/ml) under both cell growth conditions. Time 0 h refers to the starting point of cyclic equibiaxial deformation and control for respective exposure levels of MWCNT. #Significantly different from the other cell growth condition at same concentration of MWCNT (p<0.05). *Significantly different from the control for static cell growth condition (static condition at 0 µg/ml of MWCNT) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 µg/ml of MWCNT) (p<0.05). ................................................. 90

4.3 Cellular inflammation in A549 cultures grown under static or dynamic cell growth conditions during 0 (control), 5, 10, and 20 µg/ml MWCNT exposures. IL-8 was measured in the media supernatant samples of A549 cell cultures following their exposure to MWCNT’s at different levels (0, 5, 10, and 20 µg/ml) under both cell growth conditions. Time 0 h refers to the starting point of cyclic equibiaxial deformation and the control for respective exposure levels of MWCNT. #Significantly different from the other cell growth condition at same concentration of MWCNT (p<0.05). *Significantly different from the control for static cell growth condition (static condition at 0 µg/ml of MWCNT) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 µg/ml of MWCNT) (p<0.05). ................................................. 92

4.4 ROS production in A549 cells grown under static and dynamic cell growth conditions during 0 (control), 5, 10, and 20 µg/ml MWCNT exposure. ROS was measured in from the media supernatant of A549 cultures following exposure to MWCNTs at different levels (0, 5, 10, and 20 µg/ml) under both cell growth conditions. Time 0 h refers to the starting point of cyclic equibiaxial deformation and is the control for the
respective exposure levels of MWCNT. #Significantly different from the other cell growth condition at same concentration of MWCNT (p<0.05). 
*Significantly different from the control for static cell growth condition (static condition at 0 µg/ml of MWCNT) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 µg/ml of MWCNT) (p<0.05). ................................................. 94

4.5 GSH production in A549 cells grown under static and dynamic cell growth conditions during 0 (control), 5, 10, and 20 µg/ml MWCNT exposure. GSH was measured in the cell lysate of A549 cultures following the exposure of MWCNT at different levels (0, 5, 10, and 20 µg/ml) under both cell growth conditions. Time 0 h refers to the starting point of cyclic equibiaxial deformation and is the control for the respective exposure levels of MWCNT. #Significantly different from the other cell growth condition at same concentration of MWCNT (p<0.05). 
*Significantly different from the control for static cell growth condition (static condition at 0 µg/ml of MWCNT) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 µg/ml of MWCNT) (p<0.05). ................................................. 97

5.1 The effect of short-SWCNTs on A549 cell proliferation. *Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05). ................................................................. 112

5.2 The effect of long-SWCNTs on A549 cell proliferation. *Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05). ................................................................. 112

5.3 The effect of short-SWCNTs on ROS production in A549 cells. *Significantly higher in static cell growth condition than in dynamic cell growth condition (p<0.05). ................................................................. 113

5.4 The effect of long-SWCNTs on ROS production in A549 cells. *Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05). ................................................................. 113

5.5 The effect of short-SWCNTs on IL-8 expression in A549 cells. *Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05). ................................................................. 114

5.6 The effect of long-SWCNTs on IL-8 expression in A549 cells. *Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05). ................................................................. 114
LIST OF SYMBOLS, NOTATIONS, AND DEFINATIONS

Symbol Key

- $\mu$M: Micro molar
- $\mu$g: Micrograms
- ppm: Parts per million
- ng: Nano-grams
- pg: Pico-grams
- mg: Milligrams
- $\mu$l: Micro-liter
- ml: Milliliter
- mM: Millimolar
- nm: Nano-meter
- cm: Centimeter
- W: Watts

Abbreviation Key

- CDC: Center of Disease Control and Prevention
- A549: Type II airway basal epithelial cells
- CLRD: Chronic lower respiratory disease
- COPD: Chronic obstructive pulmonary disease
- ARDS: Acute respiratory distress syndrome
- VILI: Ventilator induced lung injury
- ALI: Acute lung injury
TLC  Total lung capacity
BT  Bronchial tissue
BW  Bronchial wash
DPM  Diesel particulate matter
DE  Diesel Exhaust
ROS  Reactive oxygen species
IL-8  Interleukin-8
CRP  C-reactive protein
EPA  Environmental protection agency
PM  Particulate matter
NIST  National institute of standards and technology
PAH  Polycyclic aromatic hydrocarbon
SRM  Standard research material
F-12K  Kaighn’s modification of Ham’s F-12 medium
H$_2$DCFDA  2’,7’- dichlorodihydrofluorescein diacetate
H$_2$DCF  2’,7’-dichlorofluorescin
DCF  2’,7’-dichlorofluorescein
HCl  Hydrochloric acid
NaOH  Sodium hydroxide
NaH$_2$PO$_4$  Sodium dihydrogen phosphate
ANOVA  Analyses of variance
ELISA  Enzyme-linked immunosorbent assay
GSH  Glutathione
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Single-walled carbon nanotube</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

1.1 Background

The Center of Disease Control and Prevention (CDC) has categorized chronic lower respiratory diseases (CLRD) as the fourth leading cause of death in United States with estimated 127,924 deaths for year 2007 alone (Xu et al., 2010). CLRD comprises major diseases such as chronic bronchitis, emphysema, asthma, and other chronic obstructive pulmonary disease (COPD). During the period of 1999-2007, death rates from CLRD remained almost constant, but were expected to increase steadily in coming years with an increasing number of people with respiratory diseases such as asthma and emphysema (U.S.CDC, 2010; Xu et al., 2010; Xu et al., 2009). With an increase in CLRD and growing evidence of its link with environmental and health factors such as air pollution and obesity, it has not just remained a severe problem affecting a subset of individuals, but has become an epidemic that threatens global well being (Kress et al., 1999; Ponka et al., 1994; Summer et al., 2008). To begin to think about ways to ameliorate the epidemic of respiratory disease, it is first important to understand the underlying mechanisms of CLRD in the body.

The lung serves a very important function in the human body while providing an excellent air-blood barrier. In the process of serving its function, it deals with the external environment on a regular basis which makes it prone to exposure to airborne materials like particulate matter, bacteria, viruses, air pollutants, etc. in the air. In addition to continuous exposure to the external environment, the human lung is also prone to many
chronic and acute disorders like CLRD, or disorders induced during ventilation therapy such as acute respiratory distress syndrome (ARDS), ventilator induced lung injury (VILI), acute lung injury (ALI), and so forth. Some of these disorders are well understood, but still most of them have not been well characterized because of the complex nature of the disease and the complexity of structural and cellular diversity inside the lung which hinders the analysis process. Most of these disorders are either initiated by disruption/physiological changes in the air-blood barrier or induces physiological changes inside the lung. In any condition, airway and alveolar epithelium becomes very prone to disruption and physiological changes, which increases the porosity of the air-blood barrier and eventually leads to immune system activation and expression of pathophysiological conditions related to respective diseases. Thus, the characterization of lung epithelium has remained a central theme for most research studies aimed to understand lung diseases and disorders.

With respiratory diseases being the fourth leading cause of annual deaths in the United States, any achievement in understanding the underlying mechanisms of complex respiratory diseases would have a significant impact on improving our overall understanding and treatment for these respective diseases (Kung et al., 2008).

1.2 Respiratory Research Using Whole-Animal Research Models

To study the in vivo systemic response to different CLRD conditions, various whole-animal models such as guinea pig, sheep, and mouse have been developed (Allen et al., 2009; Bates et al., 2009; Braun, 2008; Pare et al., 1979; Sarpong et al., 2003; Shapiro, 2000; Smith, 1989; Snapper, 1986; Summer et al., 2008). In most studies,
animal models have been used with the consideration of understanding the whole organ
or systemic responses and due to the higher relevance of outcomes comparable to human
studies (Wright et al., 2008). Much has been elucidated from work involving animal
models; however, using them for studies of molecular mechanisms and signaling
pathways has remained challenging and not feasible (Lankford et al., 2005).

1.3 Use of in vitro Cell Culture Models
for Respiratory Research

In vitro cell culture models which mimic the morphologic and functional
characteristics of the airway epithelium as in vivo have been established using different
cells types from the lung. A distinct advantage of such in vitro systems is their ability to
study the contribution of each individual cell type alone, excluding the influence of other
cell types. Most of the studies, carried out to understand the signaling pathway and
molecular mechanism, utilized in vitro models made out of different cell types from the
lung. The in vitro models grown under static condition have served as a good study
model due to their properties such as the case of using and controlling the cell culture
growth conditions, compatibility with cellular imaging and characterization equipment.
But the outcomes from static in vitro model-based research have not been in parallel with
most in vivo studies. In order to improve in vitro cell functionality and hence the overall
applicability of the research, different approaches such as cell growth surface
modification, co-cultures, air-liquid interface, dynamic cell growth environment, etc.
have been used in many studies (Choe et al., 2003, 2006; Pugin et al., 1998; Stoker et al.,
2008; Tomei et al., 2008; Tschumperlin et al., 2001; Tschumperlin et al., 1998; Vlahakis
et al., 2001). Such approaches have provided a good platform for studying cell
differentiation and functionality analysis under various pathological states mimicking conditions (Choe et al., 2003; Tschumperlin et al., 2001; Vlahakis et al., 2001). But considering the fact that the lung is a dynamic organ which continuously experiences complex longitudinal and circumferential mechanical stresses, \textit{in vitro} models with dynamic cell culture environments have received significant attention over the other \textit{in vitro} models for their functionality and the translationability of the results (See Chapter 2; Choe et al., 2003; Tschumperlin et al., 2001; Vlahakis et al., 2001).

Inside the human body a majority of the cells in organs continuously grow under complex nature of biomechanical forces such as compression, tension, and shear. Under various pathological conditions, especially related to lung, heart, and muscular systems, the profile biomechanical forces have been noticed to change and cause the alterations in cellular functions in surrounding cells (Suresh, 2007). Exposures to such forces in \textit{in vitro} environment have also shown to induce significant changes in cellular morphology and biochemical responses of the cells (Bershadsky et al., 2006; Suresh, 2007; Vandenburgh, 1992). These findings have highlighted the importance of studying the effects of biomechanical and biophysical force transduction and their effects in individual cell lines specific to dynamic organs.

Dynamic cell culture models with different mechanical environment simulating capabilities such as uniaxial, biaxial stretching or compression in single cell cultures or co-cultures have been developed and used for such studies (See Chapter 2; Choe et al., 2003, 2006; Pugin et al., 1998; Tschumperlin et al., 2001). The central theme for the most dynamic cell culture model development was to change the area of cell growth surface. Cell culture vessels with cell growth surface made of silastic rubber bottom were used in
these studies for the flexibility of manipulating cell growth surface. Due to their capability for simulating mechanical environment similar to physiological and different pathophysiological conditions, they make a good experimental model for studies focusing on understanding diseases related to the lung such as asthma, VILI, and COPD and nanotoxicity assessment.

Flexcell® 4000T Plus™ System (Flexcell International Corporation, NC) is a computer driven dynamic cell culture system (Figure 1.1) that uses vacuum pressure to apply cyclic or static strain to cell cultured on silastic-bottomed culture plates. The Flexcell Tension Plus system uses BioFlex 6-well plate, which is similar to a regular 6-well plate, but has cell growth surface made out of cell culture treated silastic rubber. As shown in Figure 1.1, computer controlled vacuum level pulls down the silastic cell growth surface around the loading post and implements equibiaxial elongation in cell cultures right on top of the loading post. The system is designed to control the equibiaxial surface elongation and elongation frequency. This system has provided a strong platform

Figure 1.1. Flexcell® 4000T Plus™ System, Working diagram of Flexcell® 4000T Plus™ System. Figure adapted from Flexcell® 4000T Plus™ System user manual.
Table 1.1. Relationship between equibiaxial elongations level and percentage total lung capacity (Tschumperlin et al., 1998).

<table>
<thead>
<tr>
<th>Equibiaxial Elongation Level (% Area Change)</th>
<th>Total Lung capacity (%)</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>45</td>
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<tr>
<td>10</td>
<td>60</td>
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<tr>
<td>15</td>
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for studying stretch-induced effects in variety of cell lines from dynamic organs such as lung, heart, kidney, liver, bone etc. However, the correlated between the equibiaxial elongation in this system and relevant level of biomechanical forces inside the human body has remained not well understood for all organs. Study performed by Tschumperlin et al have established the relationship between equibiaxial elongations in silastic-bottom plate with the total lung capacity (TLC) in the lung (Tschumperlin et al., 1998). The relationship between the levels of equibiaxial elongations, used in our study, with TLC are as shown in the Table 1.1.

1.4 Experimental Model Selection

With pros and cons associated with each experimental model, like small animal and \textit{in vitro} model, usage of one model over another has been preferred based on the feasibility of research objective. With comparative research analysis between different model usage, for respiratory research, it has made clear that preference should be given to cell functionality and structural complexity in addition to ease of controlling experimental environment for experimental model selection (Rothen-Rutishauser et al.,
Cell functionality is one of the most important factors in cell biology research since individual cells response and reproducibility are highly dependent on that. For respiratory research structural complexity is really important factor since lung acts as an air-blood barrier and most of the respiratory disorders cause alterations in lung’s air-blood barrier properties. Thus understanding the alterations in the status of air-blood barrier during respiratory disorder would have greater impact on characterizing respiratory disorder. Ease of manipulating experimental environment is also an important factor to study cellular behavior. \textit{In vitro} models are easy to control the experimental environment but in animal models simulating precise environment is a challenge which may affect the reproducibility of the results.

1.5 Ventilation-Induced Lung Injury

Ventilator-induced lung overdistension has been a growing concern in the management of mechanically ventilated patients. In clinical practice, mechanical ventilation is used as an effective life-saving strategy, but on the dark side it initiates injury and leaves the injured lung susceptible to rapid remodeling. Mechanical ventilation causes injury to the lung not only by the mechanical stress caused by a complex set of forces, but also by the inflammation that follows after. Typically acute respiratory distress syndrome and acute lung injury are the most commonly observed side effects of ventilator-induced lung injury (Matthay et al., 2003). It is believed that mechanical ventilation triggers or enhances the net inflammatory activity between proinflammatory and anti-inflammatory mediators. The mechanisms underlying the sensing and
conversion of inappropriate mechanical stretching into cytotoxicity, net inflammatory activity, and extracellular matrix remodeling have not been well characterized.

A negative effect of lung overdistension on net inflammatory activity, in different localized cell types inside the lung, is the most common phenomenon during a ventilative condition. Preliminary studies, including animal studies, have already shown injurious effects of mechanical ventilation on the lung by induction of inflammation following increased positive pressure (Altemeier et al., 2004; Tremblay et al., 1997). Current knowledge allows us to understand the chronological order of events, which take place under mechanical ventilative conditions: surfactant dysfunction, alveolar damage, preinflammatory mediator production, structural changes in the alveolar capillary, and changes in gene transcription (Frank et al., 2008; Reddy et al., 2007). These events are highly interconnected and orchestrate a chain reaction. Thus, understanding of each individual event enables us to better model this process.

Understanding the effects of mechanical forces on altering production of surfactants and preinflammatory mediators has been a focal point of many studies. Animal studies have clearly shown the rapid increase of proinflammatory mediators like IL-8, TNF-α, and IL-6 in response to various ventilative conditions (Altemeier et al., 2004; Tremblay et al., 1997). The findings of animal studies showed the outcome of the entire process, but failed to show the individual contributors such as the process initiators and mediators. To better understand the process and reduce the complexity of the outcome, in vitro models with stretching (Hammerschmidt et al., 2005) or compressing (Tschumperlin et al., 2000) capabilities have been used. These in vitro strategies have provided information about the cell–cell interactions, the responses of individual cell
types, and the originator and mediators of the entire process. Because A549 cells form a protective barrier at the surface of lung tissue, these cells have been the most commonly studied cell types in in vitro stretching or compressing systems. Due to exposure of mechanical forces, A549 cells have been shown to produce proinflammatory mediators such as IL-8, nitric oxide (NO), and IL-6 (Hammerschmidt et al., 2005; Ning et al., 2007). These proinflammatory mediators are known to play a crucial role in attracting immune cells and altering gene transcription by activating multiple pathways including the NF-κB pathway and amplifying the inflammation (Li et al., 2002; Nam et al., 2004).

Along with proinflammatory mediators, anti-inflammatory mediators and remodeling factors are also produced by A549 cells, but the level of production is highly dependent on the surrounding conditions inside the lung (Tillie-Leblond et al., 1999; Yamamoto et al., 2002). The remodeling factors like matrix metalloproteases (MMP) and tissue inhibitor Metalloproteases (TIMP) are also known to play an important role during inflammatory conditions by promoting the tissue remodeling activities (Haseneen et al., 2003; Sacco et al., 2004). Mechanical forces not only increase the inflammatory response, but also increase the remodeling activity in A549 cells by increasing MMP-2 and MMP-9 production (Choe et al., 2006). Although both proinflammatory and remodeling factors play a crucial role during lung overdistension, the interplay between them has not been well characterized.

1.6 Evaluation of DPM Exposure in Respiratory System

Epidemiological and lab-bench scale studies have linked air pollution with various, potentially fatal respiratory and cardiovascular conditions (Anenberg et al.,
2010; Baja et al., 2010; Kramer et al., 2010; Liao et al., 2010). As outlined by United States environmental protection agency (EPA), six common air pollutants are carbon monoxide, ozone, lead, nitrogen dioxide, particulate matter (PM), and sulfur dioxide (EPA, 2010; Mazzoli-Rocha et al., 2010; Neher et al., 1994). Of these common air pollutants, PM has been studied most extensively to understand and characterize its adverse effect on human health due to its strong association with air pollution related morbidity (Kelsall et al., 1997; Mazzoli-Rocha et al., 2010; Saldiva et al., 1995; Schwartz et al., 2001; Schwartz et al., 1990; Zanobetti et al., 2000). One of the most common forms of PM generated from human activities is DPM or diesel exhaust particles (DEP), which is frequently linked with occupational and public exposure in urban areas (Patel et al., 2010; Zuurbier et al., 2010). Due to the complex diversity in composition and nanoscale dimension, DPM can cause potentially adverse effect when humans are exposed to it for an extended period (Mazzoli-Rocha et al., 2010; Saldiva et al., 2002).

