3-2005

In Vitro Toxicity of Cadmium Oxide Particles in BRL 3A Rat Liver Cells

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THESIS

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Wright-Patterson Air Force Base, Ohio

AFIT/GES/ENV/05M-04

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Presented to the Faculty

Department of Systems and Engineering Management

Graduate School of Engineering and Management

Air Force Institute of Technology

Air University

Air Education and Training Command

In Partial Fulfillment of the Requirements for the

Degree of Master of Science in Environmental Engineering and Science

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March 2005

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Abstract

For the last decade or so, *in vitro* toxicology has been studied as a method for determining human toxicity, as well as hazard identification and characterization. One purpose of *in vitro* studies is to determine the possibility of developing systems in which the toxicity of chemicals can be quantified, without heavy reliance on animal experimentation. Specific data pertaining to cadmium toxicity in humans through use of *in vitro* methods and models is sparse, at best. There is a need to complete more studies and collect more data to study particular chemicals and their effects and to complete proper evaluation and comparison to *in vitro* studies on human cells. The need for such data is the overall reason for this thesis research and experimentation.

Toxicology *in vitro* refers to a method of exposing cell tissue directly to the chemical compound under study. In this research, rat liver cells, or hepatocytes, were cultured, exposed to cadmium oxide particles, and analyzed with an MTT assay. The assay provided information on the effects of the particles on the mitochondria of the liver cells. Once the assay was completed, data was generated using a spectroflourometer to measure the optical density of the cells, and finally a pre-formulated Excel spreadsheet. The final data output showed doses vs. percent of control cells. Experiments were carried out with doses ranging from 0.001 μg/mL to 2.5 μg/mL for 6, 12, and 24 hours. Two cell densities were also studied for each experiment, 1,000,000 cells/mL and 500,000 cells/mL, in order to ascertain the effects of toxicity on cell density.

Acknowledgements

I would like to express my sincere appreciation to my faculty advisors, Lt Col Ellen England and Dr. Charles Bleckmann for their guidance, patience, and support throughout the course of this thesis effort. Their thorough and timely advice went a long way in reducing the potential frustrations naturally encountered in an academic endeavor of this magnitude. I would also like to thank Dr. Jeff Gearhart for his encouragement and friendship while serving as an advisor during this research. Were it not for him, I may never have discovered *in vitro* toxicology. Last, but not least, I am profoundly grateful for Dr. Saber Hussain's involvement. Without his expertise, methods in *in vitro* toxicology would still be a mystery. Dr. Hussain's willingness to take time out of his extremely busy schedule; to provide mentoring and instruction in cell culture, cadmium oxide exposures, and MTT assays; is prized more than he will ever know.

I am also indebted to the many laboratory technicians and faculty of the Air Force Research Laboratory who spent their valuable time ensuring that I was never without proper supplies, cells, and mentoring.

As a result of the successful collaboration between my faculty advisors and the Air Force Research Laboratory, I have become enthusiastic about studying toxicology as much as possible in the future. My thanks to all who took part in keeping that enthusiasm alive.

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IN VITRO TOXICITY OF CADMIUM OXIDE PARTICLES IN BRL 3A RAT LIVER CELLS

I. Introduction

1.1 Overview

Predicting the overall influence of any toxic chemical on the human physiological system is difficult. The most widely accepted estimates of toxicity to humans are based on extrapolation of laboratory animal bioassay data (Carere et al., 2002) rendering them less than completely accurate. This extrapolation does not effectively take into account the manner in which humans are exposed to the chemical, environmentally or occupationally, nor the exact mechanisms of action in the human body. Since there is a lack of certainty concerning how chemicals are metabolized in the body, safety factors and conservative estimates are used in calculating human risk when extrapolating from animal toxicity data. These toxicity estimates and their assigned safety factors are then translated by regulatory enforcement agencies into human occupational or environmental exposure standards.

Another and newer method of estimating chemical toxicity to humans is through the use of *in vitro* toxicity studies. For example, some of those studies apply nanoparticles (particles in the 1-100 nanometer range) to immortalized Buffalo Rat Liver (BRL) 3A cells, namely rat hepatocytes (Hussain *et al.* 2004). *In vitro* studies are also conducted using immortalized rat lung cells or

macrophages (Hussain *et al*. 2004). These *in vitro* methods are gaining wide acceptance, although they are not seen as the ultimate and final goal for studying the toxic effects of chemicals to humans. *In vitro* toxicity of hepatocytes refers to simply applying the known toxin directly to the liver cells in the media, after the cells have attached to the plates.

Ideally, toxicity studies will eventually be conducted directly on human tissue, which has been immortalized, like the rat liver cells. Use of immortalized human tissue would negate the need to study and sacrifice animals and would likely yield data that would accurately predict the effect of chemicals on human cells and tissues.

1.2 Background

1.2.1 *In Vitro* Particle Studies, BRL 3A Cells.

In order to gain a general sense of toxicity along with a maximum quantity of data in a short period of time, the BRL 3A immortal rat liver cell line was selected as a convenient working model system to screen for particle toxicity. The selection of rat liver cells is justified, as exposure to particles is likely to have an impact on liver cells through ingestion. The BRL 3A liver cell line also provides the ability to screen several materials at a low cost in a relatively rapid time frame.

To date, there are only a few studies directly or indirectly investigating the toxic effects of materials and no clear guidelines are available to quantify these effects. However, many in vitro methods are used in the preliminary screening of compounds of pharmacological interest, or for safety assessment. In other cases,

they represent supplementary tools for gathering more information on the specific mechanisms involved in a toxic effect. These methods will provide guidance for further evaluation, if necessary, both by in vivo studies on animals, as well as on humans (Carere et al., 2002). Toxicity data concerning effects on basic cellular functions and/or structures has a good correlation with in vivo data concerning human lethal toxicity (Ekwall, 1999).

