Summer 6-1-2022

MICRO-RNA ANALYSIS OF EXTRACELLULAR VESICLES SECRETED BY ALVEOLAR MACROPHAGES AND EPITHELIAL CELLS IN RESPONSE TO CADMIUM

Anisha Bhakta
University of Texas at Tyler, abhakta3@patriots.uttyler.edu

MICRO-RNA ANALYSIS OF EXTRACELLULAR VESICLES SECRETED BY ALVEOLAR MACROPHAGES AND EPITHELIAL CELLS IN RESPONSE TO CADMIUM

by

ANISHA BHAKTA

A thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in Biotechnology
Department of Cellular and Molecular Biology

Nagarjun Venkata Konduru, PhD, DVM Thesis Advisor

School of Medical Biological Sciences

The University of Texas at Tyler
April 2022

The University of Texas at Tyler
Tyler, Texas

This is to certify that the Master's Thesis of

ANISHA BHAKTA

has been approved for the thesis requirement on
April 28th, 2022, for
The Master of Science in Biotechnology degree

Approvals:

Thesis Advisor: Nagarjun Venkata Konduru, Ph.D.

Thesis Committee Chair: Sachin Mulik, Ph.D.

Member: Hua Tang, Ph.D.

Member: Osamu Sato, Ph.D.

Chair, Department of Cellular and Molecular Biology

Dean, School of Medical Biological Sciences

ACKNOWLEDGMENTS

Completing this thesis in such a short amount of time has been a challenge, but I am grateful to have an amazing support system.

Lab Members

Nagarjun Venkata Konduru, D.V.M.Ph.D., Raju Adduri Ph.D., Karen Alzate Velasco M.S, Ravikiran Vasireddy M.S.

My mentors

Nagarjun Venkata Konduru, D.V.M.Ph.D., Raju Adduri Ph.D., Karen Alzate Velasco M.S, Buka Samten M.D., Vijay Boggaram Ph.D., Sachin Mulik Ph.D., Osamu Sato Ph.D., and the Biotechnology Faculty.

Thesis Committee

Sachin Mulik Ph.D., Hua Tang Ph.D., Osamu Sato Ph.D.

Family and Friends

Mahesh and Sangita Bhakta, Sonam Bhakta, and Pratik Patel, the rest of my family, friends, and biotechnology classmates.

TABLE OF CONTENTS

List of Figures iii

List of Tables iv

List of Abbreviations v

Abstract 1

Introduction 2

Cd exposure and adverse health effects 2

Exosome in Toxicology 3

Research Hypothesis 6

Materials and Methods 7

Cell Culture 7

Cell Viability Assay 7

THP-1 Cells Differentiation 7

Exosomes Isolation 7

Western Blotting 8

Nanoparticle Tracking Analysis 8

Cryo-Electron Microscopy 8

miRNA Sequencing 9

miRNA Sequencing Analysis 9

Statistical Analysis 9

Results 10

Characterization of Exosomes from A549 cells and THP-1 Macrophages 10

Changes in Exosome Concentration due Cd Exposure 12

miRNA Changes Exosomes from A549 cells and THP-1 Macrophages 14

Discussion 17

References 22

LIST OF FIGURES

Figure 1. Visual Representation of Cd Exposure at a Cellular Level. 5

Figure 2. Cell Viability of A549 and THP-1 after $CdCl_2$ Treatment. 10

Figure 3. Images of Exosomes from A549 and THP-1..... 11

Figure 4. Western Blot. Both cell lines exosomes were tested for surface markers. 11

Figure 5. Size Distribution of Exosomes. 12

Figure 6. NTA Results of Exosome Concentration in A549 and THP-1 Cells..... 14

Figure 7. Early Biomarker Discovery of Lung Injury Due to Inhalation of Cd..... 17

LIST OF TABLES

Table 1. Average Exosome Size per Sample. 13

Table 2. Upregulated and Downregulated miRNA from A549 exosomes. 15

Table 3. A549 miRNA Targets. 15

Table 4. miRNA Upregulated and Downregulated from THP-1 exosomes. 16

Table 5. THP-1 miRNA Targets. 16

LIST OF ABBREVIATIONS

Cd: Cadmium

ROS: Reactive Oxygen Species

DNA: Deoxyribose Nucleic Acid

RNA: Ribose Nucleic Acid

miRNA: Micro Ribose Nucleic Acid

mRNA: Messenger RNA

EVs: Extracellular Vesicles

Cryo-TEM: Cryo- Electron Microscopy

NTA: Nanoparticle Tracking Analysis

$CdCl_2$: Cadmium Chloride

nm: Nano Meters

nM: Nano Molar

PMA: Phorbol 12-Myristate 13-Acetate

NGS: Next-Generation Sequencing

mL: Milli Liter

ABSTRACT

Inhalation of cadmium (Cd) has been an environmental health concern with the increase in industrial activities and smoking. Cd exposure is known to affect several organs such as lungs, kidneys, and liver. In this study, we wanted to understand if Cd inhalation exposure can affect the lungs and other organs in the body. Exosomes are extracellular vesicles secreted by all living cells and are known to carry toxicants between organs. We investigated whether Cd exposure could affect exosome biogenesis and their composition. Cd exposure did not affect the viability of A549, a lung epithelial cell line and macrophages derived from THP-1 monocytes. There was no difference in size distribution of exosomes following Cd exposure. However, there was an increase in exosome concentration following Cd exposure in THP-1 macrophages. We observed significant difference in miRNAs composition in exosomes after Cd treatment. There were 8 and 2 differentially expressed miRNAs in exosomes from A549 cells and THP-1 macrophages, respectively. Our findings indicate that the response to heavy metal exposure varies cell to cell, The changes in exosome concentration and miRNAs indicates the exosomes may play a role in cell-to-cell communication.