Previous studies have highlighted inflammation, autonomic nervous system activity, procoagulant effects, covalent modification of cellular components, and ROS production as contributors to DPM’s adverse health effects (Li et al., 2008; Mazzoli-Rocha et al., 2010; Nel et al., 2006). Many epidemiological studies have also successfully established the correlation between DPM exposure and various adverse health effects (Kelsall et al., 1997; Kramer et al., 2010; Miller et al., 2006; Patel et al., 2010; Saldiva et al., 1995; Schwartz et al., 2001; Schwartz et al., 1990). In vivo studies performed on small animals and human volunteers have shown the occurrence of precursor events which can lead to pulmonary and cardiac disorders as suggested by epidemiological studies (Ghio et al., 2000; Gong et al., 2003a, 2003b; Holgate et al., 2003a, 2003b;
Nemmar et al., 2002; Nemmar et al., 2008; Salvi et al., 1999). Findings from in vivo studies were helpful in understanding the systematic responses of the whole exposure organ, the lung, and the body. However, they were limited to show the molecular level interaction between cells and DPM, which is equally important in understanding the chronological order of events post DPM exposure (Fischer et al., 2007). In vitro studies performed to evaluate the effect of DPM exposure on single cell type monolayers or co-cultures, mimicking the exposure boundary for different organs, have been more preferable in understanding the cellular response and determining the biomarkers for characterization (Alfaro-Moreno et al., 2008; Fischer et al., 2007; Jones et al., 2009; Mazzarella et al., 2007; Stoker et al., 2008). Many in vitro studies, conducted on lung airway epithelial cells, have shown the changes in extracellular levels of various signaling molecules such as CRP, heat shock protein-70, IL-8, interleukin-6, cyclooxygenase-2, granulocyte-macrophage colony-stimulating factor etc., under the exposure of DPM (Ahn et al., 2008; Alfaro-Moreno et al., 2008; Chirino et al., 2010; Dobrovolskaia et al., 2007; Mazzarella et al., 2007; Moller et al., 2010; Ramage et al., 2004a; Ramage et al., 2004b; Sanchez-Perez et al., 2009; Seagrave et al., 2004; Veranth et al., 2008). Results of these in vitro studies might be useful in characterizing precursory events that can be linked to whole organ (lung) response from the in vivo studies (Ahn et al., 2008; Alfaro-Moreno et al., 2008; Chirino et al., 2010; Dobrovolskaia et al., 2007; Mazzarella et al., 2007; Moller et al., 2010; Ramage et al., 2004a; Ramage et al., 2004b; Sanchez-Perez et al., 2009; Seagrave et al., 2004; Veranth et al., 2008). However, the most in vitro studies related to DPM exposure could not be well translated to in vivo studies (Fischer et al., 2007). Many possible reasons such as complex chemical
composition, variety in nanoscale size distribution of DPM, use of unreasonable exposure concentrations in experimental design, interference of DPM with biomarkers (signaling molecules) and poor functionality of in vitro models, could be at blame to explain the poor translatability of in vitro studies (Grainger, 2009; Jones et al., 2009; Moller et al., 2010; Seagrave et al., 2004). Moreover, most of the in vitro DPM-toxicity studies were performed under the static cell culture system, which might be limited in mimicking dynamic nature of tissues in vivo. Our study was performed with an additional parameter related to dynamic nature of exposed tissues in the experimental design, which included lung cells grown under the dynamic cell growth condition during the period of DPM exposure.

This dynamic in vitro culture system of human airway epithelial cells was used for the investigation of DPM effects on cell proliferation, cellular inflammatory response (IL-8), ROS production, and CRP expression. In this study, we used the concentrations of DPM in the range of 0.01 – 20 ppm. Possible atmospheric concentrations of DPM lie between 0.01 and 0.1 (not exceed 1-3 ppm). Exposure at the concentrations above 3 ppm can be the case of occupational exposure (EPA, 2010; Hesterberg et al., 2009). Our in vitro dynamic culture system improved cellular functionality, improvised a mechanical environment similar to that found in the lungs, and facilitated the altered interactions between DPM and human airway epithelial cells (See Chapter 2). This study will provide crucial information to develop viable alternatives to in vivo tests to evaluate the toxicity of DPM exposure and other air pollutants in combination in the future.
1.7 Evaluation of Carbon Nanotube Exposure in Respiratory System

The respiratory system is especially susceptible to insult by airborne toxic materials. Particles that can enter the respiratory system are broken down into three major regions: (1) dust reaching the gas exchange, or alveolar, region is called *respirable dust*; (2) dust reaching the tracheobronchial region and alveolar region is called *thoracic dust*; and (3) dust entering the nose and mouth is called *inhalable dust*. Respirable dust is smaller than about 4 μm aerodynamic equivalent diameters, thoracic dust is smaller than about 10 μm, and inhalable dust is smaller than about 100 μm. Larger airborne particles can deposit in the upper respiratory system (Baron et al., 2003). In a manufacturing environment, CNTs are handled in much larger quantities as compared to typical laboratories, subjecting the workers to a higher risk of exposure to these potentially hazardous nanoparticles. The nanotechnology community in the U.S., led by NIOSH (The National Institute for Occupational Health and Safety) and OSHA (Occupational Safety & Health Administration) is devoting efforts to issue “best practices” guide for safely working with nanomaterials. However, the development is still in its infant stage, and there is a strong need for science-based methodologies to predict the health and toxicological effects of CNTs.

Intensive studies on the toxicity of CNTs have shown that exposure to CNTs results in pulmonary inflammation (Chou et al., 2008; Lam et al., 2004; Li et al., 2007; Mitchell et al., 2007; Muller et al., 2005; Shvedova et al., 2005; Warheit et al., 2004). The inflammatory lung reactions (alveolitis) are a source of genetic lesions which could eventually lead to the development of lung cancer (Chou et al., 2008). *In vivo* studies
performed using guinea pigs and rats showed the appearance of multifocal granulomas, resulting in inflammatory reactions of the terminal and respiratory bronchial. Mild fibrosis in the alveolar septa was also observed (Helland et al., 2007). Ken Donaldson and his colleagues described three properties of CNTs associated with pathogenicity in particles. They are 1) nanoparticles showing more toxicity than larger sized particles, 2) fiber-shaped particles behaving like asbestos and other pathogenic fibers which have toxicity associated with their needle-like shape, and 3) biologically biopersistent. They also pointed out that CNTs are possibly one of the least biodegradable man-made materials ever devised (Donaldson et al., 2006). Also concerns over the increased emissions of CNTs into the environmental compartments (air, water and soil) mainly due to improper disposal of CNTs were raised (Helland et al., 2007). Recent studies for nanomaterials indicate: (1) CNTs and fullerenes have produced toxic effects on biological systems (Chin et al., 2007; Dumortier et al., 2006; Helland et al., 2007; Lam et al., 2006; Yang et al., 2006); (2) evidence that nanoparticles can translocate to bloodstream (Rothen-Rutishauser et al., 2007; Shimada et al., 2006); and (3) evidence that nanoparticles can cross blood brain barrier (Kim et al., 2007). However, studies are still preliminary, as the current in vivo and in vitro response data are difficult to extrapolate.

The airway wall exists in a mechanically dynamic environment, where different amounts of circumferential and longitudinal expansion and contraction occurred during breathing movements. In this study, we established in vitro dynamic culture system simulating normal breathing condition of our airway. This dynamic culture system of human airway epithelial cells was used for the investigation of the effect of different size
of SWCNTs on cell proliferation, cellular inflammatory response, and the level of reactive oxygen species. The different level of biological and toxicological effects was observed both in static and dynamic conditions of airway epithelial cell monolayer.

1.8 Hypothesis and Objectives

Based on the knowledge we have on the respiratory research, it is believed that use of *in vitro* model is inevitable for research studies focusing on exploring or exploiting the intracellular and extracellular signaling pathways (Huh et al., 2010). In dynamic *in vitro* model based studies, Choe et al. and Huh et al. demonstrated that use of dynamic cell growth condition, using uniaxial straining, improves the functionality of lung cells and depicts the ideal *in vitro* cell growth condition mimicking dynamic environment inside the lung (Choe et al., 2006; Huh et al., 2010). Also, Grainger et al. and others have suggested to involve the active dynamic environment in *in vitro* cell culture based models to improve the applicability of results from studies evaluating adverse effects of nano-pollutants (Grainger, 2009; Jones et al., 2009). Collectively, we hypothesized that use of *in vitro* cell culture based model, with cyclic equibiaxial elongation capabilities, for respiratory research will improve the applicability of the research outcome. The main goal of this project was to use the equibiaxially elongating *in vitro* airway epithelial cell based model (dynamic *in vitro* models) to understand and characterize the response of airway epithelial cells under different pathophysiological conditions. In this study, we used dynamic *in vitro* models for analyzing the effect of ventilative overdistention on type II airway epithelial cell (A549) monolayers under normal and pre-existing inflammatory conditions. Moreover, we utilized the dynamic *in vitro* models to evaluate
the adverse effects of nano-materials/pollutant such as diesel particulate matter (DPM), multi-walled carbon nanotube (MWCNT), and single-walled carbon nanotube (SWCNT) on A549 cell monolayers. To achieve these objectives, the experimental work of this research was divided into four specific-aims as listed below:

1. Characterizing the effect of ventilative overdistention on A549 cultures under normal and pre-existing inflammatory state using dynamic in vitro models.

2. Understanding the adverse effects of DPM exposure on A549 cells under dynamic and static in vitro cell growth conditions.

3. Observing the side effects of MWCNT exposure on A549 cells, growing under dynamic or static cell growth condition.

4. Evaluating the size, concentration, and cell growth condition (static or dynamic) induced adverse effects on A549 cells exposed to SWCNT.

In order to accomplish these aims, we used the Flexcell Tension Plus system for simulating appropriate mechanical environment for cell growth, corresponding to each pathophysiological condition. We also used various molecular and cell biology based assays to characterize the effects of each treatment on A549 cells.

1.9 Dissertation Outline

This dissertation research is focused on using dynamic cell growth model to study effects of pathological conditions such as lung-overdistention and nano-pollutant/material on A549 cell monolayers. In Chapter 2, my research is focused on characterizing the inflammatory and tissue remodeling responses under lung-overdistention with normal or pre-existing inflammatory condition in A549 cells. To mimic the extent of lung-
overdistention, the magnitude of equibiaxial elongation in Flexcell 4000T PLUS System was varied (5, 10, 15, and 20 %) while keeping the cyclic elongation frequency (0.2Hz) constant. Characterization of the inflammatory response was done by monitoring extracellular nitric oxide level. For monitoring the tissue remodeling activities, total MMP-2, TIMP-2 concentrations along with MMP-2 activity, using zymography, were measured. Further, we pre-treated A549 cells with TNF-α to implement pre-existing inflammatory state and characterizing its effects on A549 cells under lung-overdistention.

In Chapters 3, 4, and 5, our research is focused on characterizing the adverse effects from DPM, MWCNT and SWCNT exposure on A549 cells grown under both static or dynamic cell growth condition. Dynamic cell growth condition was implemented using Flexcell 4000T PLUS System to mimic mechanically active cell growth environment similar to normal breathing condition. These studies are focused on characterizing the side effects from DPM, MWCNT, and SWCNT exposure under dynamic cell growth condition and comparing them with static cell growth condition and animal model based studies. Cell growth, cellular inflammation and cellular oxidative stress indicators such as IL-8, CRP, ROS, and GSH were monitored to characterize the adverse effects.

1.10 References


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CHAPTER 2

INTERPLAY BETWEEN CYTOKINE-INDUCED AND CYCLIC EQUIBIAXIAL
DEFORMATION-INDUCED NITRIC OXIDE PRODUCTION AND
METALLOPROTEASES EXPRESSION IN HUMAN
ALVEOLAR EPITHELIAL CELLS†

2.1 Abstract

Ventilator-induced lung overdistension has been a growing concern in the management of mechanically ventilated patients. Mechanical ventilation triggers or enhances the net inflammatory and tissue-remodeling activities. Although it has been shown that proinflammatory and tissue remodeling factors play important roles during airway remodeling, the interplay between them is not well understood. Thus our objective was to study and characterize the molecular mechanism of cyclic equibiaxial deformation-induced airway inflammation and remodeling either in the presence or absence of a pre-existing inflammatory condition. This was to be done using an in vitro dynamic model, which can simulate different mechanical ventilative conditions. Type II alveolar basal epithelial cell (A549) monolayers were exposed to the different levels of mechanical ventilative conditions using the Flexcell® Tension Plus™ 4000T system, which generated the different levels of cyclic equibiaxial deformation (5, 10, 15 and 20%) at 0.2 Hz deformation frequency. The production of nitric oxide (NO), the expression of metalloprotease-2 (MMP-2) / tissue inhibitor metalloprotease-2 (TIMP-2), and the activation of MMP-2 were measured under the different levels of cyclic

† Coauthored by Hemang Patel and Soonjo Kwon. Interplay Between Cytokine-Induced and Cyclic Equibiaxial Deformation-Induced Nitric Oxide Production and Metalloproteases Expression in Human Alveolar Epithelial Cells Cell Mol Bioeng 2 (4): 615-624, 2009.
equibiaxial deformation either in the presence or absence of TNF-α. Our studies indicated that cyclic equibiaxial deformation induced production of NO and MMP-2/TIMP-2. Higher levels of cyclic equibiaxial deformation increased the expression of the active form of MMP-2. In particular, in the presence of TNF-α, the more active form of MMP-2 was detected during both cyclic equibiaxial deformation and remodeling periods.

2.2 Introduction

Ventilator-induced lung overdistension has been a growing concern in the management of mechanically ventilated patients. In clinical practice, mechanical ventilation is used as an effective life-saving strategy, but on the dark side it initiates injury and leaves the injured lung susceptible to rapid remodeling. Mechanical ventilation causes injury to the lung not only by the mechanical stress caused by a complex set of forces, but also by the inflammation that follows after. Typically acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) are the most commonly observed side effects of ventilator-induced lung injury (VILI). It is believed that mechanical ventilation triggers or enhances the net inflammatory activity between proinflammatory and anti-inflammatory mediators. The mechanisms underlying the sensing and conversion of inappropriate mechanical stretching into cytotoxicity, net inflammatory activity, and extracellular matrix remodeling have not been well characterized.

A negative effect of lung overdistension on net inflammatory activity in different localized cell types inside the lung is the most common phenomenon during a ventilative condition. Preliminary studies, including animal studies, have already shown injurious
effects of mechanical ventilation on the lung by induction of inflammation following increased positive pressure.\textsuperscript{1, 23} Current knowledge allows us to understand the chronological order of events, which take place under mechanical ventilative conditions: surfactant dysfunction, alveolar damage, proinflammatory mediator production, structural changes in the alveolar capillary, and changes in gene transcription.\textsuperscript{5, 17} These events are highly interconnected and orchestrate a chain reaction. Thus, understanding of each individual event enables us to better model this process.

Understanding the effects of mechanical forces on altering production of surfactants and proinflammatory mediators has been a focal point of many studies. Animal studies have clearly shown the rapid increase of proinflammatory mediators like IL-8, TNF-\(\alpha\), and IL-6 in response to various ventilative conditions.\textsuperscript{1, 23} The findings of animal studies showed the outcome of the entire process, but failed to show the individual contributors such as the process initiators and mediators. To better understand the process and reduce the complexity of the outcome, \textit{in vitro} models with stretching\textsuperscript{7} or compressing\textsuperscript{24} capabilities have been used. These \textit{in vitro} strategies have provided information about the cell-cell interactions, the responses of individual cell types, and the originator and mediators of the entire process. Because A549 cells form a protective barrier at the surface of lung tissue, these cells have been the most commonly studied cell types in \textit{in vitro} stretching or compressing systems. Due to exposure of mechanical forces, A549 cells have been shown to produce proinflammatory mediators such as IL-8, Nitric oxide (NO), and IL-6.\textsuperscript{7, 16} These proinflammatory mediators are known to play a crucial role in attracting immune cells and altering gene transcription by activating multiple pathways including the NF-\(\kappa\)B pathway and amplifying the inflammation.\textsuperscript{12, 15}
Along with proinflammatory mediators, anti-inflammatory mediators and remodeling factors are also produced by A549 cells, but the level of production is highly dependent on the surrounding conditions inside the lung.\textsuperscript{22, 27} The remodeling factors like matrix metalloproteases (MMP) and tissue inhibitor metalloproteases (TIMP) are also known to play an important role during inflammatory conditions by promoting the tissue remodeling activities.\textsuperscript{8, 18} Mechanical forces not only increase the inflammatory response but also increase the remodeling activity in A549 cells by increasing MMP-2 and MMP-9 production.\textsuperscript{2} Although both proinflammatory and remodeling factors play a crucial role during lung overdistension, the interplay between them has not been well characterized.

To enhance our understanding of molecular mechanisms for cyclic equibiaxial deformation–induced airway remodeling, we used an \textit{in vitro} model, which can simulate different mechanical ventilation conditions. We hypothesized that cyclic equibiaxial deformation either in the presence or absence of a pre-existing inflammatory condition would affect the net inflammatory activity and airway remodeling. The Flexcell® Tension Plus™ 4000T system was used to mimic ventilator-induced overdistension. Human type II alveolar epithelial cells (A549) were used as a model cell line for characterization of cellular response under cyclic equibiaxial deformation. Percentage area change generated by cyclic equibiaxial deformation was considered analogous to surface area change resulting from lung inflation, during mechanical ventilation. Different levels of cyclic equibiaxial deformation (5, 10, 15, and 20%) at a frequency of 0.2Hz was applied to A549 cell monolayers either in the presence or absence of inflammatory cytokine (TNF-\textgreek{a}). Under these conditions, the expression of proinflammatory (NO) and remodeling factors (MMP-2/TIMP-2) were investigated.
2.3 Materials and Methods

2.3.1 Cell Culture. Type II alveolar basal epithelial cells of human origin (A549) were purchased from ATCC (Manassas, VA). A549 is epithelial-like in morphology and originates from a human lung carcinoma patient. The cells were seeded at $3 \times 10^5$ cells/well onto six well BioFlex plates (Flexcell International, PA) containing 2 ml of F-12k culture medium, which was supplemented with 1% penicillin streptomycin (Invitrogen, CA) and 10% fetal bovine serum (Thermo Fisher Scientific, UT). Cells reached confluency in 48 hours after seeding. After reaching confluency, cells were exposed either to cyclic equibiaxial deformation or to inflammatory cytokine (TNF-α) followed by cyclic equibiaxial deformation.

2.3.2 Exposure of A549 Monolayers to TNF-α. After reaching confluency, cells were incubated in serum-free media for 24 hours before exposure to TNF-α. The cells were then exposed to TNF-α (10 ng/ml) in serum-free media for 24 hours. Following TNF-α exposure, serum was returned to the culture media. Cells were either grown in static condition (no cyclic equibiaxial deformation) as controls or exposed to cyclic equibiaxial deformation (Figure 2.1-b).

2.3.3 Cyclic Equibiaxial Deformation. The airway wall exists in a mechanically dynamic environment, where different amounts of circumferential and longitudinal expansion and contraction occurred during breathing movements or ventilation therapy. We used a physiologically relevant range of cyclic equibiaxial deformation 5, 10, 15, and 20%, which corresponds to 45, 60, 70, and 80% of the total lung capacity respectively. Flexcell® Tension Plus™ 4000T system (Flexcell International, PA) was used to equibiaxially elongate the monolayers of cells on silicone rubber bottoms of a BioFlex
Figure 2.1. (a) Experimental Scheme for cyclic equibiaxial deformation of A549 cells. Following confluency, A549 cell monolayers were incubated in serum-free media for 24 hours. The cell monolayers were then either exposed to the different levels of cyclic equibiaxial deformation for 24 hours and grown in static condition for another 48 hours or growth in static condition. Time 0 hour marks the end of serum free media incubation and refers to the starting point of cyclic equibiaxial deformation or static condition growth (for controls). (b) Experimental Scheme for cyclic equibiaxial deformation of A549 cells in presence of TNF-α. Following confluency, A549 cell monolayers were incubated in serum-free media for 24 hours before TNF-α exposure. Cell layers were exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour marks the end of TNF-α exposure and refers to the starting point of cyclic equibiaxial deformation.
plate. The cell monolayers were exposed to the different levels of cyclic equibiaxial deformation for 24 hours at frequency of 0.2Hz and then grown in static condition for another 48 hours. The 0 hour time refers to the starting point of cyclic equibiaxial deformation (Figure 2.1-a).