1.2.2 Analysis. Analysis was accomplished with the MTT assay. This assay measures the mitochondrial function of the cells after they are exposed to the cadmium oxide, the particle of choice for this study, for the specified period of time. The MTT solution contains a tetrazolium salt which is reduced into an insoluble formazan product by the mitochodria of living cells. The reduction produces a noticeable color change in the solution.

1.2.3 Cadmium. Cadmium is a non-essential element occurring naturally in the earth's crust (Verougstraete et al. 2002). Cadmium occurs in high abundance in zinc and lead ores and in phosphate fertilizers (Garrett, 2000; McLaughlin and Singh, 1999). High concentrations of cadmium are also found in sewage sludge (Resource Sciences, 1997). Agricultural uses of phosphate fertilizers and sewage sludge, and industrial uses of cadmium have been identified as a major cause of widespread dispersion of the metal at trace levels into the general environment and human foods (Satoh et al., 2002). Cadmium has high rates of soil-to-plant transference compared with other non-essential elements. On the basis of typical concentrations, vegetables and cereals contain five times more

cadmium than fruit and the levels of cadmium in oil seeds and cocoa beans are five times higher than in vegetables and cereals. General population cadmium ingestion exceeds the recommended safe intake level of 70 μg per day (WHO, 1989; IPCS, 1992) with vegetables and cereals found to be the most significant sources of cadmium in the diet. Considerable amounts of cadmium found in zinc supplements have also raised a concern for users (Krone et al, 2001).

Non-workplace exposure to cadmium has been linked to a variety of adverse health affects. These include the development and/or progression of diabetic renal complications, hypertension, osteoporosis, leukemia and cancer in several organs such as the lung, kidney, urinary bladder, pancreas, breast and prostate (IPCS, 1992; Bellinger et al, 2001; Jarup et al, 1998). Chronic exposure to low levels of cadmium in drinking water or the diet was found to cause hypertension in several experimental animal species including the monkey (Akahori et al., 1994), rat (Schroeder, 1964), rabbit and dog (Thind et al, 1970; Thind et al, 1973). Human population studies, however, have provided conflicting results regarding a role for environmental cadmium exposure in the development of hypertension. Moderate to high intakes of cadmium were found to be associated with hypotension in some studies (Nishijo and Nakagawa and 1996; Nishijo *et al*, 2000).

Although cadmium is currently considered to be a human carcinogen by the International Agency for Research and Cancer (Waalkes, Michael P., 2003), it may be inappropriate to conclude that sufficient evidence on carcinogenicity of cadmium in humans exists, due to a lack of epidemiological justification in recent

research studies (Satarug, *et al.,* 2003). Since there are inconsistent reports on the relationship of cadmium exposure with the life expectancy of people living in cadmium-polluted areas (Satoh et al, 2002), further studies are needed for clarification.

1.3 Problem Identification

Specific data pertaining to cadmium toxicity in humans through use of *in vitro* methods and models is sparse, at best. There is a need to complete more studies and collect more data to study particular chemicals and their effects and to complete proper evaluation and comparison to *in vitro* studies on human cells, when the time comes. The need for such data is the overall reason for this thesis research and experimentation.

1.4 Research Questions/Objectives

The purpose of this research was to determine the effect of cadmium oxide particles on rat hepatocytes. Specifically, the following research questions were addressed:

What are occupational and environmental sources of cadmium or cadmium oxide?

What is the cellular toxicity of cadmium oxide particles?

What is the effect of cell density on cadmium oxide toxicity?

1.5 Research Approach

The first step in this research effort was to identify occupational and environmental sources of cadmium and cadmium oxide through a literature search and discussion with knowledgeable individuals. Secondly, a literature search was

used to identify studies involving *in vitro* methods of cellular toxicity testing, specifically, studies examining cadmium. The third step in the research was to conduct laboratory experiments using rat hepatocytes and cadmium oxide particles. The particles were purchased by the Air Force Research Laboratory and made available to the researcher. Specifically, culturing of hepatocytes was through the use of Ham's F-12 media in 100 mL flasks. Once the cells had become confluent, a desired amount of cells was withdrawn from each flask and counted. Various 6-well plates were then seeded with the counted cells, starting with 1 million cells per well and working down, to achieve the desired densities. After seeding for 24 hours, the cells on plates were then dosed with a range of concentrations of cadmium oxide solution. 24–hour exposures of the cells to cadmium oxide were studied. The MTT assay wasused to determine the toxicity to cells after the exposure. Upon completion of the 24-hour experiments, 12-hour and 6-hour experiments were completed to obtain a comparison. After the assay was accomplished for each experiment, the cell mortality results were measured through use of a spectrofluorometer. The data was then transferred to a pre-formulated Excel spreadsheet for graphical output.

1.6 Scope of Research

This research has limited scope in several ways. There are few cellular toxicity studies cited in the literature using *in vitro* methods, or those specifically involving cadmium. Consequently, efficient comparisons of data between literature sources and specific studies may not be effectively accomplished. Also,

a complete and thorough analysis of the toxicity of cadmium oxide on more than hepatocyte cells is beyond the scope of this thesis. Neither tissue nor organ toxicity was addressed. Additionally, the experimental data is always subject to errors in measurement and procedure.