INTRODUCTION

Cd exposure and adverse health effects

Human exposure to toxic heavy metals such as Cd has become a cause of serious concern both in environmental and occupational settings due to increasing industrial activities and emissions. According to the World Health Organization, Cd is a known carcinogen, and the lungs are the primary gateway to inhaled heavy metals. In adults, long-term exposure to Cd in adults is linked to lung, breast, and kidney cancers [1]. Cd exposure is most common through cigarette smoking [1]. In a cohort study comprising individuals over 60 that were lifetime smokers blood Cd levels were around 1.66 micrograms per liter [2]. The blood Cd levels in former smokers and nonsmokers averaged around 0.68 and 0.51 micrograms per liter, respectively [2]. Exposure of Cd can also occur in metal processing factories due to inhaling metal fumes and dust [1]. The excretion of Cd from the body is an extremely slow process and the metal is stored over lifetime throughout the body [3]. This makes the investigations of the metal exposures significant from a toxicology standpoint.

Cd prefers the oxidation state of +2 and therefore is considered divalent metal [4]. In cells, metal transporters such as divalent cation transporter 1 (DCT1) that is primarily responsible for the uptake of Iron (Fe) could also transport other metal ions like Cd with similar charges [3]. The uptake of nonessential metals at undesired levels that do not have a naturally present biochemical and physiological functions in the body such as calcium and iron could lead to disruption of cellular homeostasis [5]. Cd affects how the body processes zinc (Zn) because they compete to bind to metallothionein (MT), a metal-binding protein relevant to Zn homeostasis [6]. Cd is also able to induce oxidative stress through the generation of reactive oxygen species (ROS) in exposed cells [4]. Conversely, the redox-inactive metals are responsible for electron transfer in

the body. Cd has the potential to induce oxidative stress by depleting the intrinsic antioxidant reserves whereby cells become more susceptible to the effects of ROS [5]. ROS causes damage to the DNA, RNA, membrane lipids, and proteins leading to cell death [5]. In cell culture models, Cd exposure has been reported to cause biochemical changes such as aberrant gene expression and signal transduction pathways [7]. At higher Cd concentrations the biosynthesis of DNA, RNA, and proteins are disrupted [6].

Exosome in Toxicology

Exosomes were first discovered by Pan and Johnstone in 1983 [8]. They are spherical and contain a lipid bilayer that are 100 nanometers in diameter and are formed by endocytosis where a substance is taken up by a cell and formed into a multivesicular body (MVB) [9, 10]. There are two paths the MVB can take, the first is being bound to a lysosome for degradation [11]. The second path the MVB can take is being secreted as an exosome. Some characteristic features to define exosomes are membrane markers such has CD63, CD9, and CD81 [9]. Few internal markers are ALIX, HSP70, and HSP90 which are internal indicators of an exosome [9].

After the discovery of exosomes, the exact role they have is still undetermined. They were previously known just to remove unnecessary constituents from cells. Exosomes are known to remove molecular constituents from cells, and also considered pivotal in intercellular communication because they are secreted and delivered to recipient cells [10]. There are other interactions that exosome secreted by one cell may have with neighboring cells. The transmembrane proteins interact with targeted cell receptors creating different types of intracellular signals [12]. Exosomes can also fuse with the plasma membrane of a recipient cell where they can deliver contents directly into the cytosol [11]. There are two potential fates of the exosome, the first is being bound to a lysosome for degradation [11]. The second is to be merged

into an endosome [11]. Exosomes are being exploited as a novel biomatrix for discovery of biomarkers and as carriers of therapeutic agents. One of the core constituents of exosomes that may play a significant role in cell-to-cell communication or response induction are the micro-RNA (miRNA).

MiRNA are considered non-coding and range from an average of 22 nucleotides made of adenine, guanine, uracil, or cytosine [13]. Unlike RNA, miRNA is highly stable and resistant to RNAse activity when circulating in bodily fluids or membrane bound vesicles [14-16]. They have the potential to inhibit gene expression by silencing mRNA during translation and can regulate cellular homeostasis [12],[8]. MiRNA can also mediate post-transcriptional gene silencing, cell proliferation, and cell differentiation [17]. Additionally, miRNA can also be involved with cell migration, disease initiation, and disease progression [16]. The circulation of EV's leads to another form of intercellular communication due to the miRNA profiles present.

A recent study found a significant association between long-term exposure to air pollution and levels of several miRNAs within exosomes isolated from blood [18]. Another study identified six miRNAs (has-miRNA 302c, has-miRNA 28-3p, has-miRNA 125a-5p, has-let- 7g, has-miRNA-181a) in circulating exosomes to be differentially expressed between the pre-and post-exposure periods in occupational workers potentially exposed to metal rich particulate matter [19]. Further, exosomes containing miRNA from stimulated macrophages promoted ROS (reactive oxygen species) generation and release of extracellular traps in neutrophils [20]. In a recent study, macrophages stimulated with oxidize LDL showed an increase in expression miRNA-146a suggesting that change in miRNA profiles could occur with environmental exposures as well as lead to an increased inflammatory response [19].

We investigated the response to Cd in similar concentrations like those of lifetime smokers. The human monocyte cell line was differentiated into macrophages to mimic the response of an alveolar macrophage when in contact to a toxicant. Human macrophages are known to be the first line of defense by digesting unwanted particles such as Cd and bacteria. The alveolar epithelial cell line A549, are the main cell line the lungs are composed, and they secrete surfactant proteins.

The current study alludes to the significance of exosomes as a response mechanism to environmental exposure. In this context, although exosomes have the potential to elicit responses in naive lung cells, their ultimate role upon secretion from toxicant-exposed cells is undetermined. In the series of experiments, we probed how exposure to Cd in macrophages and lung epithelial cells changes the response to exosome secretion and miRNA profiles (Figure 1).