2.3.4 Nitrite Measurement. The Griess Reagent system (Promega Corporation, WI) was used to measure the nitrite level in the media samples collected from all experiments. NO is highly unstable in the presence of oxygen, and is rapidly converted into \( \text{NO}_2^- \) (Nitrite) and \( \text{NO}_3^- \) (Nitrate) in liquid media. Thus, the level of nitrite measured in all media samples provided only the partial concentration of NO produced from the cells under different conditions.

2.3.5 Total MMP-2 Expression. All media samples were analyzed using Quantikine® Human/Mouse/Rat MMP-2 (total) Immunoassay (R&D Systems, MN) to detect total MMP-2 production. This assay detected both active and pro-active forms of the MMP-2.

2.3.6 Zymography for MMP-2 Activity Measurement. Gelatin-based Zymography was performed on all media supernatant samples in a 12% polyacrylamide resolving gel under nonreducing condition. Granular gelatin, dissolved in deionized water, was copolymerized in the polyacrylamide resolving gel with a final concentration of 1 mg/ml. A 6% polyacrylamide stacking gel was used. Prior to performing zymography measurement, all samples were incubated at 37 °C with 2X sample buffer (62.5 mM Tris-HCL, pH 6.8, 5% SDS, 40% Glycerol and 0.1% Bromophenol blue) in 1:1 ratio for 30 minutes. All samples mixed with sample buffer were run at 200 V for 45 minutes under constant voltage mode. After electrophoresis, gels were washed four times
at 15 minute intervals each in renaturing buffer (2.5% Triton X-100 in 50mM Tris pH 7.4, 5mM CaCl₂ and 1µM ZnCl₂) on a rotating shaker. After renaturing MMPs, the gels were incubated at 37 °C in developing solution (50mM Tris pH 7.4, 5mM CaCl₂ and 1µM ZnCl₂) overnight. Gels were stained with 0.5% Coomassie Blue R-250 in 40% ethanol and 10% Acetic acid for 1 hour. Prior to imaging, the gels were briefly destained in 40% ethanol and 10% acetic acid solution for 5 minutes. The G-Box gel imaging system (Syngene, MD) was used to analyze active and inactive forms of MMP-2. In active and active forms of MMP-2 were detected at 72 and 62 kD region, respectively.

2.3.7 Total TIMP-2 Expression. The TIMP-2 ELISA kit (EMD-Calbiochem, CA) was used to measure the level of TIMP-2 expression in media supernatant samples.

2.3.8 Total Protein Measurement. Total protein from cell lysate of all samples was measured using the BCA total protein assay (Pierce, IL).

2.3.9 Statistical Analysis. Statistical analyses were carried out using two-way analyses of variance (ANOVA) followed by Dunnett’s multiple comparison tests to determine where significance exists (p<0.05). All graphs were prepared by plotting mean data (sample size, n = 3) with corresponding standard error.

2.4 Results

2.4.1 Effect of TNF-α on Nitric Oxide Production and Cell Growth in A549 Cells Under a Static Condition (Without Cyclic Equibiaxial Deformation). Exposure of TNF-α resulted in a significant increase of nitric oxide production in A549 cells (0% stretching level, Figure 2.3-a). While A549 cell monolayers were exposed to TNF-α in a serum-free media for 24 hours, nitric oxide production significantly increased due to
inflammatory stimuli. Nitric oxide production peaked at 48 hours after TNF-α exposure (p<0.05) and remained significantly higher than the level of nitric oxide concentration (Figure 2.3-a). Total protein concentration in cell lysate samples collected from the same experiments showed the normal cell growth up to 48 hours, followed by decrease for 72 hours (Figure 2.3-b). The decreased total protein concentration in the presence of TNF-α could be due to cytotoxicity of TNF-α to A549 cells in cultures. The increased levels of cell necrosis might influence the level of inflammation and remodeling measured at the longer time points (i.e. >48 hours).10, 19

2.4.2 Effect of Cyclic Equibiaxial Deformation on Inflammatory Response of A549 Cells. Nitrite concentration was measured from media supernatant of A549 cultures following different levels of cyclic equibiaxial deformation (5, 10, 15, and 20%). Cyclic equibiaxial deformation increased nitrite concentration during the period of cyclic equibiaxial deformation and remodeling (Figure 2.2-a). Nitrite concentration at 72 hours was significantly higher than the control (at 0 hours). Total protein concentration was significantly decreased during cyclic equibiaxial deformation between 0 and 24 hours, but it returned to control level during remodeling period between 24 and 72 hours (Figure 2.2-b). Nitrite concentration and total protein concentration was not significantly changed in 0 % cyclic equibiaxial deformation (static control) during the entire period of experiments (Figure 2.2-a, and 2.2-b).

2.4.3 Effect of Cyclic Equibiaxial Deformation on Inflammatory Response of A549 Cells in the Presence Of TNF-α. Nitrite concentration was measured in media supernatant of A549 cultures following different levels of cyclic equibiaxial deformation (5, 10, 15, and 20%) in the presence of TNF-α. Nitrite concentrations were significantly
Figure 2.2. (a) Effect of cyclic equibiaxial deformation on inflammatory response of A549 cells. Nitrite concentration was measured from media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour refers to the starting point of cyclic equibiaxial deformation. *significantly higher than the control (p < 0.05). (b) Effect of cyclic equibiaxial deformation on A549 cell proliferation. Total protein concentration was measured in cell lysate of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour refers to starting point of cyclic equibiaxial deformation. *significantly higher than the control (p < 0.05).
increased immediately following exposure to cyclic equibiaxial deformation, and remained higher than the control (Figure 2.3-a). However, in the presence of TNF-α, cyclic equibiaxial deformation did not cause significant decrease in total protein concentration. In fact total protein concentration was higher than the control during the remodeling period (Figure 2.3-b). This indicates a different cell growth response as compared to the response of those that underwent cyclic equibiaxial deformation without the exposure of TNF-α (Figure 2.3-b).

### 2.4.4 Effect of Cyclic Equibiaxial Deformation on MMP-2 Expression in A549 Cell Monolayers Either in the Presence or Absence of TNF-α

The total MMP-2 (inactive and active) expression was observed in cells both exposed to cyclic equibiaxial deformation (Figure 2.4-a) and cyclic equibiaxial deformation in presence of TNF-α (Figure 2.4-b). During the first 24-hour period of cyclic equibiaxial deformation, the level of MMP-2 expression was lower than the basal level of MMP-2 expression. However, during the period of remodeling following cyclic equibiaxial deformation, the level of MMP-2 expression began to increase and continued increasing up to 72 hours following cyclic equibiaxial deformation. Cells grown in a static condition (0% stretching level, Figure 2.4-a) did not show significant change in MMP-2 expression. But cells grown in a static condition with TNF-α (0% stretching level, Figure 2.4-b) showed significant increase in the total MMP-2 expression level for 48 and 72 hours.

### 2.4.5 Effect of Cyclic Equibiaxial Deformation on MMP-2 Activation in A549 Cell Monolayers Either in the Presence or Absence of TNF-α

Without any cyclic equibiaxial deformation, only the inactive form of MMP-2 expression was detected at the
Figure 2.3. (a) Effect of cyclic equibiaxial deformation on inflammatory response of A549 cells in the presence of TNF-α. Nitrite concentration was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α (10 ng/ml). Cell monolayers were exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. * significantly higher than the control (p < 0.05). # significantly higher than other time points under the same condition (p < 0.05). (b) Effect of cyclic equibiaxial deformation on A549 cell proliferation in the presence of TNF-α. Total protein concentration was measured in cell lysate of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α. Cell monolayers were exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. * significantly higher than the control (p < 0.05).
48- and 72-hour intervals (Table 2.1 and Figure 2.5-a, 0% stretching). The active form of MMP-2 expression appeared immediately following exposure to cyclic equibiaxial deformation. Under the 10% of cyclic equibiaxial deformation, both active and pro-active forms of MMP-2 expression were observed at 24 and 48 hours (Table 2.1). At 72 hours after cyclic equibiaxial deformation, the pro-active form of MMP-2 expression prevailed under the lower levels of cyclic equibiaxial deformation (5 and 10 %). Under the higher levels of cyclic equibiaxial deformation (15 and 20%), all MMP-2 expression was detected as active forms (Table 2.1 and Figure 2.5-a). In the presence of TNF-α without cyclic equibiaxial deformation, the active form of MMP-2 was observed at both 0 and 24 hours. Thereafter, all MMP-2 expression was detected as pro-active forms (Table 2.2 and Figure 2.5-b, 0% stretching). Exposure to TNF-α alone induced the activation of MMP-2 up to 24 hours. Following the exposure of cyclic equibiaxial deformation in the presence of TNF-α, all MMP-2 expression was observed as active forms during both cyclic equibiaxial deformation and the remodeling period (Table 2.2 and Figure 2.5-b). These results were significantly different from those observed in A549 cell monolayers exposed to cyclic equibiaxial deformation alone (Table 2.1 and Figure 2.5-a).

2.4.6 Expression of TIMP-2. Cyclic equibiaxial deformation did not affect TIMP-2 expression until 24 hours but TIMP-2 expression increased significantly at 48 hours following cyclic equibiaxial deformation and remained higher for 72 hours (Figure 2.6-a). The level of TIMP-2 expression increased in a similar pattern to that observed in the expression of MMP-2 (Figure 2.4-a). Cyclic equibiaxial deformation–induced expression of TIMP-2 in the presence of TNF-α dramatically increased at 24 hours following cyclic equibiaxial deformation and remained higher up to 72 hours (p<0.05)
Figure 2.4. (a) Effect of cyclic equibiaxial deformation on MMP-2 expression in A549 cell monolayers. Total MMP-2 expression was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). The cell monolayers were exposed to different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour refers to starting point of cyclic equibiaxial deformation. *significantly higher than the control (p < 0.05). (b) Effect of cyclic equibiaxial deformation on MMP-2 expression in A549 cell monolayers in the presence of TNF-α. Total MMP expression was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α. Cell monolayers were exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to the different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. *significantly higher than the control (p < 0.05).
Table 2.1. Qualitative analysis results from zymography of media samples from A549 cells exposed to cyclic equibiaxial deformation in the absence of TNF-α. (A –Active, I-Inactive).

<table>
<thead>
<tr>
<th>Cyclic Equibiaxial Elongation Levels</th>
<th>0</th>
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<th>48</th>
<th>72</th>
</tr>
</thead>
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<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>5% None</td>
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<td>A</td>
<td>I</td>
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<td>A</td>
<td>A</td>
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<td>I</td>
</tr>
<tr>
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<td>A/I</td>
<td>A/I</td>
<td>A/I</td>
<td>A/I</td>
</tr>
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<td>A/I</td>
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<td>I</td>
</tr>
<tr>
<td>0% (Control) None</td>
<td>None</td>
<td>None</td>
<td>I</td>
<td>I</td>
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<td>0% (Control) None</td>
<td>None</td>
<td>None</td>
<td>I</td>
<td>I</td>
</tr>
</tbody>
</table>

(Figure 2.6-b). The expression of TIMP-2 significantly increased at 24 hours following stretching although the expression of MMP-2 decreased at the same time interval, especially in the presence of TNF-α (Figure 2.4-b).

2.5 Discussion

We used human type II alveolar epithelial cell monolayers with cyclic equibiaxial deformation, simulating normal breathing and ventilator conditions to study the mechanisms of ventilation-induced lung injury. In this study, we showed the effect of
different levels of cyclic equibiaxial deformation on the net inflammatory response (e.g. NO production) and the net tissue remodeling activity (e.g. expression of MMP-2 and TIMP-2), either in the presence or absence of a pre-existing condition of inflammation (e.g. exposure to TNF-α). It was observed that different levels of cyclic equibiaxial deformation on A549 cell monolayers altered the different levels of inflammatory activity and the net tissue remodeling activities, either in the presence or absence of pre-existing inflammatory stimulus, both during cyclic equibiaxial deformation (first 24 hours) and post-cyclic equibiaxial deformation (next 48 hours). Total MMP-2 expression was

Table 2.2. Qualitative analysis results from zymography of media samples from A549 cells exposed to cyclic equibiaxial deformation in the presence of TNF-α (A – Active, I – Inactive).

<table>
<thead>
<tr>
<th>Cyclic Equibiaxial Elongation Levels</th>
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<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
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<td>5%</td>
<td>A/I</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>5%</td>
<td>A/I</td>
<td>A</td>
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<td>A</td>
</tr>
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<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>10%</td>
<td>A/I</td>
<td>A</td>
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<tr>
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<td>A/I</td>
<td>A</td>
<td>A</td>
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<tr>
<td>10%</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>15%</td>
<td>A/I</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>15%</td>
<td>A/I</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>20%</td>
<td>A/I</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>20%</td>
<td>A/I</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>0% (Control)</td>
<td>A</td>
<td>A</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>0% (Control)</td>
<td>A/I</td>
<td>A</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>0% (Control)</td>
<td>A</td>
<td>A</td>
<td>I</td>
<td>I</td>
</tr>
</tbody>
</table>
Figure 2.5. (a) Effect of cyclic equibiaxial deformation on MMP-2 activation in A549 cell monolayers. Active and pro-active forms of MMP-2 were analyzed by zymography following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). Inverted view of zymography gels were presented for better visualization of the gelatinase activity. Inactive and active forms of MMP-2 were detected at 72 and 62 kD region, respectively. One of three gel pictures was presented in this figure. All results were presented in Table 2.1. (b) Effect of cyclic equibiaxial deformation on MMP-2 activation in A549 cell monolayers in the presence of TNF-α. Active or pro-active forms of MMP-2 were analyzed by zymography following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α. Inverted view of zymography gels was presented for better visualization of the gelatinase activity. One of three gel pictures was presented in this figure. All results were presented in Table 2.2.

decreased during the period of cyclic equibiaxial deformation for 24 hours, and significantly increased for 48 and 72 hours for all levels of cyclic equibiaxial deformation.
either in the presence or absence of TNF-α (Figure 2.4). TIMP-2 expression was steadily increased both during cyclic equibiaxial deformation and post-cyclic equibiaxial deformation either in the presence or absence of TNF-α (Figure 2.6). The decrease in total MMP-2 expression for the 24 hours may be due to the decrease in the total number of cells (Figure 2.3-b) during the period of cyclic equibiaxial deformation. Following cyclic equibiaxial deformation, as cell growth was restored, MMP-2 and TIMP-2 expression increased significantly to a level higher than the control. The expression level of MMP-2 shown in Figure 2.5 includes both active and pro-active forms of MMP-2. To identify active (62 kDa) and pro-active (72 kDa) forms of MMP-2 from the total expression of MMP-2, zymography was used to detect the level of the active form of MMP-2 under different levels of cyclic equibiaxial deformation either in the presence or absence of TNF-α (Figure 2.5).

Without the exposure of TNF-α and cyclic equibiaxial deformation, no detectable level of MMP-2 expression was observed with zymography. Only the inactive form of MMP-2 was detected during the remodeling period (at 48 and 72 hours) (Table 2.1 and Figure 2.5-a, 0% cyclic equibiaxial deformation). All MMP-2 expression was observed to have active form dominant during cyclic equibiaxial deformation (at 24 hour) and during the remodeling period (at 48 hour). Under 10% cyclic equibiaxial deformations, both the active and the inactive forms of MMP-2 were detected during the whole period following cyclic equibiaxial deformation. At 72 hours after cyclic equibiaxial deformation, the inactive form of MMP-2 started to appear in cultures that underwent the lower levels (5 and 10%) of cyclic equibiaxial deformation (Table 2.1 and Figure 2.5-a). As shown in Figure 2.6-a, the level of TIMP-2 expression increased during the post cyclic mechanical
strain, which might have played a role in the inactivation of MMP-2. An increased level of TIMP-2 expression has also been known to stimulate cell growth through the mediation and activation of NF-κB, which is also responsible for the expression of proinflammatory proteins, including inducible nitric oxide synthase (iNOS), which increases the level of NO production.\textsuperscript{13} In this study, we cannot rule out the possibility that A549 cells produced TNF-α in response to cyclic equibiaxial deformation and were stimulated to produce NO by secreted TNF-α.

To investigate the effects of cyclic equibiaxial deformation on cell inflammation and tissue remodeling activity under a pre-existing inflammatory condition, A549 cell monolayers were exposed to TNF-α before starting cyclic equibiaxial deformation.

TNF-α induced NO production without cyclic equibiaxial deformation as shown in Figure 2.3-a (left group of bar graph). The production of NO, the expression of MMP-2, and TIMP-2, induced by cyclic equibiaxial deformation, were different in pattern than those induced by TNF-α (Figure 2.2-a, 2.4-a, and 2.6-a). Additionally, the time at which NO production peaked was different between treatment in the presence and absence of TNF-α. There were significant differences between Fig 2a and 3a in that TNF-α-induced NO production appeared to peak at 48 hours (Fig 2a) while cyclic equibiaxial deformation-induced NO production appeared to continuously increase up to 72 hours (Fig 3a). Cyclic equibiaxial deformation in the presence of TNF-α also resulted in a similar pattern of NO production but with a significantly higher level of NO (p < 0.05). The cell growth was not significantly down-regulated during cyclic equibiaxial deformation or during the remodeling period (Figure 2.2-b). However, the exposure of TNF-α before cyclic equibiaxial deformation had a positive effect on tissue remodeling
activities, especially on the activation of MMP-2 (Figure 2.4-b, Figure 2.5-b, and Table 2.2). Cyclic equibiaxial deformation in the presence of TNF-α affected not only the expression of MMP-2, but also the activation of MMP-2. The exposure to TNF-α might have increased proteolytic activity by increasing MMP-2 and MMP-9 activity. Not only MMP expression but also TIMP expression was increased by TNF-α. TNF-α exposure alone induced an increase in the MMP-2 expression at 48 and 72 hours (0% stretching level, Figure 2.4-b). Exposure to cyclic equibiaxial deformation also induced an increase in the MMP-2 expression at 48 and 72 hours (Figure 2.4-a).