1.7 Significance

There is possibly a long road ahead for researchers to achieve statistically correct data on cadmium oxide toxicity, in order to gain more certainty for rat to human tissue extrapolations. The Air Force Research Laboratory, Wright-Patterson Air Force Base, Ohio is attempting such research. The data presented in this study will contribute to the overall research team's accomplishments and goals, potentially, ultimately influencing the occupational and environmental exposure standards set by the Air Force

1.7 Overview of Document

The remainder of this thesis consists of four chapters. Chapter two reviews literature on *in vitro* toxicity and cadmium studies in order to gain a broad perspective on the overall research to include history, concerns, and goals set forth by professional researchers and toxicologists. Chapter three provides a detailed discussion of the methodology used in this research. Next, chapter four presents the data and analytical results. Finally, chapter five discusses conclusions and significance of findings.

II. Literature Review

2.1 Introduction

This literature review describes *in vitro* and hepatocyte research and discusses the toxicology of cadmium and cadmium oxide. The review includes a general discussion of *in vitro* toxicology history and its outcomes as well as information concerning hepatocyte cell cultures. The toxicity of cadmium oxide is the focus of this research. Cadmium will be characterized with regard to its usage, carcinogenicity, and action in the body. The chapter is concluded with a summary of the revelations from the literature review.

2.2 Hepatocyte Cell Culture

Monolayer cell cultures were used in this research effort. Descriptions of these cultures were from Jakoby *et al.*(1979) and Guillouzo *et al.*(1986). Basic monolayer techniques are well established. The cells routinely studied in monolayer culture can be derived in the laboratory from primary cultures, or obtained from commercial suppliers and nonprofit organizations. Monolayer cell cultures are frequently established from single cell suspensions prepared by the dissociation of organ fragments. The cell preparation is inoculated into a culture vessel containing fluid medium and incubated in a controlled atmosphere. The viable cells then settle and attach to the inside surface of the flask (coated with substrate) within several hours. The resultant primary culture is a mixed cell population, which contains many of the cell types contained in the tissue of origin. In order to obtain a monolayer culture consisting of a single cell type, it is necessary to isolate that cell from the primary preparation. There are various methods of cell separation, which can be applied to the initial cell suspension prior to culture. Alternatively, the cell population can be enriched using *in vitro* methods that select cell

types based on their attachment or growth characteristics. In this case, hepatocytes, or liver cells, were the cells of interest. In mixed cell cultures they tend to overgrow the population and obscure other cell types.

Cell types which require attachment to a rigid substrate in order to replicate *in vitro* are classified as anchorage-dependent cells. The hepatocytes used in this study were cultures in flasks containing a substrate. The growth kinetics of cells in monolayer culture follow a characteristic pattern. The cells eventually enter a log phase of growth in which there is an exponential increase in cell number. During the log phase, the cells exhibit their highest metabolic activity. When culture conditions no longer support cell division, the population enters a stationary phase, during which the cell number remains constant. In the case of hepatocytes, the researcher would extract the cells just before the stationary phase, when the cells are said to be confluent. "Confluent" cultures occupy all available growth surface. If nutritional conditions are adequate, a confluent cell population may continue to replicate in log phase. In practice, however, cells are not used beyond the point of confluency since essential metabolites are rapidly depleted at high cell density.

The physical culture environment should be well defined and precisely controlled. For example, optimum pH of the culture medium is usually within the range of 7.2-7.5. The determination of proper pH in a culture system is dependent on the cell type. Hepatocytes specifically require a pH of 7.25. Humidity and temperature in the incubator also play an important role in the integrity of the culture environment.

Subculturing or passaging cells involves detaching the cells from their substrate and transferring them to new culture vessels. This is done when cells have utilized the growth surface available to them. Various methods can be used to remove cells from the

culture surface. One method, proven as one of the less disruptive to the cells, is dissociation with a proteolytic enzyme such as trypsin. However, enzymic dissociation can still damage cells after a period of time, so it is desirable to minimize the duration of contact between the cells and enzyme solution. For hepatocytes, 2-3 hours is the optimal contact time.

2.3 *In Vitro* Toxicity

For the last decade or so, *in vitro* toxicology has been studied as a method for determining human toxicity, as well as hazard identification and characterization. Currently, toxicological hazard and risk assessments are mainly based on highly standardized protocols for animal experimentation and exposure assessments. (Holme *et al.,* 2002) One purpose of *in vitro* studies is to determine the possibility of developing systems in which the toxicity of chemicals can be quantified, without the heavy reliance on animal experiments.

2.3.1 History

Only a few *in vitro* toxicology tests are accepted by regulators. Many others are in the pre-validation or validation phase. An area where *in vitro* tests play a key role is genetic toxicology. *In vitro* methods are also used in the preliminary screening of compounds of pharmacological interest, or for safety assessment (Blaauboer, 2001).

The three critical aspects for success of *in vitro* tests are the availability of new methods, the validation of new test procedures, and the definition of criteria of acceptance by regulators. (Carere et al., 2002).

Several in vitro cellular experimental models have been made available in the last four decades (Zucco *et al*., 1998), which are more or less representative of various cell types. Most of these cellular models are cell lines, immortal and often tumour-derived.

An *in vitro* model for skin absorption is now available and is being considered for inclusion in the OECD (Organization for Economic and Co-operation Development) guidelines. It has been considered scientifically validated by ESAC (European Surveillance of Antimicrobial Consumption) very recently. Several, *in vitro* skin irritation models are currently available and used for research. Only the protocols of three commercial kits have been judged ready for a pre-validation study (Van de Sandt *et al*., 1999).