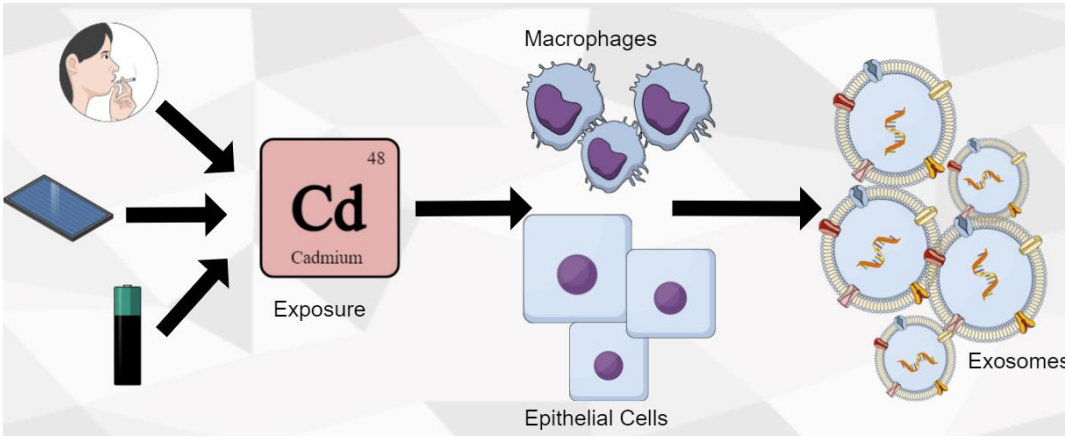

Figure 1. Visual Representation of Cd Exposure at a Cellular Level.
Cigarettes, solar panels, batteries, and other metal-based items have the potential to expose humans to Cd. The Cd exposure is being observed on the cellular level by the secretion of exosomes. The exosome amounts are being observed from healthy and exposed cells. The miRNA profiles are also being viewed for any changes due to exposure to Cd.

RESEARCH HYPOTHESIS

Hypothesis: Exposure of lung cells to Cd would alter the dynamics of exosome concentration and their miRNA cargo.

Objective 1: *To determine the differential concentration of exosomes in human monocytes differentiated into macrophages (THP-1) and alveolar type II epithelial cells (A549) in response to Cd.*

Each cell line was treated with concentration of 15 nM Cd in correspondence to blood concentrations trends reported in human populations. Following the dosage, the EVs were isolated as a function of time from the cultured media based on size-exclusion chromatography. The EVs were characterized for the presence of specific surface proteins and their concentrations were determined using nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing. Based on the experiments, we characterized the dose-EV response relationship and determined the optimum concentrations in relation to the number of EVs.

Objective 2: *To elucidate miRNA profiles of EVs secreted by alveolar macrophages and epithelial cells.*

From the isolated EVs, RNA was isolated and the miRNA profiles from the RNA were determined from Illumina Sequencing. Further, we used Ingenuity Pathway Analysis to identify the putative targets of the detected miRNAs and explore biological networks associated with them.

MATERIALS AND METHODS

Cell Culture

THP-1 and A549 cell lines were used in this study. THP-1 were be maintained in RMPI 1640 medium (Cytiva, UT, USA) containing 2 mM of L-glutamine, 25 mM HEPES, 10% exosome depleted heat inactivated fetal bovine serum (Corning, CA, USA), and penicillin/streptomycin (Corning, VA, USA). A549 cells were maintained in F-12K Kaighn's modification medium (ATCC, VA, USA) containing 2 mM L-glutamine, 10% exosome depleted heat inactivated fetal bovine serum, and Pen/Strep. Cells were passaged and maintained following ATCC guidelines.

Cell Viability Assay

A549 cells ($1x10^5$) were seeded into each well of a 96-well plate and allowed to adhere for 6 hours. The cells were treated with 5, 10, and 15 nM of $CdCl_2$ for 24 hours. The alamarBlue reagent (Bio-Rad) was added to the culture medium and incubated for 4 hours. The plate absorbance was measured at 560 nm using Synergy microplate reader (BioTek Instruments Ltd, VT, USA).

THP-1 cells ($1x10^5$) were seeded into each well of a 96-well plate and were differentiated using 65 ng/mL of PMA for 24 hours. Cell viability was measured following $CdCl_2$ treatment using the same method of A549.

THP-1 Cells Differentiation

THP-1 cells ($5x10^6$) were plated in 100 mm dishes and treated with 65 ng/mL of PMA for 24 hours to differentiate into macrophages.

Exosomes Isolation

The culture medium was centrifuged at 4000 rpm for 20 minutes to remove debris and reconcentrated to 2 mL using filtration through 100 kDa filters (Pall, Inc.). The EVs were

isolated using size exclusion chromatography, qEV2 35nm column (Izon Science) according to manufacturer's protocol. Briefly, the column was washed with 3 column volumes of degassed PBS (Corning). Then 2 mL of the filtrate was added to the column allowed to enter the column. The column reservoir was filled with degassed PBS. Following of 14 mL of void volumes was measured and 10 mL of exosomes were collected and concentrated to 200 µL using filtration through 300 kDa filters.

Western Blotting

SDS-PAGE using standard protocols as well as HSP70 (Abcam antibodies, cat# ab2787), Calnexin Rabbit PolyAb (Proteintech, cat# 10427-2-AP), and CD9 (Abcam antibodies, cat# ab22305) were performed. The protein concentration was estimated using Pierce BCA protein assay kit (Thermo Fisher Scientific) using bovine serum albumin as standard. Proteins were then detected using chemiluminescence using Clarity Max ECL substrate (Bio-Rad) on a Chemidoc-MP Gel imaging system (Bio-Rad).

Nanoparticle Tracking Analysis

To measure the exosomes, present in each cell sample at various concentrations, EVs were diluted in PBS and analyzed using Nanosight NS300 instrument (Malvern Panalytical).

Cryo-Electron Microscopy

For cryo-electron microscopy, 4 µL of the EV solution was added to a Lacey Carbon Grid (200-Mesh Electron Microscopy Sciences). The grid was plunged-frozen in liquid ethane and cooled by liquid nitrogen. The sample was imaged using a Titan Krios 300KV transmission electron microscope equipped with a post-column Gatan imaging filter and Volta Phase plate. The images obtained were analyzed with SerialEM software.

miRNA Sequencing

The exosomes were sent out for sequencing. The RNA from the exosomes were isolated and 100 ng of the RNA sample went through ligation of the 3' adapter and the excess was removed. Proceeding the 5' was ligated, respectively. Reverse transcription occurred to produce cDNA. The cDNA was size selected using Ampure XP beads. The purified product was amplified using PCR reaction using Illumina barcoded primers for 10-15 cycles. The libraries were determined in PAGE and the bands will contain exosome miRNA (150 bp) extracted and DNA were removed from the gel bands. The product was then purified using Ampure XP beads and quantified in Quantus spectrophotometer (Promega). 10 nM of the barcoded library from each sample was then sequenced on Illumina Hiseq.

miRNA Sequencing Analysis

Adapter sequences and pool quality reads were filtered. Reads obtained from sequencing were trimmed down to 30 bases using cutadapt program. The reads were aligned to human mature miRNA sequences from miRbase using Bowtie 2.1. The reads mapping to each miRNA were counted using HTSeqCounts function. The read counts were normalized across the samples using DEseq2 package. miRNAs differentially expressed between treated and untreated cells were identified using DEseq2 package. miRNAs exhibiting $\log_2$ fold-change of > 0.585, and p-value of < 0.05 were considered differentially expressed.