In the presence of TNF-α without cyclic equibiaxial deformation, the active form of MMP-2 was observed at both 0 and 24 hours. Thereafter, all MMP-2 expression was detected as pro-active forms (Table 2.2 and Figure 2.5-b, 0% stretching). Exposure of TNF-α alone induced the activation of MMP-2 up to 24 hours. Following the exposure of cyclic equibiaxial deformation in the presence of TNF-α, all MMP-2 expression was observed as the active form during both cyclic equibiaxial deformation and the remodeling period (Table 2.2 and Figure 2.5-b). This suggests that TNF-α exposure prior to cyclic equibiaxial deformation induced prolonged activation of MMP-2. Cyclic equibiaxial deformation or TNF-α exposure induced the release of inflammatory and tissue remodeling mediators through the different interconnected pathways. Mechanical deformation increases the cytoplasmic Ca^{2+} concentration through various pathways and mechanisms, which include an increase in intracellular inositol 1,4,5-trisphosphate (IP3) concentration; the activation of stretch activated calcium channels, and the repair of stretch activated plasma membrane damage. The increased concentration of cytoplasmic Ca^{2+} boosts the Calmodulin (CaM) activity and amplifies the basal level of
Figure 2.6. (a) Effect of cyclic equibiaxial deformation on TIMP-2 expression in A549 cell monolayers in the absence of TNF-α. Total TIMP-2 expression was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%). The cell monolayers were exposed to the different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. Time 0 hour refers to starting point of cyclic equibiaxial deformation. *significantly higher than the control (p < 0.05). (b) Effect of cyclic equibiaxial deformation on TIMP-2 expression in A549 cell monolayers in the presence of TNF-α. Total TIMP-2 expression was measured in media supernatant of A549 culture following different levels of cyclic equibiaxial deformation (0, 5, 10, 15, and 20%) in the presence of TNF-α. Cell monolayers are exposed to TNF-α for 24 hours and then grown in static condition (for controls) or under cyclic equibiaxial deformation. The cell monolayers were exposed to the different levels of cyclic equibiaxial deformation for 24 hours and then grown in static condition for another 48 hours. *significantly higher than the control (p < 0.05).
NO by increasing the activity of constitutive nitric oxide synthase (cNOS). On the other hand, TNF-α induced the activation of NF-κB, resulting in the induction and activation of iNOS and the release of a large amount of NO. Increased NO production following cyclic equibiaxial deformation in the presence of TNF-α (Figure 2.3-a) could be explained by the combined effect of cNOS and iNOS. Mechanical ventilation also increased tissue remodeling activities through the increased activity of metalloproteases in airway epithelial cells. Haseneen et al. have shown that mechanical stretch induced the expression of MMP (MT1-MMP) in the presence of the inducer, EMMPRIN. Little is known about how MMP-2 and TIMP-2 (5 and 10 %) are up-regulated in airway epithelial cells during and after cyclic mechanical strain in the presence of TNF-α. TNF-α may have increased the expression of MMP-2 and TIMP-2 through the activation of the NF-κB pathway or possibly through interaction with MMPs, which underwent mechanical ventilation-induced activation as shown in Figure 2.4 and 2.6.

In conclusion, our study supports the idea that exposure to cyclic equibiaxial deformation, either in the presence or absence of preexisting inflammation, positively regulates the inflammatory and net tissue remodeling activities in A549 cells. Cyclic equibiaxial deformation induced NO production and MMP-2/TIMP-2 expression. Higher levels of cyclic equibiaxial deformation increased the active form of MMP-2 in both instances. However, in the presence of TNF-α more of the active form of MMP-2 was detected during both cyclic equibiaxial deformation and remodeling periods.

2.6 References

1 Altemeier, W. A., G. Matute-Bello, C. W. Frevert, Y. Kawata, O. Kajikawa, T. R. Martin, and R. W. Glenny. Mechanical ventilation with moderate tidal volumes...


CHAPTER 3
EFFECTS OF DIESEL PARTICULATE MATTERS ON INFLAMMATORY RESPONSES IN STATIC AND DYNAMIC CULTURE OF HUMAN ALVEOLAR EPITHELIAL CELLS†

3.1 Abstract

Diesel particulate matter (DPM) possesses the potential to induce acute and chronic health issues upon occupational and daily exposure. Many recent studies have focused on understanding molecular mechanisms to depict DPM’s side effects inside the lung using static in vitro cell culture models. These studies have provided abundant fundamental information on DPM’s adverse effects on cellular responses, but these systems were limited by the absence of dynamic nature to access relevant cellular responses and functionality. We hypothesized that the exposure of DPM under dynamic environment may affect the levels of cellular inflammation and reactive oxygen species, which may be different from those under static environments. In this study, we used the dynamic cell growth condition to mimic mechanically dynamic environment similar to the normal breathing in vivo. We also used high (20, 10, and 5 ppm) and low (3, 1, 0.1, and 0.01 ppm) ranges of DPM exposure to mimic different levels of exposure, respectively. Following 24, 48, and 72 hour exposure of DPM, Interleukin-8 (IL-8), C-reactive protein (CRP), reactive oxygen species (ROS), and total amount of protein were analyzed. Our results demonstrated the distinct differences in the profiles of

inflammatory mediators (IL-8, CRP, and ROS) between the static and dynamic cell growth conditions.

3.2 Introduction

Epidemiological and lab-bench scale studies have linked air pollution with various, potentially fatal respiratory and cardiovascular conditions (Anenberg et al., 2010; Kramer et al., 2010; Liao et al., 2010). As outlined by United States environmental protection agency (EPA), six common air pollutants are carbon monoxide, ozone, lead, nitrogen dioxide, particulate matter (PM), and sulfur dioxide (EPA, 2010; Mazzoli-Rocha et al., 2010; Neher et al., 1994). Of these common air pollutants, PM has been studied most extensively to understand and characterize its adverse effect on human health due to its strong association with air pollution related morbidity (Kelsall et al., 1997; Mazzoli-Rocha et al., 2010; Saldiva et al., 1995; Schwartz et al., 2001; Schwartz et al., 1990; Zanobetti et al., 2000). One of the most common forms of PM generated from human activities is DPM or diesel exhaust particles (DEP), which is frequently linked with occupational and public exposure in urban areas (Patel et al., 2010; Zuurbier et al., 2010). Due to the complex diversity in composition and nanoscale dimension, DPM can cause potentially adverse effect when humans are exposed to it for an extended period (Mazzoli-Rocha et al., 2010; Saldiva et al., 2002).

Previous studies have highlighted inflammation, autonomic nervous system activity, procoagulant effects, covalent modification of cellular components, and ROS production as contributors to DPM’s adverse health effects (Li et al., 2008; Mazzoli-Rocha et al., 2010; Nel et al., 2006). Many epidemiological studies have also successfully established the correlation between DPM exposure and various adverse health effects
In vivo studies performed on small animals and human volunteers have shown the occurrence of precursor events which can lead to pulmonary and cardiac disorders as suggested by epidemiological studies (Ghio et al., 2000; Gong et al., 2003a, 2003b; Holgate et al., 2003a, 2003b; Nemmar et al., 2002, 2003; Salvi et al., 1999). Findings from in vivo studies were helpful in understanding the systematic responses of the whole exposure organ, the lung, and the body. However, they were limited to show the molecular level interaction between cells and DPM, which is equally important in understanding the chronological order of events post DPM exposure (Fischer et al., 2007). In vitro studies performed to evaluate the effect of DPM exposure on single cell type monolayers or co-cultures, mimicking the exposure boundary for different organs, have been more preferable in understanding the cellular response and determining the biomarkers for characterization (Alfaro-Moreno et al., 2008; Fischer et al., 2007; Jones et al., 2009; Mazzarella et al., 2007; Stoker et al., 2008). Many in vitro studies, conducted on lung airway epithelial cells, have shown the changes in extracellular levels of various signaling molecules such as CRP, heat shock protein-70, IL-8, interleukin-6, cyclooxygenase-2, granulocyte-macrophage colony-stimulating factor etc., under the exposure of DPM (Ahn et al., 2008; Alfaro-Moreno et al., 2008; Chirino et al., 2010; Dobrovolskaia et al., 2007; Mazzarella et al., 2007; Moller et al., 2010; Ramage et al., 2004b; Sanchez-Perez et al., 2009; Seagrave et al., 2004; Veranth et al., 2008). Results of these in vitro studies might be useful in characterizing pre-cursory events that can be linked to whole organ (lung) response from the in vivo studies (Ahn et al., 2008; Alfaro-Moreno et al., 2008; Chirino et al., 2010; Dobrovolskaia
et al., 2007; Mazzarella et al., 2007; Moller et al., 2010; Ramage et al., 2004b; Sanchez-Perez et al., 2009; Seagrave et al., 2004; Veranth et al., 2008). However, the most in vitro studies related to DPM exposure could not be well translated to in vivo studies (Fischer et al., 2007). Many possible reasons such as complex chemical composition, variety in nanoscale size distribution of DPM, use of unreasonable exposure concentrations in experimental design, interference of DPM with biomarkers (signaling molecules) and poor functionality of in vitro models, could be at blame to explain the poor translatability of in vitro studies (Grainger, 2009; Jones et al., 2009; Moller et al., 2010; Seagrave et al., 2004). Moreover, most of the in vitro DPM-toxicity studies were performed under the static cell culture system, which might be limited in mimicking dynamic nature of tissues in vivo. Our study was performed with an additional parameter related to dynamic nature of exposed tissues in the experimental design, which included lung cells grown under the dynamic cell growth condition during the period of DPM exposure.

The airway wall exists in a mechanically active environment, where different amounts of circumferential and longitudinal expansion and contraction occurred during breathing movements. We hypothesized that the exposure of DPM under dynamic environment may alter its interaction with cells, and affect the levels of cellular inflammation and reactive oxygen species, which may be different from those under static environments. To test our hypothesis, we used the dynamic in vitro culture system, simulating the mechanical environment similar to normal breathing condition (5% of surface elongation at the frequency of 0.2 Hz) in the lung (See Chapter 2; Tschumperlin et al., 1998). This dynamic in vitro culture system of human airway epithelial cells was used for the investigation of DPM effects on cell proliferation, cellular inflammatory
response (IL-8), ROS production, and CRP expression. In this study, we used the concentrations of DPM in the range of 0.01 – 20 ppm. Possible atmospheric concentrations of DPM lie between 0.01 and 0.1 (not exceed 1-3 ppm). Exposure at the concentrations above 3 ppm can be the case of occupational exposure (EPA, 2002; Hesterberg et al., 2009). Our in vitro dynamic culture system improved cellular functionality, improvised a mechanical environment similar to that found in the lungs, and facilitated the altered interactions between DPM and human airway epithelial cells (See Chapter 2). This study will provide crucial information to develop viable alternatives to in vivo tests to evaluate the toxicity of DPM exposure and other air pollutants in combination in the future.

3.3 Materials and Methods

3.3.1 Dynamic Cell Culture System. Type II alveolar basal epithelial cells of human origin (A549) were purchased from ATCC (Manassas, VA). A549 is epithelial-like in morphology and originates from a human lung carcinoma patient. The cells were seeded at $3 \times 10^5$ cells per well ($9.6 \text{ cm}^2$) into six well BioFlex plates (Flexcell International, PA) containing 2 ml of F-12k culture medium, which was supplemented with 1% penicillin streptomycin (Invitrogen, CA) and 10% fetal bovine serum (Thermo Fisher Scientific, UT). After cells reached confluence, they were exposed to DPM at different concentrations and grown under either static or cyclic strain (dynamic) condition. The dynamic cell growth condition was implemented using Flexcell® Tension Plus™ 4000T system, which used vacuum pressure to apply cyclic strain to cells cultured on BioFlex plates. Schematic diagram of Flexcell® Tension Plus™ 4000T system has
Figure 3.1. Schematic Diagram of Flexcell® Tension Plus™ 4000T system. A computer driven system controls the pressure of vacuum, which pulls down the silastic membrane around loading post and implements the circumferential strain in cell culture growing on top of the loading post.

been included in Figure 3.1. During the course of dynamic cell growth, cyclic stretching was applied to silastic well-bottoms of BioFlex plates to attain 5% surface elongation at the frequency of 0.2 Hz, which corresponds to 45% of the total lung capacity similar to normal breathing condition in the lung (Tschumperlin et al., 1998).

3.3.2 DPM Solution Preparation. DPM (standard reference material 1650b, SRM 1650b) was obtained from National Institute of Standards and Technology (NIST). Details of certified concentrations of selected polycyclic aromatic hydrocarbons (PAH) in SRM 1650b have been outlined on NIST’s website (www.nist.gov/srm/index.cfm). The stock solution for DPM was prepared by suspending 116.1 mg of DPM in 3 ml of sterile deionized water. To breakdown the agglomerates and achieve better suspension of DPM, stock solutions was sonicated using water-bath sonicator for 12 hours under temperature
controlled condition. To make exposure concentrations of 20, 10, 5, 3, 1, 0.1 and 0.01 ppm, appropriate amounts of stock solutions were added to complete cell culture media.

3.3.3 DPM Exposure. F-12k complete medium containing DPM at different concentration (20, 10, 5, 3, 1, 0.1 and 0.01 ppm) was added to A549 monolayers when cells were confluent. Immediately after adding DPM containing media, cells were grown in either static or dynamic conditions for different exposure time (24, 48, and 72 hours). The dynamic cell growth condition was simulated by growing cell monolayers under continuous cyclic equibiaxial deformation with 5% surface area change at 0.2 Hz, which was similar to normal breathing in vivo (Tschumperlin et al., 1998). Following each exposure time, media and cell lysate samples were collected in aliquots and immediately stored at -80°C until they were analyzed. All samples were analyzed immediately once thawed. Media supernatant samples were used to measure the level of IL-8, CRP and ROS. Cell lysate samples were used to measure the level of total protein.

3.3.4 IL-8 Measurement. IL-8 from media supernatant was measured using ELISA prepared by IL-8 human antibody pair and buffer kit (Invitrogen, CA). The concentration unit of IL-8 was picograms/milliliter (pg/ml).

3.3.5 Reactive Oxygen Species (ROS) Measurement. The ROS level was measured using de-acetylated probe 2’,7’-dichlorofluorescin (H$_2$DCF) based fluorescence assay to evaluate the level of ROS. The H$_2$DCF was prepared from 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) by alkaline hydrolysis using NaOH (Cathcart et al., 1983). 500 μl of 1 mM H$_2$DCFDA was added to 2 ml of 0.01N NaOH and hydrolyzed into H$_2$DCF at room temperature for 30 minutes. The prepared H$_2$DCF solution was neutralized by adding 10 ml of 25mM NaH$_2$PO$_4$ and pH of the solution was
adjusted to 7.4. Right after pH adjustment, 40 µM H$_2$DCF solution was kept on ice or at 4°C until used. Fresh H$_2$DCF solution was prepared before ROS measurement to avoid molecular probe deterioration.

To perform the ROS measurement, 20 µl of media supernatant sample (cell-free sample) was incubated with 50 µl of 40 µM H$_2$DCF and 130 µl of 40 mM Tris-HCl, pH 7.4 for 10 minutes at 37 °C, which was oxidized to 2’,7’-dichlorofluorescein (DCF). Level of DCF was measured using Synergy 4 series multiwell-plate fluorometer (Biotek, VT), which was set at an excitation of 488 nm and emission of 525 nm. The level of DCF (i.e. fluorescence) was correlated to the level of ROS in the media supernatant samples, collected from the experiments. To measure the interference of DPM on oxidization of H$_2$DCF to DCF, media samples with DPM concentrations at 0.01, 0.1, 1, 3, 5, 10, and 20 ppm were tested using the same procedure.

3.3.6 **Total Protein Measurement.** Cells grown on each well (9.6 cm$^2$) of BioFlex plates were lysed using 250 µl of RIPA buffer with protease inhibitors (Thermo Scientific, IL). Total amount of protein from cell lysate of each sample was measured using the BCA total protein assay (Pierce, IL) to evaluate cell proliferation. The concentration unit of total protein was micrograms/milliliter (µg/ml).

3.3.7 **CRP Measurement.** CRP level from media supernatant was measured using ELISA kit obtained from Helica Biosystems Inc, CA. The concentration unit of CRP was nanograms/milliliter (ng/ml).

3.3.8 **Statistical Analysis.** All data from IL-8, CRP and ROS measurement were normalized with total amount of protein measured from cell lysate, collected from respective samples. Statistical analyses were carried out using two-way analyses of
variance (ANOVA) followed by Dunnett’s multiple comparison tests to determine where significance exists (p<0.05). All graphs were prepared by plotting mean data (sample size, n = 3) with corresponding standard error of mean.

3.4 Results

3.4.1 Effect of DPM Exposure on Cell Growth. Total protein was measured from cell lysate to characterize the cell proliferation under static and dynamic cell growth conditions at different concentrations of DPMs. Following 24 hour exposure of DPM, A549 cell growth was not significantly different under all DPM exposure concentrations as compared to the respective controls in both cell growth conditions (Figure 3.2-a), except for DPM concentrations of 0.1, 1, 3 and 20 ppm under the dynamic cell growth condition (Figure 3.2-a, **p<0.05). Moreover, under dynamic condition at the DPM concentrations of 0.1, and 3 ppm, cell proliferation significantly decreased as compared to the static condition (Figure 3.2-a, *p<0.05). Following 48 hour exposure of DPM, A549 cell growth was not significantly different as compared to the respective controls under both cell growth condition, except at 0.1 ppm under dynamic cell growth condition (Figure 3.2-b, **p<0.05). However, after 72 hour exposure of DPM, A549 cell growth under static cell growth condition significantly decreased at 0.1, 1, 3, 5, 10 and 20 ppm as compared to the control (static condition at 0 ppm of DPM) (Figure 3.2-c, +p<0.05). At lower concentrations (0.01 – 1 ppm) of DPM under the static cell growth condition following 72 hour exposure, the decrease in cell proliferation was dose-dependent (Figure 3.2-c). However, cell proliferation under dynamic condition following 72 hour exposure significantly decreased at 1 and 5 ppm of DPM (Figure 3.2-c, **p<0.05). Also, at the
Figure 3.2. Cell proliferation in A549 cultures grown under static or dynamic cell growth condition exposed at 0 (control), 0.01, 0.1, 1, 3, 5, 10, and 20 ppm of DPM. Total protein level was measured from cell lysate to characterize the cell proliferation during the exposure to different concentrations of DPM under static and dynamic cell growth conditions. a) 24 hours exposure of DPM, b) 48 hours exposure of DPM, and c) 72 hours exposure of DPM. * Significantly different from the static condition at each concentration of DPM (p<0.05). **Significantly different from the control for static cell growth condition (static condition at 0 ppm of DPM) (p<0.05). ***Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 ppm of DPM) (p<0.05).
concentrations of 0.1, 1, and 20 ppm under the dynamic condition following 72 hour exposure, cell proliferation remained higher than that in static cell growth condition at same concentrations of DPM (Figure 3.2-c, *p<0.05).