In genetic toxicology, the primary objectives of *in vitro* tests are identification of potential germ cell or somatic cell mutagens (potential carcinogens). In general, at least two *in vitro* tests, one at the gene level in bacteria and one at the chromosome level in mammalian cells, are considered necessary for chemicals of limited or absent human exposure (Carere *et al*., 1995). In the case of chemicals for which direct human exposure is expected, several testing strategies have been proposed with 3-4 tests at the gene and at the chromosome level, often also including an *in vivo* assay (Carere *et al*., 1995; Muller *et al*., 1999; COM, UK, 2000).

The recently updated guideline for food additives by the E.C. Scientific Committee on Food (Carere *et al.*, 2002) recommends the following standard battery of three *in vitro* tests:

1. A test for the induction of gene mutations in bacteria.
2. A test for the induction of gene mutations in mammalian cells.

3. A test for induction of chromosomal aberrations in mammalian cells.

In general, this battery is considered sufficiently sensitive to detect the most genotoxic compounds.

2.3.2 Risk Assessment and Hazard Identification

Toxicological risk evaluation traditionally relies on a wide array of animal experiments. The day-to-day practice of these procedures have led to the relatively safe use of chemicals in industry, or as agrochemicals, drugs, household chemicals, or cosmetics. However, large numbers of laboratory animals have been used and distress caused to many of these animals (Anonymous, 1986). Besides the ethical objections against the use of animals, there is also a scientific motivation for criticizing the heavy reliance on animal data, which has always been prone to some degree of uncertainty.

In vitro systems have been extremely useful in studying the molecular basis of a chemical's biological activity, including its mechanisms of toxic action (Blaauboer et al., 1998). Knowledge of a compound's mechanism of toxic action, either derived from *in vitro* studies or based on its structure, can be the basis of hazard identification. However, a hazard assessment cannot easily be made without further knowledge of the compound's behavior in the integrated system of an intact organism. Thus, results obtained from in vitro studies in general are often not directly applicable to the *in vivo* situation. One of the most obvious differences between the situation *in vitro* and *in vivo* is the absence of processes regarding absorption, distribution, metabolism and excretion that govern the exposure of the target tissue of the organism *in vivo* (Flint, 1990; Blaauboer et al., 1990).

From this description, it is clear that the information derived from *in vitro* toxicity studies will be useful for studying toxic mechanisms of chemicals on the cellular and

molecular level. However, this information then needs to be interpreted taking into account the compounds' behavior.

Walton *et al*., (1999) reviewed the application of *in vitro* data in the derivation of the acceptable daily intake of food additives and concluded that *in vitro* metabolism and mechanistic studies in isolation have limited value for the prospective toxicological classification and characterization of food additives. However, in conjunction with detailed short- and long-term *in vivo* studies, tests can be used in the calculation of both inter-species and inter-individual variability in the toxicokinetics and toxicodynamics of food additives. Thus, *in vitro* studies may assist in removing some of the uncertainty involved in extrapolating toxicity from the most sensitive test species to humans.

2.3.3 Extrapolation From High to Low Dose

Often there are differences in the toxicodynamics of a chemical revealed at high doses compared to that operating at lower doses. One example is bis(tri-n-butylin)oxide (TBTO), mainly used as a biocide in crop protection and antifouling paints for large ships. *In vitro* studies showed low concentrations of TBTO effectively inhibit DNA synthesis and cell proliferation (Vandebriel et al., 1999). At higher concentrations of TBTO, the cells die (Stridh et al., 1999). These *in vitro* findings parallel *in vivo* findings in the rat. There is also an example of *in vitro* studies supporting *in vivo* studies in the case of paracetamol, showing the mechanism of action at both high and low doses (Davies *et al*., 1986).

2.3.4 Conclusion

In vitro studies can never, in isolation, determine the exact level of zero adverse toxicity for threshold compounds or risk specific doses for non-threshold compounds.

However, *in vitro* systems will aid in the identification of the most sensitive species or strain. Also, *in vitro* systems are good models for studying qualitative and quantitative species differences in toxicity. Further, *in vitro* systems are excellent models for characterization of the mode of action/mechanism for critical effects, but findings need to be validated *in vivo*. Finally, *in vitro* systems will aid in the extrapolation from high to low dose and from experimental animal to humans.

2.4 Cadmium

Cadmium is an abundant transition metal of worldwide concern because it accumulates in the environment as a result of its numerous industrial uses. In humans, non-occupational exposure results predominantly from the consumption of contaminated food, from smoking as a result of the uptake of cadmium from tobacco plants in contaminated soil, and from inhalation of polluted air. Cadmium has an extremely long biological half-life that essentially makes it a cumulative toxin and, at present, there is no proven effective treatment for chronic cadmium intoxication. Occupational exposure to cadmium is associated with lung cancers in humans, while impacts on organs, such as the prostate, have not been established (Waalkes, 2000). Due to multiple regulations on the use of cadmium compounds, their industrial uses, and further dispersion in the environment, have been sharply curtailed in the last 10 years. However, cadmium is an extremely persistent element, which, once introduced to the environment, will persist for centuries (Filipi et al., 2003).

As far as environmental sources, plants can take up cadmium from soil, and fish can take it up from water. Phosphate fertilizers and sewage sludge may also increase cadmium levels in soils, which can increase it in crops (Saturug et al., 2003). The largest

source of cadmium is from the burning of fossil fuels (coal and oil) or incineration of municipal waste materials (Waalkes, 2002). The US Environmental Protection Agency has listed cadmium 7th on the superfund hazardous list.