Targetscan was used to identify potential miRNA targets of differentially expressed miRNAs.

Statistical Analysis

A student t-test was used for comparing the exosome concentration and miRNA expression between samples using a p-value cut off value of 0.05.

RESULTS

Characterization of Exosomes from A549 cells and THP-1 Macrophages

We investigated whether Cd exposure affected viability of A549 cells and THP-1 macrophages at various concentrations. A549 cells and THP-1 cells differentiated with 65 ng/mL of PMA were treated with 5 nM, 10 nM, and 15 nM concentrations of $CdCl_2$ for 24 hours. After 24 hours of treatment with $CdCl_2$. We observed a minimal decrease in viability of A549 cells and THP-1 macrophages following Cd treatment. Viability of A549 was 98.89% at 5 nM, 98.25% at

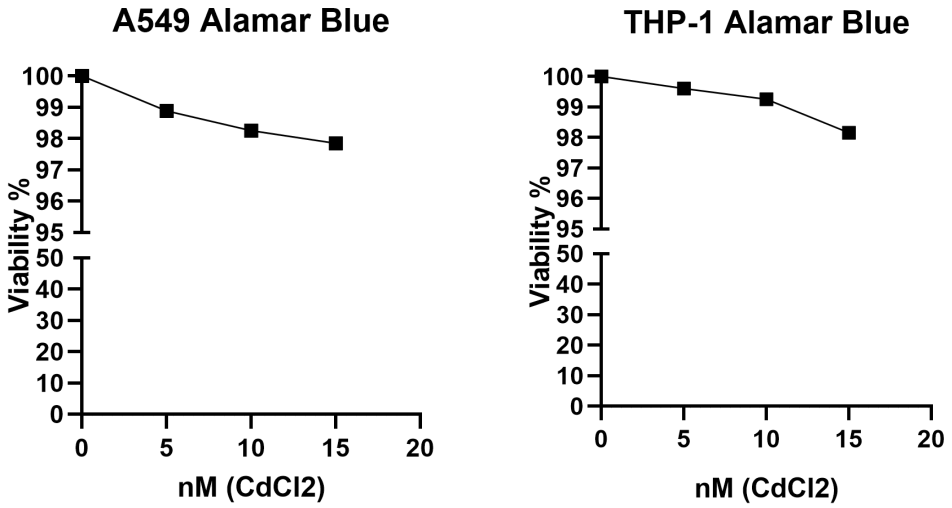

Figure 2. Cell Viability of A549 and THP-1 after $CdCl_2$ Treatment.
Following treatment of $CdCl_2$ media was removed from cells and replaced with fresh media containing 5% alamarblue reagent. Each plate was read at 570 nm and 600 nm. (A) A549 cell line graph shows the viability of a starting value of 40,000 cells after treatment of $CdCl_2$ at 0, 5, 10, 15 nM. (B) THP-1 cell line graph shows the viability of the starting value of 100,000 cells differentiated with 65 ng/mL of PMA after treatment of $CdCl_2$ at 0, 5, 10, 15 nM.

10 nM, and 97.86% at 15 nM (Figure 2). Similar results were observed with THP-1 macrophages, viability at 5 nM was 99.61%, at 10 nM was 99.25%, and at 15 nM was 98.16% (Figure 2). These results suggest that Cd exposure did exhibit cytotoxicity.

We isolated exosomes from cultures media from A549 cells and THP-1 macrophages using size-exclusion chromatography. To determine the purity of the exosome isolates, we measured size of the vesicles and detected the presence of bonified exosome protein markers. First, to determine size, we performed Cryo-TEM imaging of exosome isolates (Figure 3). The size of the majority of vesicles present in our preparations ranged between 75 nm - 200 nm in diameter, the vesicles are spherical in shape, and contain an intact lipid bilayer (Figure 3).

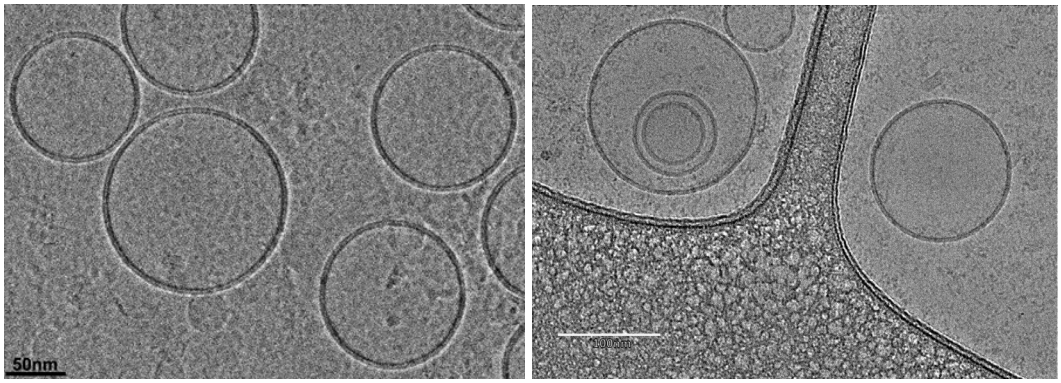

Figure 3. Images of Exosomes from A549 and THP-1.
The exosome solution was suspended on a Lacey Carbon Grid and Imaged using a Titan Krios 300 KV transmission electron microscope. (a) Exosomes range from 75 nm to 150 nm and have a double lipid membrane and circular shape. (b) The visual image of an exosomes enclosed on the Lacey Carbon Grid.