3.4.2 Effect of DPM Exposure on Cellular Inflammation. IL-8 was measured from culture media to characterize the inflammatory response of cells exposed to different concentrations of DPM under static and dynamic cell growth conditions. The total amount of protein obtained from the cell lysate of each sample was used to normalize the IL-8 measurement of same sample, to compensate for the variation in IL-8 measurement resulting from the different number of cells in different cell growth conditions. In case of 24 hour exposure of DPM, extracellular level of IL-8 in A549 cells was significantly higher in the dynamic cell growth condition than the static condition at all concentrations of DPM (0 -20 ppm) (Figure 3.3-a, *p<0.05). Especially at DPM concentrations of 0.1 and 1 ppm under the dynamic cell growth condition, the level of IL-8 was significantly higher than the control (the dynamic condition at 0 ppm of DPM) (Figure 3.3-a, **p<0.05). After 48 hours of exposure, significant increase in IL-8 was observed only at DPM concentrations above 0.01 ppm in A549 cell cultures grown under the dynamic cell growth conditions (Figure 3.3-b, *p<0.05). However, there was no concentration effect on extracellular levels of IL-8 in both static and dynamic conditions, in case of 48 hour exposure (Figure 3.3-b). As exposure time increased up to 72 hours, the levels of IL-8 was dramatically changed between different concentrations and significantly higher than each control in both static and dynamic growth conditions (Figure 3.3-c, +p<0.05, and **p<0.05). Extracellular IL-8 levels in A549 cell cultures were significantly higher under each DPM exposure concentrations as compared to
**Figure 3.3.** Cellular Inflammation in A549 cultures grown under static or dynamic cell growth condition exposed at 0 (control), 0.01, 0.1, 1, 3, 5, 10, and 20 ppm of DPM. IL-8 was measured from culture media to characterize the inflammatory response of cells exposed to different concentrations of DPM under static and dynamic cell growth conditions. Total amount of protein obtained from cell lysate of each sample was used to normalize the IL-8 measurement of same sample, to compensate the variation of IL-8 measurement resulted from different number of cells at different cell growth conditions. a) 24 hours exposure of DPM, b) 48 hours exposure of DPM, and c) 72 hours exposure of DPM. *Significantly different from the static condition at each concentration of DPM (p<0.04). †Significantly different from the control for static cell growth condition (static condition at 0 ppm of DPM) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 ppm of DPM) (p<0.05).
controls for respective cell growth conditions (Figure 3.3-c, +p<0.05, and **p<0.05). The level of IL-8 was steadily increased at lower concentrations up to 1 ppm, and its level was decreased at higher concentrations above 1 ppm in both static and dynamic conditions. Especially at concentrations of 1, 3, and 5 ppm, the extracellular levels of IL-8 in A549 cell cultures grown under the dynamic condition was significantly higher than the static condition (Figure 3.3-c, *p<0.05). Only for the highest concentration (20 ppm), IL-8 level in cells grown under the static condition was significantly higher than the dynamic condition (Figure 3.3-c, *p<0.05).

3.4.3 Characterization of Acute Inflammatory Response. CRP levels were measured from culture media to characterize the acute-phase inflammatory response in A549 cells exposed to different concentrations of DPM under both cell growth conditions. The total amount of protein obtained from the cell lysate of each sample was used to normalize the CRP measurement of same sample, to compensate for the variation in CRP measurement resulting from the different number of cells in different cell growth conditions. In case of 24 hour exposure of DPM, extracellular level of CRP in A549 cells steadily increased at lower concentrations below 1 ppm, and decreased at higher concentrations above 1 ppm in the static conditions. No significant changes in CRP levels were observed in the dynamic growth condition. Extracellular levels of CRP in A549 cells grown under the static condition were significantly higher than the control at all concentrations, except 5 ppm (Figure 3.4-a, +p<0.05). During this period of exposure, significant changes in CRP levels were observed in the static condition. At the concentrations of 1, 3, and 5 ppm, CRP levels were significantly higher in the static condition than the dynamic condition (Figure 3.4-a, *p<0.05). Following exposure of 48
Figure 3.4. CRP level in A549 cells grown under static and dynamic cell growth condition exposed at 0 (control), 0.01, 0.1, 1, 3, 5, 10, and 20 ppm of DPM. CRP was measured from culture media to characterize the inflammatory response of cells exposed to different concentrations of DPM under static and dynamic cell growth conditions. Total amount of protein obtained from cell lysate of each sample was used to normalize the CRP measurement of same sample, to compensate the variation of CRP measurement resulted from different number of cells at different cell growth conditions. a) 24 hours exposure of DPM, b) 48 hours exposure of DPM, and c) 72 hours exposure of DPM. *Significantly different from the static condition at each concentration of DPM (p<0.05). †Significantly different from the control for static cell growth condition (static condition at 0 ppm of DPM) (p<0.05).
hours, there was no significant change in extracellular levels of CRP at any concentrations in either static or dynamic conditions (Figure 3.4-b). After 72 hours of exposure, extracellular levels of CRP were not significantly different in A549 cells exposed to different DPM concentrations under the dynamic cell growth condition when compared to the control (Figure 3.4-c). During this period of exposure, the only significant change in CRP level was observed at 20 ppm under the static cell growth condition as compared to the control (Figure 3.4-c, \( p<0.05 \)). At concentrations of 3 and 20 ppm, levels of CRP were significantly higher in the static cell growth condition than the dynamic cell growth condition. Also, when the levels of CRP were compared among three different exposure durations at same concentrations of DPM under both cell growth conditions, CRP levels were significantly higher during 24 hours exposure than during longer exposure time (\( p<0.05 \), no statistical markers shown in figure).

3.4.4 Characterization of Oxidative Stress Through ROS Measurement. ROS levels were measured from culture media to characterize the oxidative stress in A549 cells exposed to different concentrations of DPM under both cell growth conditions. The total amount of protein obtained from the cell lysate of each sample was used to normalize the ROS measurement of the same sample, to compensate for the variation of ROS measurement resulting from the different number of cells at different cell growth conditions. The level of ROS steadily increased at lower concentrations below 3 ppm, and decreased at higher concentrations above 3 ppm in both static and dynamic conditions (Figure 3.5-a). The levels of ROS at concentrations of 0.1, 1, and 3 ppm was significantly higher under both cell growth conditions when compared to respective controls (Figure 3.5-a, \( p<0.05 \), and \( **p<0.05 \)). At concentration of 5 ppm, ROS levels
Figure 3.5. ROS production in A549 cells grown under static and dynamic cell growth condition exposed at 0 (control), 0.01, 0.1, 1, 3, 5, 10, and 20 ppm of DPM. ROS was measured from culture media to characterize the inflammatory response of cells exposed to different concentrations of DPM under static and dynamic cell growth conditions. Total amount of protein obtained from cell lysate of each sample was used to normalize the ROS measurement of same sample, to compensate the variation of ROS measurement resulted from different number of cells at different cell growth conditions. a) 24 hours exposure of DPM, b) 48 hours exposure of DPM, and c) 72 hours exposure of DPM. *Significantly different from the static condition at each concentration of DPM (p<0.05). †Significantly different from the control for static cell growth condition (static condition at 0 ppm of DPM) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 ppm of DPM) (p<0.05).
were significantly higher than the control in the static cell growth condition (+p<0.05). Following 48 hours of exposure, the ROS level was higher in the dynamic condition than in the static condition at 3ppm of DPM (Figure 3.5-b, *p<0.05). In the dynamic cell growth condition during this period of exposure, significant changes were observed at concentrations of 0.1, 1, and 3 ppm (Figure 3.5-b, **p<0.05). After 72 hours exposure, DPM concentrations of 1, 3, and 5 ppm induced significantly higher levels of ROS as compared to control under the dynamic cell growth condition (Figure 3.5-c, **p<0.05). Under the static cell growth condition, DPM concentrations of 0.1, 1, 3, 5, and 10 ppm induced significantly higher levels of ROS as compared to the control (Figure 3.5-c, +p<0.05). Also, 0.1 ppm exposure concentration induced significantly higher levels of ROS under the static cell growth condition than the dynamic cell growth condition (Figure 3.5-c, *p<0.05). When the levels of ROS were compared among three different exposure durations at same concentrations of DPM under both cell growth conditions, ROS levels were significantly higher during 24 hours exposure than during longer exposure time (p<0.05, no statistical markers shown in figure). ROS measurement was not affected by the presence of DPM in the media.

3.5 Discussion

To evaluate the inflammatory effect of DPM on the respiratory system with more realistic in vitro models (viable alternative to in vivo model), we used a dynamic cell growth system. The levels of total protein, IL-8, CRP and ROS were measured to characterize cell growth, cellular inflammation and oxidative stress, respectively. A549 cells were used as a model cell line. Even though A549 cell lines lack several key
physiological features (e.g. tight junction formation, cilia formation, etc.) of airway type II cells, it has been well characterized and used to study airway diseases and cellular inflammation. A dynamic environment was used to implement the cell growth environment inside the lung, with its varying amounts of circumferential and longitudinal expansion and contraction occurred during breathing movements. It is important to utilize the dynamic cell growth condition for evaluation of interactions between cells and nanomaterials since the mechanical environment may facilitate epithelial uptake of nanomaterials through the altered interaction between cells and nanoparticles (Chambers et al., 2007; Huh et al., 2010).

It was observed that the dynamic cell growth condition induced significantly higher levels of inflammation in A549 cell cultures exposed to DPM at various concentrations. Cells grown under the dynamic cell growth condition following 24 and 48 hours exposure showed elevated levels of IL-8 at all concentrations of DPM, except at 0 and 0.01 ppm post 48 hours exposure (Figure 3.3-a, and 3.3-b, *p<0.05). Following 72 hours of exposure under both cell growth conditions, IL-8 levels were significantly higher at all DPM exposure concentrations as compared to controls (Figure 3.3-c). After 24 hours of exposure, extracellular IL-8 level was significantly higher in controls for the dynamic cell growth condition as compared to control for the static condition (Figure 3.3-a). This difference could be a result of an initial increase of inflammation induced by the implementation of dynamic cell growth condition following confluence (Yamamoto et al., 2001). Studies based on human subjects reported significant increases in IL-8 expression in bronchial tissues (BT) and bronchial wash (BW) cells after two separate 1 hour exposure to diesel exhaust (DE) (Salvi et al., 2000), which was similar to
inflammatory response observed in the dynamic cell growth condition after 24 hour exposure in this study (Figure 3.3-a). In case of longer exposure to DPM (72 hours in this study), extracellular levels of IL-8 were significantly elevated in a dose-dependent manner at lower concentrations (up to 1 ppm) of DPM in A549 cell cultures exposed under both cell growth conditions as compared to controls (Figure 3.3-c, + p<0.05 and **p<0.05). The dose-dependent increase in IL-8 levels was not observed at higher concentrations of DPM (above 1 ppm) for the longer period of exposure (Figure 3.3-c). This may be due to the interferences caused by possible interaction between DPM and IL-8 or to DPM’s agglomerate formation at higher concentrations, which may lead to size dependent effect (Seagrave et al., 2004). Also, during the longer exposure period, only three concentrations (1, 3, and 5 ppm) induced significantly higher levels of IL-8 under the dynamic cell growth conditions than the static cell growth condition (Figure 3.3-c, *p<0.05). Longer durations of exposure might have provided enough time for DPM to interact with cells under both cell growth conditions. The increased levels of extracellular IL-8, under longer exposure period, can be related with neutrophil migration and mucin production as a precursory event to remove DPM particles at sites of exposure in the lung (Bautista et al., 2009; Kunkel et al., 1991). Small animal studies on mice and rats have reported the increase in neutrophil migration and in as little as 48 hours after the DEP exposure (Harkema et al., 2004; Nemmar et al., 2008). Also, the studies conducted on human subjects indicated the increase in IL-8 expression and neutrophil levels in BT and BW following two separate 1 hour exposure to DE (Salvi et al., 1999, 2000), which was parallel to the results from DPM exposure under the dynamic cell growth condition (Figure 3.3).
A549 cell cultures treated under the dynamic cell growth condition, following the DPM exposure for all exposure duration, showed no significant change in extracellular levels of CRP. Only A549 cells grown under the static cell growth conditions had significantly higher levels of CRP following 24 hours exposure at all concentrations of DPM, except for 5 ppm, as compared to control (Figure 3.4-a, \( +p<0.05 \)). Also, at concentrations of 1, 3, and 5 ppm, cells grown under the static condition showed significantly higher levels of CRP as compared to the dynamic condition (Figure 3.4-a, \( *p<0.05 \)). Following 48 and 72 hours exposure, extracellular levels of CRP was not significantly changed in both conditions for all concentrations of DPM, except 3 and 20 ppm, at which significantly higher levels of CRP were induced under the static cell growth condition following 72 hours exposure (Figure 3.4-c, \( *p<0.05 \)). Post 72 hours exposure, significantly higher level of CRP was detected only at 20 ppm (Figure 3.4-c, \( +p<0.05 \)). Ramage et al. showed increased CRP expression in the intra-/extracellular environment of *in vitro* A549 cell cultures upon exposure to carbon particles and particulate matter (PM-10), which was consistent with CRP level in cells grown under the static condition during DPM exposure (Ramage et al., 2004a; Ramage et al., 2004b). However, the study conducted on human subjects by Carlsten et al. showed no change in CRP levels post DE exposure. This finding was similar to what we observed in cells grown under the dynamic condition following DPM exposure (Carlsten et al., 2007). This discrepancy in CRP levels between *in vitro* and *in vivo* studies could be explained by the observation of the results between static and dynamic growth conditions, providing the possibility that the dynamic environment may facilitate altered interactions between cells and DPM.
ROS levels in A549 cells exposed to 0.1, 1, and 3 ppm showed significant increases under both cell growth conditions following 24 hours exposure as compared to their respective controls (Figure 3.5-a, **p<0.05, and +p<0.05). Following other exposure times (48, and 72 hours), significant increases in ROS level were observed only at a couple of concentrations under either static, dynamic or both conditions as compared to the control. For any pair-wise comparison, the mean difference in ROS level was not as high as post 24 hour exposure (Figure 3.5-b, and 3.5-c). Results from our study were in parallel to both in vitro and in vivo studies demonstrating PM, DEP, DE and DPM’s ability to induce oxidative stress through increased ROS production and related damage in exposed cells and tissues (Chirino et al., 2010; Mazzoli-Rocha et al., 2010; Moller et al., 2010; Sanchez-Perez et al., 2009).

As previously reported, the increased level of ROS is strongly correlated with the increased level of CRP (Ramage et al., 2004a), similar correlations between ROS and CRP (correlation coefficient (r) =0.83) was found at low concentration range (0.1, 1, and 3 ppm) following 24 hour exposure in the static cell growth condition (Figure 3.4-a and 3.5-a, +p<0.05). Under the static cell growth condition, at the rest of the concentrations for which CRP levels were significantly elevated (0.01, 10, and 20 ppm), ROS levels were also elevated higher than the control (p<0.07), but were not significantly different due to small marginal differences. This correlation between CRP and ROS could be explained by the oxidative stress dependent CRP expression in A549 cells (Ramage et al., 2004a). Under the dynamic cell growth condition, there was no clear correlation between ROS and CRP (correlation coefficient (r) = 0.47). CRP levels were not significantly different from control under the dynamic condition (Figure 3.4-a), whereas ROS levels
were significantly higher than the control following 24 hours of exposure (Figure 3.5-a, 0.1, 1, and 3 ppm **p<0.05). The correlation between ROS and CRP levels might have been altered as a result of the inhibitory effect of dynamic condition on CRP expression level. Existing studies showed the link between the increased level of ROS and NF-κB activation (Chou et al., 2008; Ghosh et al., 1998), which can initiate the IL-8 expression (Ye et al., 2009) and CRP expression (Zhang et al., 1995). As we mentioned the relationship among IL-8, CRP, and NF-κB, and the relationship between ROS and NF-κB, the significant increase in ROS level can trigger the increased expression of IL-8 and CRP, which should increase in parallel to each other. In our results, under static cell growth condition during 24, and 48 hour exposure, only extracellular level of CRP increased in accordance to the increase in ROS levels, whereas under dynamic condition, only extracellular level of IL-8 increased in accordance to the increase in ROS levels. During 72 hour exposure, when IL-8 level increased in accordance to the increase in ROS levels, the CRP level remained unchanged under both cell growth conditions, except for 3, and 20 ppm under static condition. Even though IL-8 and CRP get expressed through the same transcription factor, NF-κB, they might be dependent on the different cell growth environment and the level of inflammatory markers expressed. Our results strongly demonstrated the distinct differences in the profiles of inflammatory mediators such as IL-8, CRP, and ROS between static and dynamic cell growth conditions used for measuring the adverse effect of DPM.

The dynamic cell growth system together with the static cell growth system yielded several important findings: 1. All IL-8 levels in the dynamic condition were significantly higher than those of the static condition except for concentrations of 0 and
0.01 ppm after 48 hour exposure. 2. Although no significant increase in IL-8 levels was detected in the static condition as DPM concentration increased, a significant increase in IL-8 was observed in the dynamic condition following 24 hours of exposure, especially at low concentrations of DPM (0.1 and 1 ppm) (Figure 3.3-a). 3. The correlation between ROS and CRP was found at low concentration range (0.1, 1, and 3 ppm) following 24 hour exposure in the static cell growth condition. 4. The dynamic condition facilitated altered interactions between DPM and A549 cells, yielding levels of IL-8, ROS, and CRP similar to those observed in in vivo studies. In conclusion, the dynamic cell growth system used in this study provided important changes in cellular responses that were not found in the static cell growth system. The dynamic cell growth systems can be considered as viable alternative to in vivo test system in combination with existing in vitro static cell growth systems to evaluate the cellular responses of DPM on the respiratory system.

3.6 References


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CHAPTER 4
ALTERNATIVE ESTIMATION OF HUMAN EXPOSURE TO MULTI-WALLED CARBON NANOTUBES USING DYNAMIC CULTURE OF AIRWAY EPITHELIAL CELLS

4.1 Abstract

Multi-walled carbon nanotubes (MWCNTs) possess significant potential to revolutionize many fields of research and technology development with their unique properties. Due to the rapidly increasing demands for use and production, continued evaluation of the potential health risks associated with exposure to nanomaterials is essential to ensure their safe handling. Many studies have emphasized investigation of the effects of nanomaterials on the respiratory system using either animal or in vitro cell culture models. Animal studies provide important findings about systemic responses to MWCNT exposure in lung tissues (Mercer et al., 2010; Sakamoto et al., 2009; Shvedova et al., 2008). In vitro studies also provide a considerable amount of fundamental information about MWCNT-induced effects on different types of lung cells (Hirano et al., 2008, 2010; Ye et al., 2009). However, the cell culture systems used in these studies were limited by the absence of the dynamic cell growth environment present in actual lung tissues. We hypothesized that MWCNT-induced cellular responses such as proliferation, inflammation, and oxidative stress under a dynamic cell growth environment may differ from those under a static cell growth environment. In this study we used a dynamic cell growth condition to mimic the mechanically dynamic environment of the lung and to characterize expression of interleukin (IL-8), reactive oxygen species (ROS), glutathione...
(GSH), and the total amount of protein for three days following exposure to MWCNTs at different concentrations. Our results demonstrated the distinct differences in the levels of inflammatory response and oxidative stress between static and dynamic cell growth conditions.