2.4.1 Chemical Characteristics

Table 2.1 is extracted from the NIOSH Pocket Guide to Chemical Hazards, 1997 and discusses attributes of cadmium.

Table 2-1. Extract from NIOSH Pocket Guide to Chemical Hazards, 1997

Chemical Name	**Synonym/Trade Name**	**Physical Description**	**Incompatibilities and Reactivities**
Cadmium dust (as Cd)	Cadmium metal; Cadmium, others depending on compound	Silver-white, blue-tinged, lustrous, odorless solid	Strong oxidizers, elemental sulfur, selenium, tellurium
Cd (Metal)	Cadmium metal; Cadmium, others depending on compound	Silver-white, blue-tinged, lustrous, odorless solid	Strong oxidizers, elemental sulfur, selenium, tellurium
Cadmium fume (as Cd)	Cadmium monoxide, cadmium oxide fume, cadmium	Odorless, yellow-brown, finely divided particulate dispersed in air	Not applicable

2.4.2 Mutagenicity

Cadmium and cadmium compounds are suspected human carcinogens and are ubiquitous in the environment. Cadmium has been classified as a human carcinogen by the International Agency for Research on Cancer (IARC). The carcinogenic mechanisms of cadmium remains largely unknown since direct mutagenic effect is weak in bacterial and standard mammalian cell mutation assays. Cadmium has been shown to induce chromosomal aberrations, sister chromatoid exchanges, DNA strand breaks, and DNA-protein crosslinks in various mammalian cell species. Cadmium has also been reported to

be co-mutagenic in combination with either UV irradiation or other chemical carcinogens.

In a study conducted by Filipi *et al*., (2003), using a human-hamster hybrid cell line, cadmium chloride was found to induce a dose and time dependent increase of mutations in the cells. In a 5 hour exposure, 34 mutants per 10^5 survivors per μM $CdCl_2$ and 147 mutants per 10^5 survivors per μM $CdCl_2$ in a 24 hour exposure were observed.

There is evidence that cadmium interferes with several DNA repair mechanisms including the repair of oxidative DNA damage (Hartwig, 1994). These and the aforementioned results indicate that cadmium is a potent mutagen in cultured mammalian cells. In the human-hamster hybrid cells it induced mutations at concentrations comparable to those found in the environment and at the levels that can accumulate in the human body (IARC,1993).

2.4.3 Other Health Hazards

Cadmium can enter the bloodstream by absorption from the stomach or intestines after ingestion of contaminated food or water, or by absorption from the lungs after inhalation. Once in the body, cadmium is very strongly retained. So even small doses build up significantly during continuous exposure (Elliot at al., 2000). The amount of cadmium needed to cause adverse health effects depends on the physical form (Satoh et al., 2003).

Table 2.2 is extracted from the NIOSH Pocket Guide to Chemical Hazards, 1997 and lists the known hazards of cadmium.

Table 2-2. Extract from NIOSH Pocket Guide to Chemical Hazards, 1997.

Chemical Name	*Symptoms*	*Target Organs*	*Route*
Cadmium dust (as Cd)	Pulmonary edema, cough, chest tightness, headaches, chills, muscle aches, nausea, vomiting, diarrhea, mild anemia	Respiratory system, kidneys, prostate, blood	Inhalation, Ingestion
Cd (Metal)	Pulmonary edema, cough, chest tightness, headaches, chills, muscle aches, nausea, vomiting, diarrhea, mild anemia	Respiratory system, kidneys, prostate, blood	Inhalation, Ingestion
Cadmium fume	Pulmonary edema, cough, chest tightness, headaches, chills, muscle aches, nausea, vomiting, diarrhea, mild anemia	Respiratory system, kidneys, prostate, blood	Inhalation

2.4.4 Occupational Exposure

The IARC noted several constraints influencing their evaluation of cadmium, which, to a large extent, was based on animal experiments where the animals predominantly were exposed to inhaled or injected cadmium. The epidemiological evidence originates from occupationally exposed workers, with sparse historical data on exposure to cadmium, rarely accounting for confounding factors (such as smoking) (Elliot et al., 2000).

In a study conducted by Aizenburg et al., 2000, personal exposure of workers performing abrasive blasting operations (a common method of corrosion control and surface preparation) at four U.S. Air Force facilities was monitored. Airborne dust was generated through these operations and were inhaled by the workers. The aerosols from the dust were found to contain 25 metals, including cadmium. Even workers wearing respirators and blasting helmets were exposed to high cadmium levels (relative to the Threshold Limit Value) because of the presence of large cadmium-plated parts and several small cadmium-plated subsections and rivets. Overall, the measured 8-hr Time Weighted Average for airborne concentrations was higher than the Permissible Exposure Limits (PEL) of 0.005 mg/m^3 for nine out of ten tasks.

In the "Rapid Guide to Hazardous Chemicals in the Workplace" (Lewis, 1994), cadmium is listed with various safety profiles. Pure cadmium in the form of dust is a confirmed human carcinogen. The Time Weighted Average (TWA) according to OSHA PEL is 5 μg Cd/m^3. For cadmium compounds, in the form of dust and salts, the safety profile and TWA are the same as that for pure cadmium.

According to Levy and Wegman, (1995), construction workers also encounter cadmium in the work place. Welding and cutting pipe can produce metallic dusts and fumes. The authors state that cadmium exposure occurs in many other industrial processes, and can be monitored with either urine or blood concentrations. Cadmium is also commonly obtained as a by-product from smelting of zinc, lead, or copper ores and is used in metal plating, pigments, batteries, and plastics (Saturug et al., 2003).