Further, we performed immunoblotting of exosome markers. We detected presence of exosome markers such as HSP70, ALIX, TSG101 (internal markers) and CD9 and CD81 (surface markers) suggesting that our vesicle preparations are enriched with exosomes (Figure 4). In addition, we observed that Calnexin, an endoplasmic reticulum protein, was absent in our

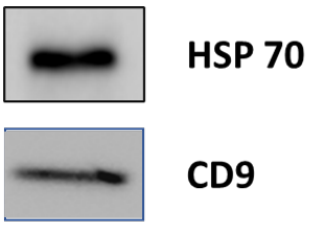

Figure 4. Western Blot. Both cell lines exosomes were tested for surface markers.
The specific surface markers were tested for are HSP70 and ALIX. (a) Bands for HSP70 and ALIX on A549 exosomes. (b) Bands of HSP70 and ALIX on exosomes from THP-1 macrophages.

vesicle preparations suggesting that our exosomes are free of membrane contaminations from other cellular components (Figure 4).

Changes in Exosome Concentration due Cd Exposure

To investigate whether the Cd exposure affects number of exosomes released by cells, we determined the exosome concentrations in the cultured media of A549 cells and THP-1 macrophages using NTA analysis. We performed this experiment at a $CdCl_2$ concentration of 15 nM. NTA analysis measures size and concentration of the exosomes. Based on NTA, size of majority of exosomes in our isolates was ranging from 75 nm-195 nm in diameter (Figure 5), confirming results obtained by Cryo-TEM (Figure 3). There was no difference is size of exosomes between treated and untreated A549 cells and THP-1 macrophages (Figure 5). The mean sizes of exosomes from treated and untreated A549 cells are 174.4 nm and 131.16 nm, respectively (Table 1). Similarly, mean sizes of exosomes from treated and untreated THP-1 macrophages are 177.5 nm and 128.28 nm, respectively (Table 1).

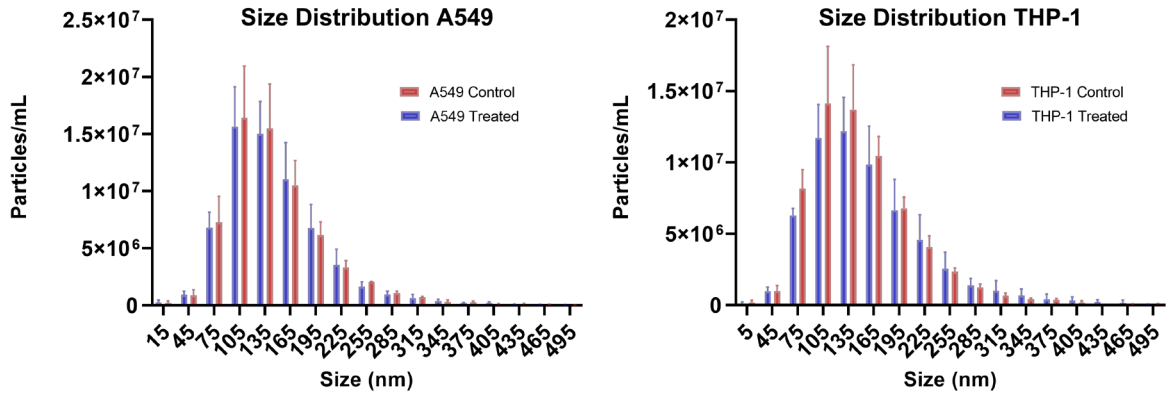

Figure 5. Size Distribution of Exosomes.
The size distribution is measures by particle per mL. The error bars represent the standard deviation n=3. (a) A549 control in compared to cells treated with 15 nM of $CdCl_2$. (b) THP-1 control compared size distribution compared to cells treated with 15 nM of $CdCl_2$.

Table 1. Average Exosome Size per Sample.

Cells Type and Group	Average Diameter (nm)	Concentration (particles/mL)
A549 Untreated Group 1	147.1	1.8×10^{6}
	120.4	1.8×10^{6}
A549 Untreated Group 2	133	2.9×10^{6}
	113.8	2.9×10^{6}
A549 Untreated Group 3	141.5	3.3×10^{6}
A549 Treated Group 1	145.4	2.9×10^{6}
	117.9	3.0×10^{6}
A549 Treated Group 2	149.3	2.6×10^{6}
	120.2	2.6×10^{6}
	306.1	3.9×10^{5}
THP-1 untreated Group 1	133.5	3.1×10^{6}
THP-1 untreated Group 2	149.9	2.1×10^{6}
THP-1 untreated Group 3	147.6	2.1×10^{6}
	118.5	1.9×10^{6}
	91.9	1.5×10^{6}
THP-1 treated Group 1	132.4	2.3×10^{6}
THP-1 treated Group 2	157.7	2.6×10^{6}
	313.4	7.0×10^{7}
THP-1 treated Group 3	165.5	1.9×10^{6}
	118.5	1.5×10^{6}

The mean sizes of exosomes from treated and untreated A549 cells were 6.5×10^{9} particles/mL and 6.4×10^{9} particles/mL, respectively (Figure 6). There was no difference is concentration of exosomes between treated and untreated A549 cells (Figure 6) suggesting that Cd exposure does not affect the number of exosomes release by A549. The mean sizes of exosomes from treated and untreated THP-1 macrophages were 4.43×10^{10} particles/mL and 1.9×10^{10} particles/mL,