4.2 Introduction

Rapid advancement in the field of nanotechnology has given birth to various types of nanomaterials with unique mechanical, thermal, and electrical properties. Carbon nanotubes (CNTs) have shown tremendous potential for use in diverse applications due to their unique electrical and mechanical properties. CNTs have been used in a variety of applications in electrical, biomedical, and mechanical engineering fields. Due to their promising industrial and medical applications, the demands for CNT production has steadily increased in the last few years and are expected to dramatically increase in the near future. It was reported that the global market for CNT production in 2009 was $103 million, which has been projected to reach $1 billion by 2014 with a compound annual growth rate of 58.9% (Oliver, 2010). MWCNTs consist of many hollow cylinders of carbon atoms inside one another which enhance their mechanical, thermal and electronic properties by increasing the higher carbon atoms integration and augmenting surface area. As the demand for MWCNT production increases, natural eco-system contamination and human exposure through occupational and medical applications are also expected to rise. Nanoscale size and non-degradability of MWCNTs poses a significant threat to all eco-systems, especially to humans (Oberdorster, 2010; Pacurari et al., 2010; Pauluhn, 2011). In recent animal studies, the high penetrative nature, long
retention time, and capability of initiating pathological response of MWCNTs in the lung was paid serious attention for evaluating the effects of MWCNT exposure (Ellinger-Ziegelbauer et al., 2009; Han et al., 2010; Kobayashi et al., 2010; Mercer et al., 2010; Pauluhn, 2010; Sakamoto et al., 2009).

A majority of studies have emphasized an understanding of MWCNT-induced effects on either the respiratory or dermal system. Recent animal studies have highlighted the higher retention of MWCNTs in the alveolar region after 6 month exposure and probability of penetrating to nearby tissues (Kobayashi et al., 2010; Mercer et al., 2010; Pauluhn, 2011). A majority of the animal studies focused on investigating the long term effects of MWCNT exposure, and showed the highly penetrating nature of MWCNTs and an increased macrophage assisted clearance in conjunction with elevated inflammation levels (Ellinger-Ziegelbauer et al., 2009; Kim et al., 2010; Kobayashi et al., 2010; Pauluhn, 2010; Reddy et al., 2010). Other studies demonstrated increased levels of cytotoxic and inflammatory response even within a day following the exposure of MWCNTs (Han et al., 2010; Mercer et al., 2010; Porter et al., 2010). These observations showed the significant adverse effects of MWCNT exposure and suggests a high probability of increased severity in the future due to a prolonged clearance process, injury to the macrophage plasma membrane, and a higher penetration rate (Hirano et al., 2008; Kobayashi et al., 2010; Pauluhn, 2010). The systemic approach for MWCNT exposure evaluation using animals helped to characterize the whole lung response but is limited in its investigating of MWCNT interactions with each individual cell type in the lung. The in vitro cell culture models provided fundamental information regarding MWCNT-induced effects on individual cell types in the lung. Lung epithelial cells act as
a barrier at the interface between the surrounding air and lung tissues and respond to exogenous particles such as air-pollutants including CNTs. MWCNTs have induced a variety of effects including increased inflammatory response, DNA damage, and cellular apoptosis in A549 and normal human bronchial epithelial cells (BEAS-2B) (Hirano et al., 2010; Ravichandran et al., 2010; Tabet et al., 2009; Tsukahara et al., 2011). These studies, using specific cell types, have provided abundant information on the response of epithelial cells to external perturbations, but these systems are limited by the absence of the dynamic cell growth environment as found inside lung tissues (Grainger, 2009). The lung exists in a mechanically active environment, where different amounts of circumferential and longitudinal expansion and contraction occur during breathing movements. Patel and co-workers recently showed the differences in cellular responses to air pollutants between dynamic and static cell growth environments, and demonstrated that implementing dynamic cell growth conditions was a more close approximation of in vivo conditions (See Chapter 3). Such differences might have resulted from the altered interactions between cells and air pollutants under a mechanically active cell growth environment.

In our study, we evaluated the cellular responses to MWCNT exposure under both static and dynamic cell growth environments and at different concentrations (5, 10 and 20 μg/ml) of MWCNTs. We hypothesized that MWCNT exposure under the dynamic cell growth environment may alter its interaction with cells and affect the levels of cell proliferation, cellular inflammation, reactive oxygen species (ROS), and glutathione (GSH). To test out hypothesis, we used Flexcell Tension Plus 4000T system (Flexcell International, PA) to simulate a dynamic cell growth environment similar to normal
breathing condition (5% of equibiaxial surface elongation at the frequency of 0.2 Hz) in the lung (See Chapter 2; Tschumperlin et al., 1998). The dynamic in vitro culture system of A549 cells was used to investigate MWCNT-induced effects on cell proliferation, interleukin 8 (IL-8), ROS, and GSH. This study will provide an alternative approach to evaluate nanoparticle-induced effects on human respiratory systems and a detailed insight for the development of a viable alternative to existing static in vitro or in vivo tests.

4.3 Materials and Methods

4.3.1 Cell Culture. Type II alveolar basal epithelial cells of human origin (A549) were purchased from ATCC (Manassas, VA). A549 is epithelial-like in morphology and originates from a human lung carcinoma patient. The cells were seeded at $3 \times 10^5$ cells/well onto six well BioFlex plates (Flexcell International, PA) containing 2 ml of F-12k culture medium, which was supplemented with 1% penicillin streptomycin (Invitrogen, CA) and 10% fetal bovine serum (Thermo Fisher Scientific, UT). Once cells reached confluence, after 48 hours of seeding, they were exposed to MWCNT at 5, 10, and 20µg/ml and then grown in static or cyclic equibiaxial deformation (dynamic) conditions. The dynamic cell growth condition was implemented using a Flexcell Tension Plus 4000T system, which used vacuum pressure to apply cyclic strain to cells cultured on BioFlex plates. A schematic diagram of the Flexcell® Tension Plus™ 4000T system has been included in Figure 4.1. During the course of dynamic cell growth, cyclic stretching was applied to silastic well bottoms of BioFlex plates to attain 5% surface elongation at the frequency of 0.2Hz, which corresponds to 45% of the total lung capacity, similar to normal breathing conditions in the lung (Tschumperlin et al., 1998).
Figure 4.1. Schematic Diagram of Flexcell® Tension Plus™ 4000T system. A computer driven system controls the pressure of vacuum, which pulls down the silastic membrane around loading post and implements the circumferential strain in cell culture growing on top of the loading post.

4.3.2 MWCNT Solution Preparation. MWCNT (length: 0.5-2 µm, outer diameter: 20-30 nm, inner diameter: 5-10 nm, and purity: >95 weight percentage (wt %)) was obtained from Cheap Tubes Inc., VT. The stock solution for MWCNT was prepared by suspending 50.5 mg of MWCNT in 30 ml of sterile deionized water with 10.08 mg of polyvinylpyrrolidone (PVP). To breakdown the agglomerates and achieve a better suspension of MWCNTs, the stock solution was sonicated at 60 watts for 30 minutes at 30 second intervals on ice. To achieve exposure concentrations of 5, 10, and 20 µg/ml appropriate amounts of stock solutions were added to complete cell culture media. Right
before the exposure study, cell culture media solutions were sonicated for 5 minutes at 30 seconds intervals on ice to make uniform suspensions.

4.3.3 MWCNT Exposure Study. F-12k complete medium containing MWCNT at different concentration (5, 10, and 20 µg/ml) was added to A549 monolayers when confluent. Immediately after adding MWCNT containing media, cells were grown in either static or dynamic conditions for various exposure times (24, 48, and 72 hours). The dynamic cell growth condition was simulated by growing cell monolayers under a continuous cyclic equibiaxial deformation with 5% surface area change at 0.2 Hz, which was similar to normal breathing in vivo (Tschumperlin et al., 1998). After each exposure time, media and cell lysate samples were collected and stored in aliquots at -80°C until analyzed. Media supernatant samples were used to measure the level of IL-8 and ROS whereas cell lysate samples were used to measure the total protein concentration and GSH.

4.3.4 IL-8 Measurement. IL-8 from media supernatant was measured using ELISA prepared by the IL-8 human antibody pair and buffer kit (Invitrogen, CA). The unit of IL-8 measurement was picograms/milliliter (pg/ml).

4.3.5 Reactive Oxygen Species (ROS) Measurement. The ROS level was measured using de-acetylated probe 2′,7′-dichlorofluorescin (H$_2$DCF) based fluorescence assay to evaluate the level of ROS. The H$_2$DCF was prepared from 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) by alkaline hydrolysis using NaOH (Cathcart et al., 1983). 500 µl of 1 mM H$_2$DCF-DA was added to 2 ml of 0.01N NaOH and hydrolyzed into H$_2$DCF at room temperature for 30 min. The prepared H$_2$DCF solution was neutralized by adding 10 ml of 25 mM NaH$_2$PO$_4$ and adjusting the pH of the
solution to 7.4. Right after pH adjustment, the 40 µM H$_2$DCF solution was kept on ice or at 4°C until used. Fresh H$_2$DCF solution was prepared before each ROS measurement to avoid molecular probe deterioration. To perform the ROS measurement, 20 µl of media supernatant sample (cell-free sample) was incubated with 50 µl of 40 µM H$_2$DCF and 130 µl of 40 mM Tris–HCl, pH 7.4 for 10 min at 37°C, which initiated ROS facilitated H$_2$DCF oxidization to 2′,7′-dichlorofluorescein (DCF). Level of DCF was measured using Synergy 4 series multiwell-plate fluorometer (Biotek, VT), which was set at an excitation of 488 nm and emission of 525 nm. The level of DCF (i.e. fluorescence) was correlated to the level of ROS in the media supernatant samples, collected from the experiments. To measure the interference of MWCNT on the oxidization of H$_2$DCF to DCF, fresh MWCNT added media samples with MWCNT concentrations at 5, 10, and 20µg/ml were tested using same procedure.

4.3.6 GSH Measurement. Levels of GSH were measured in the cell lysate samples using GSH-Glo™ Glutathione Assay (Promega, WI) to check the oxidative stress level. The unit of GSH measurement was micro-molar (µM).

4.3.7 Total Protein Measurement. Cells grown in each well (9.6 cm$^2$) of the BioFlex plates were lysed using 250 µl of RIPA buffer with protease inhibitors (Thermo Scientific, IL). The total amount of protein from the cell lysate of each sample was measured using a BCA total protein assay (Pierce, IL) to evaluate cell proliferation. The unit of total protein was micrograms/milliliter (µg/ml).

4.3.8 Statistical Analysis. All data from IL-8, ROS and GSH measurements were normalized with the total amount of protein measured from cell lysate collected from respective samples. Statistical analyses were carried out using two-way analyses of
variance (ANOVA) followed by Dunnett’s multiple comparison tests to determine where significance exists (p < 0.05). All graphs were prepared by plotting mean data (sample size, n = 3) with the corresponding standard error of mean.

4.4 Results

4.4.1 Effect of MWCNT Exposure on A549 Cell Growth. Total protein concentration was measured at 24, 48, and 72 hours following exposure of A549 monolayers to different concentrations of MWCNTs (0, 5, 10, and 20 μg/ml) under static or dynamic conditions (Figure 4.2). A549 cell proliferation still increased as MWCNT concentrations increased following 24 hour exposure under both cell growth conditions (Figure 4.2(a), *p<0.05 and **p<0.05). The dynamic cell growth condition significantly enhanced A549 cell proliferation in the control group (0 μg/ml) following a 24 hour exposure (Figure 4.2(a), # p<0.05). A549 cell proliferation under 5 μg/ml of MWCNTs in dynamic cell growth condition was not higher than the control, and not different from that in static cell growth condition. However, A549 cell proliferation at higher concentrations (10 and 20 μg/ml) of MWCNTs was significantly different between static and dynamic cell growth conditions following 24 hour of exposure (Figure 4.2(a), #p<0.05). Following a 48 hour, MWCNT exposure did not induce any significant change in A549 cell growth as MWCNT concentrations increased under both cell growth conditions (Figure 4.2(b)). However, cell proliferation at 5 and 10 μg/ml of MWCNTs in the dynamic cell growth condition was significantly higher than that in the static cell growth condition (Figure 4.2(b), #p<0.05). Following 72 hours of exposure, A549 cell proliferation significantly decreased in both cell growth conditions at all concentrations
of MWCNTs (Figure 4.2(c), *p<0.05 and **<0.05). However, the A549 cell proliferation in the dynamic cell growth condition remained significantly higher than that in the static cell growth condition following 72 hours of exposure to MWCNTs at all concentrations (Figure 4.2(c), #p<0.05).

4.4.2 Effect of MWCNT Exposure on Cellular Inflammation. The level of IL-8 was measured at 24 hour intervals from the media supernatant in A549 cultures following exposure to all concentrations of MWCNTs under either static or the dynamic cell growth condition, to characterize the level of MWCNT-induced inflammation (Figure 4.3). After a 24 hour exposure of A549 cells to MWCNT, IL-8 levels increased at 5 µg/ml of MWCNTs and decreased at 10 and 20 µg/ml of MWCNTs when compared to the control group under both cell growth conditions (Figure 4.3(a), *p<0.05, and **p<0.05), except for 10 µg/ml under the dynamic cell growth condition. A549 cells grown in the dynamic cell growth condition induced a significantly higher level of IL-8 than that observed in the static cell growth condition following 24 hours of exposure to MWCNTs at 10, and 20 µg/ml (Figure 4.3(a), # p<0.05). Following 48 hours of exposure to MWCNTs, IL-8 levels in the static cell growth condition were not significantly changed as compared to the control (Figure 4.3(b)), while IL-8 levels in the dynamic cell growth condition significantly increased at all concentrations of MWCNTs when compared to the controls in the dynamic (Figure 4.3(b), **p<0.05) and static (Figure 4.3(b), #p<0.05) cell growth conditions. After 72 hours of exposure to MWCNTs at all concentrations, IL-8 levels significantly increased in both cell growth conditions as compared to the controls (Figure 4.3(c), *p<0.05, and **p<0.05). Similar to the 48 hour
**Figure 4.2.** Cell growth of A549 under static or dynamic cell growth conditions during 0 (control), 5, 10, and 20 µg/ml MWCNT exposure. Total protein concentration was measured in each cell lysate of the A549 culture following the exposure to MWCNT at different levels (0, 5, 10, and 20 µg/ml) under both cell growth conditions. Time 0 h refers to the starting point of cyclic equibiaxial deformation and control for respective exposure levels of MWCNT. #Significantly different from the other cell growth condition at same concentration of MWCNT (p<0.05). *Significantly different from the control for static cell growth condition (static condition at 0 µg/ml of MWCNT) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 µg/ml of MWCNT) (p<0.05).
exposure, following 72 hours of exposure to MWCNTs, IL-8 levels in the dynamic cell growth condition was significantly higher than those in the static cell growth condition (Figure 4.3(c), #p<0.05).

**4.4.3 Characterization of Oxidative Stress Through ROS Measurement.** The level of ROS was measured in media supernatant taken from A549 cultures exposed to different concentrations of MWCNTs under either static or dynamic cell growth conditions to characterize the cellular oxidative stress (Figure 4.4). Following a 24 hour exposure of A549 cells to MWCNTs, ROS levels in the dynamic cell growth condition significantly increased as compared to the control (Figure 4.4(a), **p<0.05), while ROS levels in the static cell growth condition did not increase significantly, except those at 20 µg/ml of MWCNTs, as the compared to the control (Figure 4.4(a), *p<0.05). No significant differences in ROS levels were observed between dynamic and static cell growth conditions following a 24 hour exposure to MWCNTs. Following a 48 hour exposure of A549 cells to MWCNTs, ROS levels in the static growth condition significantly increased as compared to the control (Figure 4.4(b), *p<0.05), while ROS level in the dynamic cell growth condition did not increase significantly, except at 20 µg/ml of MWCNTs (Figure 4.4(b), #p<0.05). Under the dynamic cell growth condition, only ROS level from MWCNT exposure concentrations of 20 µg/ml was significantly higher than the control after 48 hours of exposure (Figure 4.4(b), **p<0.05). Following 72 hour exposure of A549 cells to MWCNTs in static cell growth condition, only 20 µg/ml of MWCNTs induced a higher level of ROS than the control (Figure 4.4(c), *p<0.05). Moreover, ROS levels in the static cell growth condition at 10 and 20 µg/ml of MWCNTs were significantly higher than those in the dynamic cell growth condition,
Figure 4.3. Cellular inflammation in A549 cultures grown under static or dynamic cell growth conditions during 0 (control), 5, 10, and 20 µg/ml MWCNT exposures. IL-8 was measured in the media supernatant samples of A549 cell cultures following their exposure to MWCNT’s at different levels (0, 5, 10, and 20 µg/ml) under both cell growth conditions. Time 0 h refers to the starting point of cyclic equibiaxial deformation and the control for respective exposure levels of MWCNT. #Significantly different from the other cell growth condition at same concentration of MWCNT (p<0.05). *Significantly different from the control for static cell growth condition (static condition at 0 µg/ml of MWCNT) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 µg/ml of MWCNT) (p<0.05).
following 72 hours of exposure to MWCNTs (Figure 4.4(c), #p<0.05).