2.5 Summary

Exposure to cadmium fumes and dust has been associated with lung cancers and various cadmium compounds have displayed genotoxic potential. Further efforts are necessary to define more precisely the risks of cancer from cadmium exposure and its target sites in humans. Exposure can also lead to many other health concerns. There is no real rationale to the belief that cadmium acts the same way in all target tissues, and it is quite plausible that multiple, target tissue- specific mechanisms apply (Waalkes, 2003). Obviously, this heavy metal has become a considerable environmental and occupational concern. Unfortunately, there are no proven effective treatments for chronic cadmium intoxication (Goering, et al., 1994). More research is needed, not only understand the behavior of cadmium in various environments, but also to know how to effectively deal with all exposure levels to the human body.

III. Methodology

3.1 Introduction

The primary goal of this research effort was to determine cadmium oxide particle toxicity to rat liver cells in culture. The research was carried out by conducting laboratory experiments with cadmium oxide solutions, using an *in vitro* toxicity method with analysis using an MTT assay. Cadmium was chosen because of the availability of research on the particles as well as a lack of solid conclusions as to the effects cadmium has on the human body. Cadmium oxide was also chosen for exposures of the cells because the Air Force Research Laboratory is already pursuing studies using this particular compound. The appeal of the *in vitro* method is the novelty of an emerging field of study. While there have been numerous studies conducted using *in vitro* techniques, there is still a lack of research data in this area compared to other toxicity studies.

The overall laboratory experiments consist of three general steps; cell culture preparation, exposure of cells to cadmium oxide particulates, and analysis of cell mortality using the MTT assay. These steps are detailed later in the chapter. First, assumptions made in conducting the research are detailed, then lab equipment described, components of media listed, and the final data output technique described. The methodology will conclude with a brief summary.

3.2 Assumptions

Several key assumptions were made at the beginning of the laboratory experiments.

1) Precedents set forth by professional researchers in previously conducted *in vitro* toxicity tests were accurate.
2) Cadmium oxide concentrations in solutions were selected based upon previous work and applied during this experiment. The concentrations calculated by former professional researchers were assumed to be accurate for the purposes of this study.
3) Despite the lack of solid evidence as to all the effects of cadmium on the human body, cadmium oxide was treated as a suspected carcinogen in this study.
4) The concentrations of dose-metrics have the same cancer-causing potential in humans as in rats, an assumption generally accepted by both regulatory agencies and researchers.

3.3 *In Vitro* Testing

There are some examples of *in vitro* studies that indicate comparative *in vitro* studies using cellular systems from experimental animals and humans can aid in the extrapolation of animal data to humans (Holme et al., 2002). Further *in vitro* results may reduce uncertainties in these quantitative extrapolations. Further, *in vitro* models have been shown, to some extent, to be excellent models for characterization of the mode of action for critical effects, however, the findings still need to be validated by *in vivo* methods, a long established procedure.

In vitro experiments involve a methodology for studying toxic effects, which is currently being employed in various studies to include cellular systems, genetics, and

stem cell research (Zucco *et al.,* 1998*;* Kulyk *et al.,* 2000). For this particular study, only immortalized rat liver cells were used.

The method of experimentation involved first culturing the cells, in an effort to achieve the appropriate cell density for each experiment. The cell densities required were 1,000,000 cells/mL and 500,000 cells/mL. Both densities were used at the same time for each experiment, for comparison. Therefore, for each experiment, one control was run for each of two cell densities. Each was at least done in triplicate. Once the 6-well plates were properly seeded with these cells (cells attached to the bottom of each well in the plate), the growth media was suctioned out, leaving the cells attached to the bottom of the plates. Immediately, the required doses of cadmium oxide solutions were applied directly to the cells in the plates. The plates were then incubated for the specified period of time unique to the particular experiment. After the end point had been achieved, the cadmium oxide solution was suctioned out, again leaving the cells attached to the plates. After which, the MTT assay (discussed later in the chapter) was applied to measure the mitochondrial function of any living cells remaining after the exposures.

3.4 Cellular Line/System Used

Through numerous research studies previously performed at the Air Force Research Laboratory, the suggested cell line for this study was the BRL-3A (Buffalo Rat Liver) cell line. For the purposes of this study, the actual process of retrieving the cells out of the animal and immortalizing them was not observed. The liver cells were stored and transported from the vivarium (located in a separate section of the Air Force Research Laboratory) to the appropriate lab in a nitrogen tank. There, the tubes of cells

were extracted from the tank by hand and allowed to thaw in the incubator, to prepare for culture.

3.5 Cell Culture

Once the cells had been thawed, they were placed in a specified number of 100 mL flasks, the number being unique to the particular experiment. Each flask was then trypsinized (injected with trypsin and Ham's F-12 media with serum), to allow the cells to attach to the walls of the flasks and grow (Guillouzo, *et al.,* 1986).

Throughout the process of culturing cells, it became necessary to continuously trypsinize the cells, in an effort to prevent them from overgrowing (becoming too confluent) in the flasks, and also to extract cells to use for the exposures and/or split the cells into a higher number of flasks.

The trypsinization process was completed as follows:

1. Growth media suctioned from each flask
2. 10 mL of Phosphate Buffered Saline (PBS) was injected into each flask. The PBS was allowed to coat and wash the cells briefly (a few seconds) before being suctioned out.
3. 1.5 mL of trypsin was added and also allowed to thoroughly coat the cells. The flasks were incubated with the trypsin for 3-4 minutes. This allowed the cells to detach from the walls of the flask as well as each other.
4. 5 mL of Ham's F-12 growth media was added to each flask.
5. Media was sprayed up and down the walls of the flask to re-suspend the cells more uniformly at the bottom of the flask and in the media.