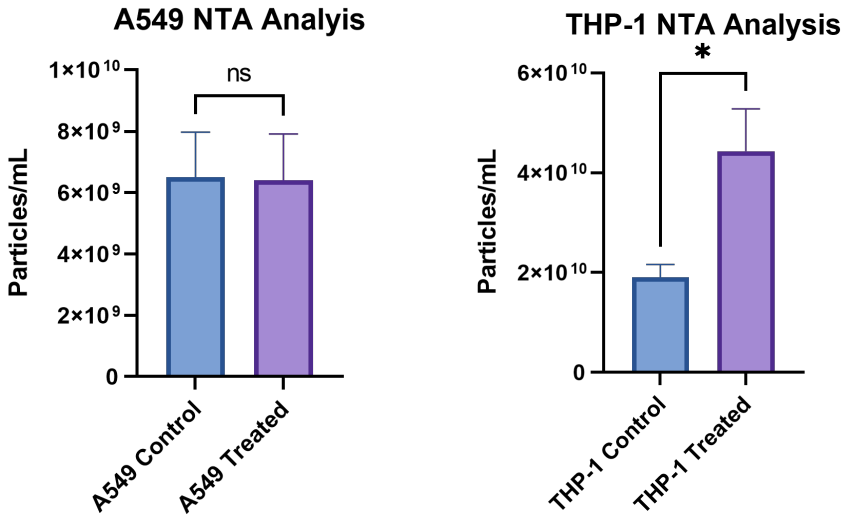

Figure 6. NTA Results of Exosome Concentration in A549 and THP-1 Cells. exosome concentration is shown in particles per milliliter. The error bars represent standard deviation (n=3). (a) The A549 untreated control cells in comparison to A549 treated with 15nM $CdCl_2$ cells. (b) The THP-1 untreated control in comparison to THP-1 treated with 15 nM $CdCl_2$. The asterisk represents statistical significance.

respectively (Figure 6). The exosome concentration has increased by 2.3-fold following treatment with $CdCl_2$ in THP-1 macrophages ($p < 0.05$) (Figure 6).

miRNA Changes Exosomes from A549 cells and THP-1 Macrophages

A total of 864 miRNAs were found in across 12 samples (3 samples each of treated and untreated A549 cells and THP-1 macrophages). As expected, many of the 864 miRNAs were not detected in all samples. A total 427 miRNAs were excluded from further analysis as they were detected in less than 50% of the samples. Differential expression was performed for A549 and THP-1 separately to identify miRNAs upregulated and downregulated in treatment compared to control using DESeq2 package. In A549, 8 differentially expressed miRNAs were identified including 5 downregulated and 3 upregulated in the exosomes from cells treated with 15 nM of $CdCl_2$ compared to those from untreated cells (Table 2).

Table 2. Upregulated and Downregulated miRNA from A549 exosomes.

miRNA	Base Mean	Log_2 Fold-Change	Standard Error	*p*-Value
hsa-miR-3200-3p	52.43	-5.52	1.88	0.003
hsa-miR-21-5p	14.78	6.15	2.29	0.007
hsa-miR-6880-5p	6.49	-3.68	1.64	0.025
hsa-miR-4429	3.94	-4.24	2.03	0.037
hsa-miR-4723-3p	10.74	-3.98	1.94	0.040
hsa-miR-518a-3p	18.00	5.21	2.59	0.044
hsa-miR-520f-5p	18.00	5.21	2.59	0.044
hsa-miR-4784-3p	1.45	-4.06	2.06	0.048

Micro RNA is known to regulate gene expression by targeting miRNAs in a sequence specific manner. By identifying mRNAs that are targeted by these 8 miRNAs we enable to predict pathways dysregulated in A549 cells following Cd exposure. Using TargetScan database we predicted that these 8 miRNAs have 9630 potential targets altogether. Table 3 shows predicted number of targets for each miRNAs.

Table 3. A549 miRNA Targets.

miRNA	Targets
hsa-miR-3200-3p	433
hsa-miR-21-5p	471
hsa-miR-6880-5p	1498
hsa-miR-4429	3206
hsa-miR-4723-3p	3183
hsa-miR-518a-3p	240
hsa-miR-520f-5p	390
hsa-miR-4784-3p	209

In THP-1 macrophages, 2 differentially expressed miRNAs were identified including one downregulated and one upregulated in the exosomes from cells treated with 15 nM of $CdCl_2$ compared to those from untreated cells (Table 4).

Table 4. miRNA Upregulated and Downregulated from THP-1 exosomes.

miRNA	Base Mean	Log_2 Fold-Change	Standard Error	p-Value
hsa-miR-6722-3p	2.61	-4.92	2.04	0.016
hsa-miR-4749-5p	3.91	5.12	2.34	0.029

Similar to A549, we predicted targets for the 2 miRNAs from THP-1 macrophages and found a total of 6607 potential targets (Table 5). Table 5 shows all the predicted targets or each of the miRNAs.

Table 5. THP-1 miRNA Targets.

miRNA	Targets
hsa-miR-6722-3p	1901
hsa-miR-4749-5p	4706

DISCUSSION

Exosomes are secreted by cells under physiological and pathological conditions [21]. They may be formed to remove excess molecular constituents or particles with no physiological role from cells to maintain homeostasis [20]. They have the potential to be used for much more than possible drug delivery vehicles and cell to cell communication. They also have the potential to provide protection against cellular stress and cell death. A definitive reason and physiological significance for the secretion of exosomes remains unclear. Identifying the nature of exosomes could help determine how cellular responses promote a disease. This means that understanding why and how these exosomes are secreted could provide an early indicator disease formation before the severity of the disease progresses uncontrollably. This information could also be used to potentially prevent the development of a disease by identifying cellular markers which could be identified prior to the onset of symptoms. More specifically, exosomes may have the potential to provide early indications of lung effects from Cd exposure (Figure 9). Early detections of fibrosis, lung cancer, pleural effusions, etc. can be further studied in many ways from increase in

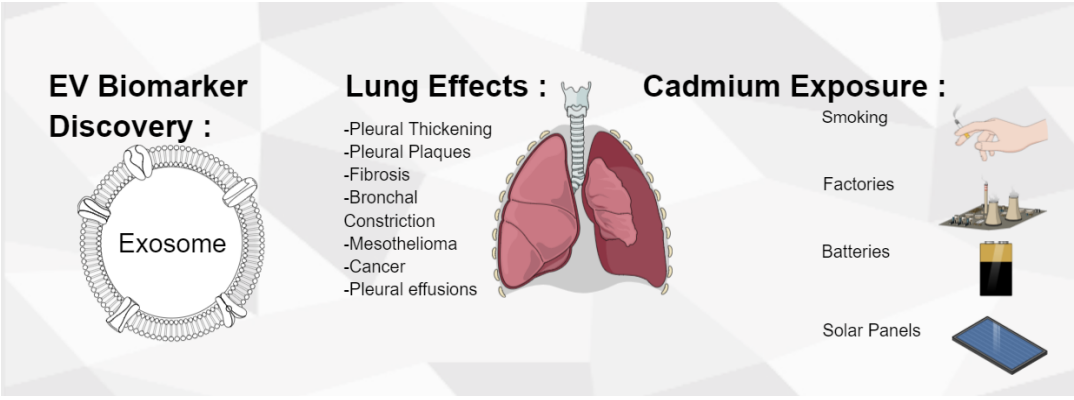

Figure 7. Early Biomarker Discovery of Lung Injury Due to Inhalation of Cd.
Cd inhalation through a duration of time can lead to a series of lung injuries. Exosome can possibly provide information to determine these injuries before a point of severity.

exosome secretion. Changes in specific miRNA profiles contained in the exosomes could be another indicator for early detection of various lung diseases to increase the expression of certain genes.