4.4.4 Characterization of Oxidative Stress Through GSH Measurement. Intracellular levels of GSH were measured from the cell lysate of A549 cultures exposed to different concentrations of MWCNTs in static or dynamic cell growth conditions to characterize intracellular oxidative stress (Figure 4.5). Following 24 hours of exposure to MWCNTs, GSH levels increased in both cell growth conditions as compared to each control, except samples grown in dynamic conditions at 5 µg/ml of MWCNTs (Figure 4.5(a), *p<0.05 and **p<0.05). Under 10 and 20 µg/ml concentrations of MWCNTs exposure, in the dynamic cell growth condition induced a significantly higher level of GSH than those in the static cell growth condition after a 24 hour exposure period to MWCNTs (Figure 4.5(a), #p<0.05). Following 48 hours of exposure to MWCNTs, 10 and 20 µg/ml of MWCNTs in both static and dynamic cell growth conditions induced significantly lower levels of GSH than the control of each cell growth condition (Figure 4.4(b), *p<0.05 and *p<0.05). However, GSH levels in the dynamic cell growth condition were significantly higher than those in the static cell growth condition (Figure 4.5(b), #p<0.05). Following 72 hour exposure to MWCNTs, GSH levels in the dynamic cell growth condition significantly decreased as compared to the control (Figure 4.5(c), **p<0.05), while GSH levels in the static cell growth condition significantly increased as compared to the control, except under 5 µg/ml of MWCNTs exposure (Figure 4.5(c), *p<0.05). Overall levels of GSH in the static cell growth condition were significantly higher than those in the dynamic cell growth condition following 72 hours of exposure (Figure 4.5(c), #p<0.05).
Figure 4.4. ROS production in A549 cells grown under static and dynamic cell growth conditions during 0 (control), 5, 10, and 20 µg/ml MWCNT exposure. ROS was measured in from the media supernatant of A549 cultures following exposure to MWCNTs at different levels (0, 5, 10, and 20 µg/ml) under both cell growth conditions. Time 0 h refers to the starting point of cyclic equibiaxial deformation and is the control for the respective exposure levels of MWCNT. #Significantly different from the other cell growth condition at same concentration of MWCNT (p<0.05). *Significantly different from the control for static cell growth condition (static condition at 0 µg/ml of MWCNT) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 µg/ml of MWCNT) (p<0.05).
4.5 Discussion

In this study, we introduced an alternative method for evaluation of MWCNT-induced effects on cellular responses such as cell proliferation, inflammatory responses, and oxidative stress by using a dynamic cell growth condition which served as more realistic in vitro model (viable alternative to in vivo model). Our results supported our initial hypothesis that exposing A549 cells to MWCNTs in a dynamic cell growth environment would alter their interactions and affect the levels of cell proliferation, cellular inflammation, oxidative stress (ROS and GSH). A dynamic cell growth environment was established to mimic the circumferential and longitudinal expansion and contraction that occurs inside the lung during normal breathing. A dynamic cell growth environment may provide a suitable condition for facilitating the interaction between nanomaterials and cells, which might alter the uptake of nanomaterials, and hence their effect on cells, similar to that seen in vivo (Chambers et al., 2007; Grainger, 2009; Huh et al., 2010; See Chapter 3). We used the Flexcell Tension Plus system to implement 5% cyclic equibiaxial elongation, which is equivalent to 45% of total lung capacity and the amount of stretching experienced during normal breathing (Tschumperlin et al., 1998). Moreover, the equibiaxial elongation frequency was set as 0.2 Hz, which is corresponding to the normal human breathing rate. Depending on the shape, density, size, and breathing pattern, nanomaterials can get deposited in alveolar regions and therefore the use of A549 cells in this study increased its applicability (Lam et al., 2004; Mercer et al., 2010; Pauluhn, 2011; Shinozaka et al., 2010; Shvedova et al., 2008). The MWCNT powder, purchased from Cheap Tubes Inc., VT (SKU # 030404), contained about 5 wt % of impurities including carbon black (3.34 wt %), iron (0.24 wt %), nickel (0.94 wt %),
and chlorine (0.47 wt %). Thus under the highest MWCNT exposure concentration (20 µg/ml) in this study, the cell growth medium contains 0.668 µg/ml of carbon black, 0.094 µg/ml of chlorine, 0.048 µg/ml of iron, and 0.188 µg/ml of nickel.

The results from this study indicated that MWCNT exposure induced significant changes in cell growth, cellular inflammation, and oxidative stress in A549 cell cultures under in both cell growth conditions (Figure 4.2, 4.3, 4.4, and 4.5). In both cell growth conditions, A549 cell growth proliferation significantly increased under all MWCNT concentrations following 24 hours of exposure as compared to the respective controls, except at 5 µg/ml of MWCNTs in the dynamic cell growth condition (Figure 4.2(a)). Increased cell proliferation and decreased IL-8 levels (Figure 4.3(a) might have been related to the increased level of GSH during the 24 hour exposure to MWCNTs (Figure 4.5(a)). Kang et al. and Horton et al. have demonstrated a strong connection between A549 cell proliferation and intracellular GSH content, which could explain the increased cell proliferation during their 24 hour exposure to MWCNTs (Horton et al., 1997; Kang, 1994; Kang et al., 1990; Kang et al., 1994). Similarly, the intracellular GSH inhibits IL-8 expression by inhibiting nuclear factor-kappaβ (NF-κβ) activation (Biswas et al., 2005; Jafari et al., 2004). The possibility of an interaction between IL-8, MWCNTs, and nanomaterials, should not be ruled out as an explanation for the results of the 24 hour cell exposure to MWCNTs (Herzog et al., 2009; Seagrave et al., 2004). In the dynamic cell growth condition, A549 cell proliferation was significantly lower while the IL-8 level expression was significantly higher at higher concentrations of MWCNTs (10, and 20 µg/ml) than those in static cell growth condition following 24 hours of exposure. During this exposure, the levels of ROS significantly increased (Figure 4.4(a), **p<0.05) in the
Figure 4.5. GSH production in A549 cells grown under static and dynamic cell growth conditions during 0 (control), 5, 10, and 20 µg/ml MWCNT exposure. GSH was measured in the cell lysate of A549 cultures following the exposure of MWCNT at different levels (0, 5, 10, and 20 µg/ml) under both cell growth conditions. Time 0 h refers to the starting point of cyclic equibiaxial deformation and is the control for the respective exposure levels of MWCNT. #Significantly different from the other cell growth condition at same concentration of MWCNT (p<0.05). *Significantly different from the control for static cell growth condition (static condition at 0 µg/ml of MWCNT) (p<0.05). **Significantly different from the control for dynamic cell growth condition (dynamic condition at 0 µg/ml of MWCNT) (p<0.05).
dynamic cell growth condition. Increased levels of ROS might be related to the decreased cell proliferation and the increased IL-8 level. Increased levels of ROS have been shown to reduce cell viability and increase NF-κβ mediated IL-8 upregulation (Chou et al., 2008; Monteiller et al., 2007; Ye et al., 2009).

Following 48 hour exposure to MWCNTs, cell proliferation was not significantly changed as MWCNT concentration increased in each cell growth condition (Figure 4.2(b)). However, A549 cell proliferation in the dynamic cell growth condition was significantly higher than that in the static cell growth condition (Figure 4.2(b), #p<0.05). Similar trends were observed in the IL-8 (Figure 4.3(b), #p<0.05) and GSH levels (Figure 4.5(b), #p<0.05), which were significantly higher in the dynamic cell growth condition than the static cell growth condition. In both cell growth conditions, GSH levels decreased as MWCNT concentration increased and were significantly lower at higher MWCNT concentrations (10, and 20 µg/ml) (Figure 4.5(b), *p<0.05, and **p<0.05). While GSH levels were decreasing, ROS levels were increasing in the static cell growth condition (Figure 4.4(b), *p<0.05), which indicated the increase in oxidative stress. However, ROS levels in the dynamic cell growth condition remained significantly lower at all concentrations of MWCNTs (Figure 4.4(b), #p<0.05), except for those at 20 µg/ml of MWCNTs at which ROS levels were significantly higher than the control, but not significantly different from that at same concentration of MWCNTs in static cell growth condition. Increased levels of oxidative stress might have down-regulated cell proliferation in the static cell growth condition.

Following 72 hour exposure to MWCNTs, A549 cell proliferation was significantly lower in both cell growth conditions than the respective controls (Figure
A549 cell proliferation was significantly higher in the dynamic cell growth condition (Figure 4.2(c), #p<0.05). A549 cell proliferation in the static cell growth might have been down-regulated by the increased levels of ROS (Figure 4.4(c), *p<0.05) and IL-8 (Figure 4.3(c), *p<0.05). Similarly, the decreased cell proliferation in the dynamic cell growth condition might have been due to the reduced levels of GSH (Figure 4.5(c), **p<0.05) and increased levels of IL-8 (Figure 4.3(c), **p<0.05). During the same exposure time, the levels of IL-8 significantly increased in both cell growth conditions, which might have resulted from the prolonged MWCNT exposure (Figure 4.3(c), *p<0.05, and **p<0.05). In the dynamic cell growth condition, the level of IL-8 was significantly higher than that in the static cell growth condition (Figure 4.3(c), #p<0.05).

A549 cell proliferation generally decreases as the concentration of MWCNTs increases during a longer exposure time (48 and 72 hours) in both cell growth conditions. During the same exposure time (48 and 72 hours), A549 cell proliferation in the dynamic condition was significantly higher. IL-8 levels increased as the concentration of MWCNTs increased during a longer exposure time (72 hours) in both cell growth conditions (Figure 4.3). Increased levels of IL-8 can be related to neutrophil migration and mucin production as precursory events to remove MWCNTs from sites of inflammation in the lung (Bautista et al., 2009; Kunkel et al., 1991; See Chapter 3). Dynamic cell growth conditions facilitated a significant increase in IL-8 levels following 48 hours of exposure (Figure 4.3(b), **p<0.05, and #p<0.05), which paralleled results from previous animal studies indicating the recruitment of neutrophils in bronchoalveolar lavage (BAL) fluid within 48 hour of MWCNT exposure (Han et al., 2010; Inoue et al.,
ROS levels decreased over the exposure duration in the dynamic cell growth condition, whereas it consistently increased in the static cell growth condition. After an initial increase in ROS levels following 24 hours of exposure to MWCNTs, the ROS levels dropped down for subsequent time periods in the dynamic cell growth condition (Figure 4.4), which was in parallel to the results from animal study (Han et al., 2010). Similarly, GSH levels decreased over the exposure duration in the dynamic cell growth condition (Figure 4.5). There was no such trend in GSH level in static cell growth condition. Our results strongly demonstrated the distinct differences in MWCNT-induced effects on cell proliferation, IL-8, ROS, and GSH between static and dynamic cell growth conditions. Interestingly, ROS and IL-8 levels in dynamic condition were found to be in parallel to the results from animal studies.

The dynamic cell growth system together with static cell growth system yielded several important findings: (1) All MWCNT exposure concentrations used in this study induced adverse effects on A549 cell proliferation. Cell proliferation in the dynamic cell growth condition was higher than the static cell growth condition during 48 and 72 hour exposure (Figure 4.2). (2) IL-8 levels were significantly higher in the dynamic cell growth condition than the static cell growth condition, except those at 5 µg/ml of MWCNTs after 24 hours of exposure (Figure 4.3). (3) Over the exposure duration ROS and GSH levels decreased under dynamic cell growth conditions (Figure 4.4 and 4.5). In conclusion, the dynamic cell growth system used in this study provided important changes in cellular responses that were not found in the static cell growth system and were similar to previous animal studies. The dynamic cell growth system can be considered a viable alternative to in vivo test systems in combination with existing in
vitro static cell growth systems to evaluate the cellular responses of MWCNTs on the respiratory system.

4.6 References


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CHAPTER 5
EFFECT OF DYNAMIC ENVIRONMENT ON THE INTERACTION BETWEEN SINGLE-WALLED CARBON NANOTUBE AND HUMAN AIRWAY EPITHELIAL CELL MONOLAYER

5.1 Abstract

Despite their great use for engineering and medical applications, nanomaterials may have adverse consequences upon accidental exposure and medical applications due to their nanoscale size, composition and shape. Like many nanomaterials, carbon nanotubes (CNTs) have been well explored for many proven applications, but very little explored to understand their potential toxic effects. It is crucial to develop viable alternatives to in vivo tests to evaluate the toxicity of engineered CNTs. To evaluate the CNT-mediated toxicity in a novel dynamic in vitro model, which can simulate normal breathing condition, two different sizes (short: OD 1-2 nm, length 0.5-2 μm; long: OD 1-2 nm, length 5-30μm) of single-walled carbon nanotubes (SWCNTs) were used at different concentrations (5, 10, and 20 μg/ml) along with different exposure time (24, 48, and 72 hours) both static and dynamic environments. Dynamic environment facilitated interaction between SWCNTs and A549 monolayer and cellular responses are significantly different from those under static condition. Short-SWCNTs decreased reactive oxygen species (ROS) at higher concentrations under longer exposure, but cells exposed to long-SWCNTs at all concentrations induced a significant decrease under longer exposure time. Long-SWCNTs induced much higher level of IL-8 expression, especially under longer exposure time.
5.2 Introduction

The respiratory system is especially susceptible to insult by airborne toxic materials. Particles that can enter the respiratory system are broken down into three major regions: (1) dust reaching the gas exchange, or alveolar, region is called \textit{respirable dust}; (2) dust reaching the tracheobronchial region and alveolar region is called \textit{thoracic dust}; and (3) dust entering the nose and mouth is called an \textit{inhalable dust}. Respirable dust is smaller than about 4 $\mu$m aerodynamic equivalent diameters, thoracic dust is smaller than about 10 $\mu$m, and inhalable dust is smaller than about 100 $\mu$m. Larger airborne particles can deposit in the upper respiratory system (Baron et al., 2003). In a manufacturing environment, CNTs are handled in much larger quantities as compared to typical laboratories, subjecting the workers to a higher risk of exposure to these potentially hazardous nanoparticles. The nanotechnology community in the U.S., led by NIOSH (The National Institute for Occupational Health and Safety) and OSHA (Occupational Safety & Health Administration) is devoting efforts to issue “best practices” guide for safely working with nanomaterials. However, the development is still in its infant stage, and there is a strong need for science-based methodologies to predict the health and toxicological effects of CNTs.

Intensive studies on the toxicity of CNTs have shown that exposure to CNTs results in pulmonary inflammation (Chou et al., 2008; Lam et al., 2004; Li et al., 2007; Mitchell et al., 2007; Muller et al., 2005; Shvedova et al., 2005; Warheit et al., 2004). The inflammatory lung reactions (alveolitis) are a source of genetic lesions which could eventually lead to the development of lung cancer (Chou et al., 2008). \textit{In vivo} studies performed using guinea pigs and rats showed the appearance of multifocal granulomas,
resulting in inflammatory reactions of the terminal and respiratory bronchioles. Mild fibrosis in the alveolar septa was also observed (Helland et al., 2007). Ken Donaldson and his colleagues described three properties of CNTs associated with pathogenicity in particles. They are 1) nanoparticles showing more toxicity than larger sized particles, 2) fiber-shaped particles behaving like asbestos and other pathogenic fibers which have toxicity associated with their needle-like shape, and 3) biologically biopersistent. They also pointed out that CNTs are possibly one of the least biodegradable man-made materials ever devised (Donaldson et al., 2006). Also concerns over the increased emissions of CNTs into the environmental compartments (air, water and soil) mainly due to improper disposal of CNTs were raised (Helland et al., 2007). Recent studies for nanomaterials indicate: (1) CNTs and fullerenes have produced toxic effects on biological systems (Chin et al., 2007; Dumortier et al., 2006; Helland et al., 2007; Lam et al., 2006; Yang et al., 2006); (2) evidence that nanoparticles can translocate to bloodstream (Rothen-Rutishauser et al., 2007; Shimada et al., 2006); and (3) evidence that nanoparticles can cross blood brain barrier (Kim et al., 2007). However, studies are still preliminary, as the current in vivo and in vitro response data are difficult to extrapolate.

The airway wall exists in a mechanically dynamic environment, where different amounts of circumferential and longitudinal expansion and contraction occurred during breathing movements. In this study, we established in vitro dynamic culture system simulating normal breathing condition of our airway. This dynamic culture system of human airway epithelial cells was used for the investigation of the effect of different size of SWCNTs on cell proliferation, cellular inflammatory response, and the level of
reactive oxygen species. The different level of biological and toxicological effects was observed both in static and dynamic conditions of airway epithelial cell monolayer.

5.3 Materials and Methods

5.3.1 Cell Culture. Type II alveolar basal epithelial cells of human origin (A549) were purchased from ATCC (Manassas, VA). A549 is epithelial-like in morphology and originates from a human lung carcinoma patient. The cells were seeded at $3 \times 10^5$ cells/well onto six well BioFlex plates (Flexcell International, PA) containing 2 ml of F-12k culture medium, which was supplemented with 1% penicillin streptomycin (Invitrogen, CA) and 10% fetal bovine serum (Thermo Fisher Scientific, UT). After cells reached confluency, in 48 hours of seeding, they were exposed to SWCNT-short/long at 20, 10, and 5µg/ml and then grown in either static or cyclic equibiaxial deformation (dynamic) condition. The dynamic cell growth condition was implemented using Flexcell Tension Plus 4000T system, which used vacuum pressure to apply cyclic strain to cells cultured on BioFlex plates.

5.3.2 Cyclic Equibiaxial Deformation. We used a physiologically relevant range of cyclic equibiaxial deformation (5%), which corresponds to 45% of the total lung capacity (Tschumperlin and Margulies, 1998). Flexcell® Tension Plus™ 4000T system (Flexcell International, PA) was used to equibiaxially elongate the monolayers of cells on silicone rubber bottoms of a BioFlex plate. Schematic diagram of Flexcell® Tension Plus™ 4000T system has been included in Figure 1. During the course of dynamic cell growth, cyclic stretching was applied to silastic well bottoms of BioFlex plates to attain 5% surface elongation at the frequency of 0.2Hz.
5.3.3 **Short/Long-SWCNT Solution Preparation.** Short-SWCNTs (length: 0.5-2 µm, outer diameter: 1-2 nm, inner diameter: 0.8-1.6 nm, and purity: >90%) and long-SWCNTs (length: 5-30 µm, outer diameter: 1-2 nm, inner diameter: 0.8-1.6 nm, and purity: >90%) were obtained from Cheap Tubes Inc., VT. The stock solution for short-SWCNTs was prepared by suspending 50.5 mg of short-SWCNTs in 30 ml of sterile deionized water with 10.08 mg of Polyvinylpyrrolidone (PVP). In similar way, stock solution for long-SWCNTs was prepared by suspending 51.38 mg of short-SWCNTs in 30 ml of sterile deionized water with 10.2 mg of Polyvinylpyrrolidone (PVP). To breakdown the agglomerates and achieve better suspension of SWCNTs, both stock solutions were sonicated at 60 watts for 30 minutes at 30 second intervals on ice. To achieve exposure concentrations of 5, 10, and 20 µg/ml appropriate amounts of stock solutions were added to cell culture media. Cell culture media solutions were then sonicated for 5 minutes at 30 seconds intervals on ice right before the exposure study.

5.3.4 **Exposure of Short/Long-SWCNT to A549 Cells.** Following confluency, A549 monolayers were treated with different concentrations (5, 10, and 20 µg/ml) of short and long-SWCNTs in F-12k medium for 24, 48, and 72 hours either in static or dynamic condition. The levels of IL-8, ROS and total protein concentration were monitored, following exposure of SWCNTs with different concentration, exposure time, and size.

5.3.5 **Total Protein Measurement.** Total protein concentration from cell lysate was measure using BCA total protein assay (Thermo Scientific, IL) to quantify the amount of A549 cells.
5.3.6 IL-8 Measurement. IL8 secretion in media supernatant was measured using ELISA kit (Invitrogen, CA) to check the level of inflammation in A549 cells.

5.3.7 Reactive Oxygen Species Measurement. The ROS level was measured using de-acetylated probe 2′,7′-dichlorofluorescin (H$_2$DCF) based fluorescence assay to evaluate the level of ROS. The H$_2$DCF was prepared from 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) by alkaline hydrolysis using NaOH (Cathcart et al., 1983). 500 µl of 1 mM H$_2$DCF-DA was added to 2 ml of 0.01N NaOH and hydrolyzed into H$_2$DCF at room temperature for 30 min. The prepared H$_2$DCF solution was neutralized by adding 10 ml of 25 mM NaH$_2$PO$_4$ and adjusting the pH of the solution to 7.4. Right after pH adjustment, 40 µM H$_2$DCF solution was kept on ice or stored at 4°C until used. Fresh H$_2$DCF solution was prepared before each ROS measurement to avoid molecular probe deterioration. To perform the ROS measurement, 20 µl of media supernatant sample (cell-free sample) was incubated with 50 µl of 40 µM H$_2$DCF and 130 µl of 40 mM Tris–HCl, pH 7.4 for 10 min at 37°C, which initiated ROS facilitated H$_2$DCF oxidization to 2′,7′-dichlorofluorescein (DCF). Level of DCF was measured using Synergy 4 series multiwell-plate fluorometer (Biotek, VT), which was set at an excitation of 488 nm and emission of 525 nm. The level of DCF (i.e. fluorescence) was correlated to the level of ROS in the media supernatant samples, collected from the experiments. To measure the interference of short/long-SWCNT on oxidization of H$_2$DCF to DCF, fresh short/long-SWCNT added media samples with short/long-SWCNT concentrations at 5, 10, and 20µg/ml were tested using same procedure.
5.3.8 **Statistical Analysis.** Statistical analyses were carried out using two-way analyses of variance (ANOVA) followed by Dennett’s multiple comparison tests to determine where significance exists (p<0.05).