6. A desired amount of media containing cells was withdrawn from the flasks and either placed in new flasks, or counted and placed into 6-well plates for exposure, or both.
7. Each remaining flask, including old flasks, then received an injection of 10 mL of Ham's F-12 growth media. The amount of media added to the exposure plates depended on what amount of media containing cells was originally added to the plates. This amount would be determined by counting the cells using a matrix plate under the microscope. Which of these options would be utilized varied from day to day, depending on the demands of the experiments.
8. All flasks and plates were then incubated for 3-4 hours, to allow the cells to attach to the walls of the flasks and/or the bottom of the plates.
9. The final step of the process was to suction out all media with the trypsin, and inject the same amount of fresh Ham's F-12 growth media because trypsin becomes toxic to the cells after a period of time and inhibits cell growth. Cells were then allowed to grow until the next appointed time to reaccomplish the entire process.

Trypsinization was accomplished as frequently as necessary to prevent cells from becoming too confluent. The number of flasks could be increased at any time, thus increasing the availability of cells for experiments, through the trypsinization process.

3.6 Lab Set-Up

---Spectromax 190 – The spectrofluorometer (or Spectromax) was used in the data analysis, after completing the assay. The Spectromax measures fluorescence, light

emitted by certain substances (in this case, cells) resulting from the absorption of incident radiation (Spectromax 190 Operator's Manual). The machine employs the use of an excitation spectral scan, which measures fluorescence at a single wavelength across a spectrum of excitation wavelengths. The default value reported for each well in the plate is the excitation wavelength of maximum fluorescence. An integrated software program is used to control and collect data from the instrument and display the data in tabular form on the computer screen.

---Microscope Analysis- Use of the microscope showed the affects of the toxin on exposed cells versus the control cells. However, this would not be sufficient data on its own.

---Media- A description of all media used in the study is listed in Table 3-1.

Table 3-1. Media used for cultures.

Media Name	Ingredients				
Ham's F-12 Growth Media (pH 7.25)	900 mL de-ionized water	1.2g sodium bicarbonate	1 L Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham	15 mL Penecillin/Streptomyacin	30 mL Fetal Bovine Serum
Ham's F-12 Exposure Media (pH 7.25)	900 mL de-ionized water	1.2g sodium bicarbonate	1 L Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham	15 mL Penecillin/Streptomyacin	
Acidified IPA (Isopropanol)	200 mL de-ionized water	1.65 mL HCL	300 mL isopropanol		
PBS (Dulbecco's Phisphate Buffered Saline)	900 mL de-ionized water	1 L PBS concentrated powder			
Trypsin	90 mL PBS	10 mL stock trypsin			
Tetrazolium Salt MTT	20 mL PBS	100 mg MTT Sigma cat. No. M-5655			

Additional equipment was used during the experiments including a Corning pH Meter 220 (AAMRL 10422) used for preparing Ham's F-12 media, an incubator

maintained at 37 degrees Celsius, and utensils to include 6- well plates, electric pipetter, hand pipetters, 100 mL flasks, 96-well plates

3.7 Exposures

3.7.1 Cadmium Oxide Solutions

Raw cadmium oxide particles were made into a 1 mg/mL stock solution. Two experiments were conducted, one with high-concentration range doses, one with lower concentrations. The amount of media and stock solution used for each concentration is shown in Table 3-2 and 3-3.

Table 3-2. Media and stock solutions used to prepare exposures.

Concentrations for high-dose range (µg/mL)	
0	10 mL exposure media
0.1	1 uL of 1 mg/mL stock solution in 10 mL exposure media
0.25	2.5 uL of 1 mg/mL stock solution in 10 mL exposure media
0.5	5 uL of 1 mg/mL stock solution in 10 mL exposure media
1.0	10 uL of 1 mg/mL stock solution in 10 mL exposure media
2.5	25 uL of 1 mg/mL stock solution in 10 mL exposure media

Table 3-3. Media and stock solutions used to prepare exposures.

Concentrations for low-dose range (µg/mL)	
0	10 mL exposure media
0.001	10 ul of 1 ug/mL stock solution (made with serial dilution from 1 mg/mL stock) in 10 mL exposure media
0.01	10 uL of 10 ug/mL stock solution (made with serial dilution from 1 mg/mL stock)10 mL exposure media
0.1	10 uL of 100 ug/mL stock solution (made with serial dilution from 1 mg/mL stock)10 mL exposure media
0.5	5 uL of 1 mg/mL stock solution in 10 mL exposure media
1.0	25 uL of 1 mg/mL stock solution in 10 mL exposure media

After the 6-well plates were seeded with the desired cell densities, the growth media was suctioned out and replaced with 1 mL of cadmium oxide solution (one concentration per well).

3.7.2 End Points

The experiments started with 24-hour incubations with the high and low dose ranges. This was followed with experiments of 12-hour incubations with both dose ranges. Each dose range was applied to three 6-well plates for each end point. This entire experiment was then repeated at least twice more, for a total of not less than 9 plates per dose range per end point. The results of the first experiments dictated which end point to study next. For example, if the high-dose 12-hour experiment showed similar results to the 24-hour experiment, then the next end point would be at 6 hours. The same method was applied to the lower dose range. The purpose of these end points was to make an attempt to discover the amount of time at which the cadmium oxide would start to become toxic between 0 and 24 hours after exposure.