Exosomes are not the only extracellular vesicles produced by a cell. Microvesicles can also form and range in size from 100-1000 nm [22]. Size exclusion chromatography is the preferred method for isolation of exosomes due to this method's ability to produce high yield of quality sample. Although column chromatography yields the purest sample, there are some limitations. Due to the overlap in similarities of size focusing on column chromatography is not enough of an indication of the sample purely being comprised of exosomes.

To determine the purity of the samples, cryo-TEM, western blot, and NTA analysis help distinguish what the sample is more highly concentrated of. Cryo-TEM provides images of exosomes from A549 cells and THP-1 macrophages. Both images shown that the average size of the exosomes are 75-150 nm in size, and they have a prominent lipid bilayer (Figure 3). The surface markers HSP70 and CD9 are further indicators of the sample being mostly comprised of exosomes (Figure 4). Microvesicles do not contain these classic surface makers and their half-life is considered to be only a few hours [21]. Therefore, the sample would contain very low traces of microvesicles. The size distribution of the exosomes from the A549 and THP-1 cells could help determine the purity of the samples. The exosome size range of those from A549 and THP-1 cell lines are on average 105-165 nm, which is the optimal size range of exosomes (Figure 6). With all these further experiments, it was confirmed that the sample is mostly enriched in exosomes.

To further understand the cellular response to Cd alamar-blue assay was used to assess the cell viability and the results shown near 100% cell viability (Figure 2). Although, alamar-blue

assay can determine if the cells are viable, it does not give any further indication of how the cells are reacting to $CdCl_2$. NTA analysis provides the concentrations of exosomes in particles per mL. Two groups of A549 were observed, untreated (control) and treated with 15 nM of $CdCl_2$. There was no significant increase in exosome concentration in A549 cells, but there was an increase in exosome concentration in THP-1 macrophages treated with 15 nM of $CdCl_2$ (Figure 5). Two groups of THP-1 cells were examined, and all were differentiated into macrophages using PMA. The first group was THP-1 control (untreated), the second was treated with 15 nM of CdCl2, but the macrophages remained inactivated (Figure 5). Reasons for the increase in exosomes with the cells that were treated could be that they were monocytes differentiated to macrophage. The increase in exosomes could also be due to the cells going through oxidative stress (Figure 5) [21]. The cells may also remove unnecessary constituents as mentioned previously, leading to an increase in exosome concentration. What can be concluded is the response to heavy metals in terms of exosome concentration varies cell to cell.

Determination of changes in miRNA profiles in exosomes secreted by cells in response to Cd was the major objective of this study. We found that there were only 8 miRNAs that were differentially expressed in the treated and untreated A549 samples (Table 2) and 2 miRNAs for THP-1 macrophages (Table 4). The 8 miRNAs from A549 cells have 9630 targets (Table 3) and the 2 miRNAs from THP-1 macrophages have 6607 targets (Table 5). The targets refer to the mRNAs that the miRNAs bind to. The miRNA targets were predicted based on confidence level provided in TargetScan software [23]. The miRNA binds to the 3' UTR target of the mRNA and suppresses gene expression [24]. They can also activate gene expression under certain conditions [23]. The changes in miRNA profiles also have the potential as biomarker to be used for certain diseases.

Out of the 8 miRNAs differentially expressed in A549 there were 2 miRNA that had targets associated with the lungs. Hsa-miR-21-5p is involved in regulating the expression of SMAD7 which promoted the progression of non-small cell lung cancer [25]. SMAD7 induces tumorigenicity by blocking TGF-beta-induced growth inhibition and apoptosis [25]. Hsa-miR-4429 can negatively regulate β-catenin expression leading to a mitigated malignant presence of adenocarcinoma in the lungs [26]. B-catenin is involved in tumor suppression [26].

The other miRNAs from A549 also had some known associated targets. Hsa-miR-3200-3p targets Ca2+/calmodulin dependent kinase 2a (CAMK2A) overexpression leading to metastasis of glioma [27]. It also downregulates CAMK2A leading to regulation of Ras/Raf/MEK/ERK pathway [27]. Hsa-miR-6880-5p is potentially a biomarker for pancreatobiliary cancer prognosis [28]. Hsa-miR-518a-3p expression could target TMEM2 and lead to deregulation of TNBC cells which are associates with triple-negative-breast cancer [29]. Hsa-miR-518a-3p is also involved in colorectal cancer because it inhibits NIK which is involved in the NF-κB pathway [30]. Currently there are no publications for hsa-miR-4784-3p, hsa-miR-520f-5p, and hsa-miR-4723-3p.

Out of the 2 differentially expressed miRNA in THP-1 macrophages there was only one publication of associated target. Hsa-miR-4749-5p inhibits RCF2 signaling enhancing temozolomide cytotoxicity to glioblastoma [31]. RCF2 is involved in heat stress–responsive gene regulation [31]. Currently there is no publications on hsa-miR-6722-3p on their predicted targets.

The small number of changes in miRNA of exosome from A549 cells and THP-1 cells could be due the treatment of $CdCl_2$ over a short period of time. Based on the present findings, we speculate that evaluations over a duration longer than 24 hours may be needed to see significant

changes in miRNA profiles. Future directions of the studies would also need to include transition from the in vitro model system to an animal model. The animal model would provide a better understanding on what changes may occur with exosomes due to long-term exposure to the heavy metal compared to the 24-hour study on a cellular level. Furthermore, the biological significance and toxicological pathways could be better understood if the entire lung could be evaluated for a response to Cd.