5.4 **Results**

5.4.1 **Effect of short-SWCNT on A549 Cell Growth.** Following exposure of short-SWCNTs, A549 cell proliferation increased over 24 hours and thereafter decreased in both cell growth conditions. A549 cell proliferation was significantly higher for all short-SWCNT concentrations at 72 hours under dynamic cell growth condition. A549 cell proliferation was significantly higher in dynamic cell growth condition in the absence of SWCNTs. Following exposure of SWCNT at different concentrations (5, 10, and 20 mg/ml), cell proliferation remained higher in dynamic cell growth condition up to 72 hour (Figure 5.1, *p<0.05).

5.4.2 **Effect of long-SWCNT on A549 Cell Growth.** In both cell growth conditions, A549 cell proliferation decreased over 24 hours and thereafter increased up to 72 hours following exposure of long-SWCNTs at all concentrations. There was no significant difference in A549 cell proliferation between both cell growth conditions, except that after 72 hours of exposure. A549 cell proliferation in dynamic cell growth condition was significantly higher than that in static cell growth condition following 72 hour exposure of SWCNTs at all concentrations (Figure 5.2, *p<0.05).

5.4.3 **Effect of short-SWCNT on ROS Production.** ROS production was not significantly different between static and dynamic conditions. Significantly higher level
Figure 5.1. The effect of short-SWCNTs on A549 cell proliferation. *Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05).

Figure 5.2. The effect of long-SWCNTs on A549 cell proliferation. *Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05).
Figure 5.3. The effect of short-SWCNTs on ROS production in A549 cells. * Significantly higher in static cell growth condition than in dynamic cell growth condition (p<0.05).

Figure 5.4. The effect of long-SWCNTs on ROS production in A549 cells. * Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05).
Figure 5.5. The effect of short-SWCNTs on IL-8 expression in A549 cells. * Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05).

Figure 5.6. The effect of long-SWCNTs on IL-8 expression in A549 cells. * Significantly higher in dynamic cell growth condition than in static cell growth condition (p<0.05).
of ROS was observed only in static cell growth condition following 72 hour exposure of SWCNTs at 20 μg/ml (Figure 5.3, *<0.05).

5.4.4 Effect of long-SWCNT on ROS Production. In both cell growth conditions, ROS level increased right over 24 hours following exposure of long-SWCNTs and remained higher than the respective controls upto 72 hours. ROS level in static cell growth condition was significantly higher after 48 and 72 hour exposure of long-SWCNTs at all concentrations, except after 48 hour exposure of SWCNTs at 5 μg/ml (Figure 5.4, *p<0.05).

5.4.5 Effect of short-SWCNT on IL-8 Expression. The IL-8 expression increased over 72 hours, but no significant changes in IL-8 were observed as exposure time increased at all concentrations of short-SWCNTs. In addition, there is no significant difference in IL-8 levels between static and dynamic cell growth conditions. Significantly higher level of IL-8 was observed only in dynamic cell growth condition following 24 hour exposure of short-SWCNTs at all concentrations of short-SWCNTs (Figure 5.5, *p<0.05).

5.4.6 Effect of long-SWCNT on IL-8 Expression. In both cell growth conditions, IL-8 expression increased following 48 hour exposure long-SWCNTs at all concentrations. IL-8 level was significantly higher in dynamic cell growth condition following 48 and 72 hours exposure of long-SWCNTs at all concentrations of SWCNTs(Figure 5.6, *p<0.05).
5.5 Discussion

Although nanomaterials have enormous biological and medical potentials, they may induce potential risk to human health. More fundamental investigation about the toxicity and safety of nanomaterials is still required. Toxicity of nanomaterials should be assessed in vivo, but in vivo toxicity assessment is generally limited. In this study, we used a dynamic cell growth system, one of the viable alternatives to in vivo, to evaluate the adverse effects of SWCNTs on human respiratory systems. Biological or toxicological effects of SWCNTs are largely depending on several important factors such as size, dose, shape, phase, and fate. Especially, CNTs have high aspect ratio in structure and may induce more curvature effects under dynamic environments in our body (Donaldson et al., 2011). We observed that the dynamic environment facilitated interaction between SWCNTs and A549 monolayer and induced significant changes in cellular responses such as cell proliferation, oxidative stress, and inflammatory response.

A549 cell proliferation decreased over 72 hours following exposure of short-SWCNTs in both cell growth conditions (Figure 5.1). However, A549 cell proliferation increased over 72 hours following exposure of long-SWCNTs in both cell growth conditions (Figure 5.2, *p<0.05). No significant changes in ROS levels were induced in both cell growth conditions following exposure of short-SWCNTs at all concentrations, except following 72 hour exposure at 20 μg/ml concentrations (Figure 5.3, *p<0.05). However, ROS levels significantly increased over 24 hours and remain higher following exposure of long-SWCNTs at all concentrations in both cell growth conditions (Figure 5.4). Moreover, the ROS level was significantly higher in static cell growth condition following 48 and 72 hour exposure of long-SWCNTs at concentrations, except at 5 μg/ml
Pulskamp et al. and Liu et al. demonstrated that ROS level increased with the increased metal impurity of SWCNTs (Liu et al., 2008; Pulskamp et al., 2007). Ren et al. and Teeguarden et al. outlined the SWCNT exposure induced ROS production (Ren et al., 2009; Ren and Zhong, 2010; Teeguarden et al., 2011). Either metal impurity or SWCNT itself could induce the changes in ROS level, but the effects of type, size and composition of SWCNTs on ROS level are still unclear (Liu et al., 2008; Pulskamp et al., 2007; Ren et al., 2009; Ren and Zhong, 2010; Teeguarden et al., 2011). Our study showed that the size of SWCNTs could significantly change ROS level (Figure 5.3 and Figure 5.4).

IL-8 levels also did not significantly change following exposure of short-SWCNTs at all concentrations in both cell growth conditions, except after 24 hour exposure in dynamic condition (Figure 5.5). However, IL-8 level increased after 48 hour exposure of long-SWCNTs in dynamic cell growth condition and after 72 hour exposure of long-SWCNTs in static cell growth condition (Figure 5.6). Some studies showed that SWCNTs induced a higher level of IL-8, which was similar to the level of IL-8 observed following exposure to long-SWCNTs (Chou et al., 2008; Jacobsen et al., 2009; Lam et al., 2006; Shvedova et al., 2008). Others demonstrated that SWCNTs induced fewer changes in inflammatory responses, which was similar to the level of IL-8 observed following exposure to short-SWCNTs (Pulskamp et al., 2007). Recent studies also demonstrated the adverse effects of wall size (single or multiple), synthesis method, composition, and metal impurity of CNTs (Herzog et al., 2007; Jacobsen et al., 2009; Kaiser et al., 2008; Pulskamp et al., 2007; Teeguarden et al., 2011). Different level of pathological conditions induced by asbestos was also dependent upon its size and shape.
We could observe the size-dependent effects of SWCNTs on cell proliferation, IL-8 expression, and ROS production in this study.

In our study, we demonstrated the effects of two different sizes of SWCNTs on cell proliferation, oxidative stress, and inflammatory response in two different cell growth conditions. The results indicated that dynamic environment facilitated interaction between SWCNTs and A549 monolayer and cellular responses are significantly different from those under static condition. Short-SWCNTs decreased reactive oxygen species (ROS) at higher concentrations under longer exposure, but cells exposed to long-SWCNTs at all concentrations induced a significant decrease under longer exposure time. Long-SWCNTs induced much higher level of IL-8 expression, especially under longer exposure time.

5.6 References


Donaldson, K., Murphy, F., Schinwald, A., Duffin, R., Poland, C.A., 2011. Identifying the pulmonary hazard of high aspect ratio nanoparticles to enable their safety-by-design. Nanomedicine (Lond) 6, 143-156.


CHAPTER 6
CONCLUSION AND FUTURE DIRECTIONS

6.1 Findings

In this study we used an alternative *in vitro* cell culture system, using dynamic cell growth environment, to stimulate mechanically active cell growth environment similar as *in vivo*. The dynamic cell culture system provided a platform to study pathological condition resulting from changes in biomechanical environment with the freedom studying desired cell type. In this study we utilized dynamic cell culture system to study its applicability for researching respiratory disorders such as ventilator induced lung overdistention and nano-scaled pollutant exposure on A549 cell cultures.

The first part of the study was focused on learning the effect lung overdistention on A549 cell cultures with or without pre-inflammatory condition. It was observed that the dynamic cell growth condition positively regulated the net inflammatory and tissue remodeling activities. Moreover, in the presence of pre-inflammation dynamic cell culture prolonged the inflammatory and tissue remodeling activities. The simultaneous production of NO and activation of tissue remodeling factors such as MMP-2 and TIMP-2 indicated an interplay between inflammatory and tissue remodeling activities triggered by increasing level of equibiaxial deformation. While this study highlights induction of inflammatory and tissue remodeling activities by increasing levels of equibiaxial deformation, further research is required to thoroughly understand the underlying pathway explaining these effects.
The second part of the study was focused on utilizing dynamic cell cultures as an alternative *in vitro* estimation method for characterizing the side effects from DPM, MWCNT, and SWCNT exposure to A549 cells. The improvements in the outcome of these exposure studies were observed in dynamic A549 cell cultures.

In DPM exposure study, it was observed that under dynamic cell growth condition A549 cell cultures produced IL-8 and ROS levels, which were in parallel to results from animal studies. Along with these, we also observed the distinct effects on A549 cell growth, inflammation and ROS levels upon DPM exposure at very low and practically relevant concentrations. Similarly, we observed the changes in IL-8 and ROS production profiles were in parallel to *in vivo* studies when A549 cell cultures grown under dynamic conditions were exposed to MWCNT. The SWCNT exposure study also highlighted the differences in IL-8, ROS and cell proliferation levels induced by dynamic cell growth environment as compared to the outcomes from static cell growth condition. Along with different cell growth induced effects we were also able to notice a strong size dependent effects in SWCNT exposure study. The short-SWCNT reduced the A549 cell growth without any change in ROS and IL-8 production. On the other hand, long-SWCNT did not have a specific effect on A549 cell growth but ROS and IL-8 levels showed significant increase under both cell growth conditions.

### 6.2 Future Directions

#### 6.2.1 Exploring the cell-mechanotransduction pathway linking equibiaxial deformation with tissue remodeling activities in A549 cells.

Our study, as described in chapter 2, suggested an equibiaxial deformation induced cellular inflammation and tissue
remodeling activity increase (See Chapter 2). The remodeling factors like matrix metalloproteases (MMP) and tissue inhibitor metalloproteases (TIMP) are known to play an important role for changing the lung permeability and facilitate host cell migration (Haseneen et al., 2003; Sacco et al., 2004). The current literature has enabled us to link an equibiaxial deformation with MT1-MMP expression in the presence of EMMPRIN (Foda et al., 2001; Haseneen et al., 2003). This suggests the increase in MMP expression in cell treated with an equibiaxial deformation. However, an equibiaxial deformation induced MMP-2 activation and TIMP-2 expression as observed in our study are not characterized yet. Thus discovering the underlying mechanism, responsible for MMP-2 activation and TIMP-2 expression will have a significant impact on understanding ventilator induced lung injury.

6.2.2 Inclusion of co-culture with dynamic cell culture system. In this study we utilized dynamic cell culture system to stimulate biomechanical environment similar as in physiological and pathological conditions in the lung. In parallel to other studies we also noticed the improvement in A549 cell functionality, when grown under appropriate biomechanical environment (See Chapter 2; Tschumperlin et al., 1998; Tschumperlin et al., 2000). The cellular functionality and hence the applicability of the dynamic cell culture model can be further increased by using 3D co-culture. Choe et al have demonstrated similar approach by integrating a co-culture model, mimicking lung wall, with a dynamic system capable of uniaxial deformation (Choe et al., 2006). Integration of 3D co-culture with dynamic cell culture system capable of equibiaxial deformation will increase the cellular functionality and tissue level representation of the system. They will
make a suitable model for many studies such as pollutant and nanomaterial exposure evaluation, wound healing study, drug penetration study, etc.

6.3 References


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APPENDIX A

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Hemangkumar Patel

EDUCATION

May 2011  PhD Biological Engineering, Utah State University (USU), Logan, UT  
Dissertation: Characterization and application of in vitro dynamic airway cell culture models in Professor Soonjo Kwon’s lab. (GPA 3.8/4.0)

2005  MS Biological Engineering, Utah State University, Logan, UT  
Thesis: A combined strategy to reduce restenosis for vascular tissue engineering applications in Professor Kytaï Nguyen’s lab. (GPA 3.67/4.0)

2002  BE Biomedical Engineering, University of Mumbai, Mumbai, India  
Technical Report: Heart rate measurement using remote infrared sensor (GPA 3.5/4.0)

PATENT AND INVENTION DISCLOSURE

- Ultrasonic Method for Monitoring Cell Cultures, Inventors: Doyle T, Patel H, Goodrich J and Kwon S. Invention disclosure was approved on October 24, 2010.

REFEREED JOURNAL ARTICLES (PUBLISHED)


**REFEREED JOURNAL ARTICLES (IN PREPARATION)**


**CONFERENCE PROCEEDINGS**


**CONFERENCE PODIUM PRESENTATION**


**CONFERENCE POSTER PRESENTATION**


RESEARCH EXPERIENCE

Utah State University, Logan, UT

Graduate Student in Professor Soonjo Kwon’s lab, 2006-Present

- Optimized the experimental setups to study the inflammatory and tissue remodeling responses under ventilator induced overdistention using lung airway epithelial cells
- Examined the role of dynamic cell growth condition on evaluating the side effects of diesel particulate matter and carbon nanotubes
- Designed the cell culture system for continuous ultrasound monitoring
- Determined the role of cell stiffness on ultrasonic detection of malignant breast mammary epithelial cells
- Proposed and implemented interdisciplinary collaborative research projects
- Introduced and established various molecular and cell biology techniques
- Trained and mentored four graduate and seven undergraduate students
- Managed research lab

Graduate Student in Professor Kytai Nguyen’s Lab, 2004-2005

- Developed a tissue engineered biodegradable cardiovascular implant to support rapid re-endothelialization and inhibit restenosis
- Compared different ECM proteins and protein immobilization techniques for poly (L-lactic acid) thin film surface modification to promote endothelial cell adhesion and proliferation
- Manufactured biodegradable nanoparticles to develop the site specific drug delivery vehicle
- Optimized the size, concentration and exposure duration for nanoparticle mediated drug delivery research
- Explored different cell culture medium formulations for primary rat bone marrow cells differentiation into endothelial, smooth muscle and osteoblast cells

TEACHING EXPERIENCE

Utah State University, Logan, UT

Teaching Assistant for Tissue Engineering Class (BIE 5890), 2005-2010

- Designed lab assignments to improve student involvement
- Trained groups of up to 20 students on cell culture, cell biology and molecular biology techniques
- Helped students with lab reports, experimental trouble shooting and final projects
- Graded lab reports
Teaching Assistant for Instrumentation for Biological System Class (BIE 3000), 2004

- Assisted with lab module design and execution
- Trained groups of up to 30 students on variety of instruments and measurement techniques
- Helped students with trouble shooting and final project
- Taught lab sessions and graded lab reports

INTERNERSHIP EXPERIENCE

Siemens Ltd., Goa, India

Company Trainee, 2001

- Assisted in production of mobile x-ray machine units (MULTIMOBIL 1.6/2.5, Siemens Ltd.)
- Assisted in quality check process
- Led the team of company trainees to achieve monthly production targets
- Participated in occupational safety awareness week
- Trained junior trainees

LAB SKILLS

- Mammalian cell culturing, rat and mice dissection, 3D cell cultures and co-cultures
- Isolating primary cells from rat such as aortic endothelial and smooth muscle cells, bone marrow mesenchymal stem cells, and pancreatic islet cells
- Culture the cells using Flexcell tension system, shear stress study using parallel plate flow chamber
- Trained to work with human biopsy tissue samples, animal tissue sectioning
- Conceptualization, design, synthesis and characterization of biomaterial scaffolds
- Biomatel surface modification, drug loaded polymer thin film coating, protein/growth factor coating using different crosslinking/adsorption techniques
- Improving cell adhesion and proliferation on biomaterial scaffold surface
- Measuring surface and mechanical properties of polymer scaffold, hydrogel, tissue biopsies & cells
- Nanoparticle synthesis - characterization & antibody conjugation
- Biocompatibility, polymer degradation, drug release rate evaluation and modeling
- Immunofluorescence, histological staining, image analysis and quantification
- Mammalian cell transfection, plasmid expression using E.coli and purification
- ELISA, SDS-PAGE, western blot, zymography, protein band characterization and quantification
- DNA & RNA extraction, RT-PCR dsDNA and total protein quantification assays
- Design of experiment, data analysis, protocol design and method development, documentation
COMPUTER SKILLS
Microsoft office, Stat View, Prism, KaleidaGraph, SAS, Adobe Photoshop, MATLAB, Simulink, Mac OS

TECHNICAL SKILLS
Particle size analyzer, AFM, SEM, UV/Vis plate reader, Fluorescence/Luminescence plate reader, Flexcell Tension Plus system, fluorescence microscopy, Cryostat, Instron, PCR Arrays

AWARDS AND SCHOLARSHIPS
- 2nd Prize at Institute of Biological Engineering regional conference, Utah 2010
- PhD student grant, Women and Gender Research Institute, USU, UT 2010
- Student travel award, Biomedical Engineering Society 2005, 2010
- Graduate Student Senate student travel award, USU, UT 2005-2009
- Honorable mention at Institute of Biological Engineering regional meeting 2009
- 3rd Prize at Seventh Annual Intermountain Paper and Poster Symposium, Utah 2005

EXTRACURRICULAR ACTIVITIES
- Organized Biomedical Session, IBE regional conference, Utah 2010
- Founding member and President, BMES student chapter, USU, UT 2010
- Session Chair, IBE Regional Conference, USU, UT 2009
- President, Child Rights and You chapter, USU, UT 2008-2010
- Vice President, Indian Students Association, USU, UT 2008-2009
- Graduate Student Representative, Biological Engineering Department, USU, UT 2004-05
- Student Member, Biomedical Engineering Society 2004 - Present
- Student Member, Institute of Biological Engineering 2008 - Present