REFERENCES

1. Waalkes, M.P., *Cadmium carcinogenesis.* Mutat Res, 2003. **533**(1-2): p. 107-20.
2. Lener, M.R., et al., *Blood cadmium levels as a marker for early lung cancer detection.* J Trace Elem Med Biol, 2021. **64**: p. 126682.
3. Bernhoft, R.A., *Cadmium toxicity and treatment.* ScientificWorldJournal, 2013. **2013**: p. 394652.
4. Pizzino, G., et al., *Cadmium-Induced Oxidative Stress Impairs Glycemic Control in Adolescents.* Oxid Med Cell Longev, 2017. **2017**: p. 6341671.
5. Ballatori, N., *Transport of toxic metals by molecular mimicry.* Environ Health Perspect, 2002. **110 Suppl 5**: p. 689-94.
6. Genchi, G., et al., *The Effects of Cadmium Toxicity.* Int J Environ Res Public Health, 2020. **17**(11).
7. Suwazono, Y., et al., *Biological half-life of cadmium in the urine of inhabitants after cessation of cadmium exposure.* Biomarkers, 2009. **14**(2): p. 77-81.
8. Pan, B.T. and R.M. Johnstone, *Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor.* Cell, 1983. **33**(3): p. 967-78.
9. Wallace, D.R., et al., *Toxic-Metal-Induced Alteration in miRNA Expression Profile as a Proposed Mechanism for Disease Development.* Cells, 2020. **9**(4).
10. Simons, M. and G. Raposo, *Exosomes--vesicular carriers for intercellular communication.* Curr Opin Cell Biol, 2009. **21**(4): p. 575-81.
11. Bellingham, S.A., B. Guo, and A.F. Hill, *The secret life of extracellular vesicles in metal homeostasis and neurodegeneration.* Biol Cell, 2015. **107**(11): p. 389-418.

12. Munich, S., et al., *Dendritic cell exosomes directly kill tumor cells and activate natural killer cells via TNF superfamily ligands.* Oncoimmunology, 2012. **1**(7): p. 1074-1083.

13. Fay, M.J., et al., *Cadmium Nephrotoxicity Is Associated with Altered MicroRNA Expression in the Rat Renal Cortex.* Toxics, 2018. **6**(1).

14. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10513-8.

15. Glinge, C., et al., *Stability of Circulating Blood-Based MicroRNAs - Pre-Analytic Methodological Considerations.* PLoS One, 2017. **12**(2): p. e0167969.

16. Sanz-Rubio, D., et al., *Stability of Circulating Exosomal miRNAs in Healthy Subjects.* Sci Rep, 2018. **8**(1): p. 10306.

17. Zhang, J., et al., *Exosome and exosomal microRNA: trafficking, sorting, and function.* Genomics Proteomics Bioinformatics, 2015. **13**(1): p. 17-24.

18. Rodosthenous, R.S., et al., *Ambient particulate matter and microRNAs in extracellular vesicles: a pilot study of older individuals.* Part Fibre Toxicol, 2016. **13**: p. 13.

19. Bollati, V., et al., *Microvesicle-associated microRNA expression is altered upon particulate matter exposure in healthy workers and in A549 cells.* J Appl Toxicol, 2015. **35**(1): p. 59-67.

20. Zhang, Y.G., et al., *Exosomes derived from oxLDL-stimulated macrophages induce neutrophil extracellular traps to drive atherosclerosis.* Cell Cycle, 2019. **18**(20): p. 2674-2684.

21. Kalluri, R. and V.S. LeBleu, *The biology, function, and biomedical applications of exosomes.* Science, 2020. **367**(6478).

22. Stahl, A.L., et al., *Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases.* Pediatr Nephrol, 2019. **34**(1): p. 11-30.

23. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.

24. O'Brien, J., et al., *Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation.* Front Endocrinol (Lausanne), 2018. **9**: p. 402.

25. Li, X. and X. Wu, *MiR-21-5p promotes the progression of non-small-cell lung cancer by regulating the expression of SMAD7.* Onco Targets Ther, 2018. **11**: p. 8445-8454.

26. Zhou, S., et al., *MiR-4429 Alleviates Malignant Behaviors of Lung Adenocarcioma Through Wnt/beta-Catenin Pathway.* Cancer Biother Radiopharm, 2021.

27. Wang, H., et al., *MicroRNA-3200-3p targeting CAMK2A modulates the proliferation and metastasis of glioma in vitro.* Bioengineered, 2022. **13**(3): p. 7785-7797.

28. Lee, K.T., *The Clinical Utility of MicroRNA as a Prognostic Biomarker of Pancreatobiliary Cancers.* Gut Liver, 2016. **10**(5): p. 663-4.

29. Gan, L., et al., *miR-518a-3p Suppresses Triple-Negative Breast Cancer Invasion and Migration Through Regulation of TMEM2.* Technol Cancer Res Treat, 2020. **19**: p. 1533033820977523.

30. Qu, L.L., et al., *Downregulation of miR-518a-3p activates the NIK-dependent NF-kappaB pathway in colorectal cancer.* Int J Mol Med, 2015. **35**(5): p. 1266-72.

31. Ho, K.H., et al., *Xanthohumol regulates miR-4749-5p-inhibited RFC2 signaling in enhancing temozolomide cytotoxicity to glioblastoma.* Life Sci, 2020. **254**: p. 117807.

Vita

After graduating from Tucumcari High School in Tucumcari, New Mexico in 2014. Anisha Bhakta entered Texas Tech University in Lubbock, Texas as an undergraduate student majoring in Chemistry. She graduated with a Bachelor of Science in Chemistry in May 2019. In August of that 2019 she entered the Biotechnology Certification graduate program at the Texas Tech University and received her certification in May 2020. In August of that year, she entered the Biotechnology graduate program at the Health Science Center at the University of Texas at Tyler where she performed her thesis work in the lab of Nagarjun Venkata Konduru, D.V.M.Ph.D. Anisha received her Master of Science in Biotechnology degree in April 2022 and has plans to further education while pursuing a career in biotechnology industry.

This thesis was typed by Anisha Bhakta