3.8 MTT Assay

After all experiments were completed, the MTT assay was performed. The MTT is useful for assaying cell survival and proliferation after exposure to the toxin. MTT assay represents the mitochondrial function of the cells. (Romijn *et al.,* 1988) This assay is a colorimetric assay system, which measures the reduction of a tetrazolium component into an insoluble formazan product by the mitochondria of viable cells. Cells with functional mitochondria convert the tetrazolium dye into its reduced form. The amount of the resulting bluish-purple color is directly proportional to the number of viable cells. The color change is measured spectrophotometrically in the Spectromax 190 micro plate reader. The MTT procedure is listed following.

3.8.1 MTT Procedure

1. Suction media from plates
2. Add 1 mL MTT solution and incubate for 20-30 minutes
3. Suction MTT solution
4. Add 1 mL acidified IPA to the plates
5. Shake the plates for 15 minutes on VWR Vortexer to obtain homogeneous staining
6. Transfer 200 uL from each well to a corresponding well in 96-well plate
7. Read the plate in Spectramax 190 micro plate reader

After the assay was completed, the researcher observed cell death via the color change before reading the plate in the Spectromax 190. The data from the Spectromax was then converted into the final graphical output.

3.9 Data Output

After the Spectromax 190 generated the data from each experiment, the data was then entered into a pre-formulated Excel spreadsheet. The formulas converted the raw data into graphical output, measuring concentrations of cadmium oxide solutions against percent of control cells on the axes. The graphs depicted both cell densities and were generated the same way for all experiments and end points.

3.10 Summary

The three general steps for these experiments were conducted as identically as possible for each experiment. The method used was a pre-existing method carried out by the Air Force Research Laboratory in on-going studies. There have already been studies conducted in this laboratory using *in vitro* techniques, however, researchers still have a long way to go to reaching definitive conclusions. The experiments conducted for this thesis research will hopefully provide additional data for AFRL and contribute to their growing efforts in the *in vitro* field.

IV. Data Description and Analysis

4.1 Introduction

This chapter will present the data gathered during the execution of the methodology. The information resulting from each individual end point and dose range, from highest to lowest is presented first. For example, data will be analyzed first from the 24 hour experiments with the higher dose range, followed by the lower dose range at the same end point. The 12 hour and 6 hour data will be presented in the same fashion. A combination of all the data at each end point and dose range will also be presented and analyzed. Each graph will include confluent cell toxicity (1,000,000 cells/mL) and semiconfluent cell toxicity (500,000 cells/mL). A discussion of the data will follow this section. The chapter will conclude with a brief summary.

4.2 Cadmium Oxide Toxicity Data

The following graphs will be presented in the order in which the experiments were completed. The graphs depict the dose range at which the cells were exposed, versus the percent of the unexposed control cells remaining after exposure. From chapter three, each set of data is a result of the MTT assay.

4.2.1 Toxicity over 24 Hours

The following data reflect the effects of CdO exposure on rat hepatocyte cells over a period of 24 hours. The data was generated following completion of the MTT assay. The data is presented in order from the higher dose range (0.1 – 2.5) μg/mL to the lower dose range (0.001 – 1) μg /mL.

4.2.1.1 High Dose Range

24-hour high dose range results are shown in Figure 4-1. An anomalous positive activity is observed at 0.1 µg/mL for the confluent cells (compared to later experiments). For both cell densities, the toxicological affects seemed less adverse at 0.5 µg/mL than at 0.25 µg/mL. With the exception of these observations, a somewhat smooth dose-response curve can be seen for both cell densities. On average, the semiconfluent cells show more resistance to the toxicant than the confluent cells. For example, at 2.5 µg/mL, semiconfluent shows 3.0% of control cells while confluent shows 1.6% of control.

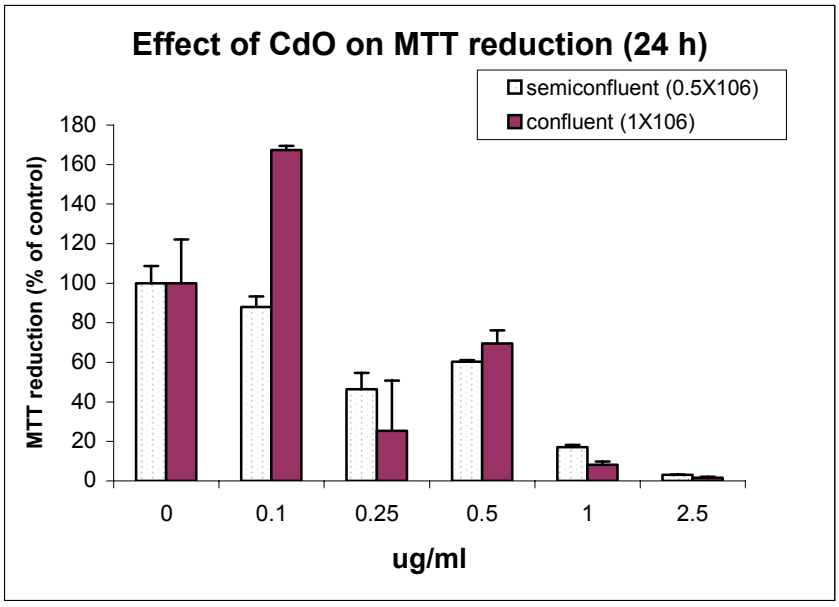

Figure 4-1. Effect of CdO, with doses ranging from 0.1 – 2.5 µg /mL, on hepatocytes following a 24 hour exposure ending on 26 Oct 04.

More 24-hour high dose results are shown in Figure 4-2. The confluent cells seem to show more resistance to the toxin than the semiconfluent cells. Once again, 0.5 µg/mL was observed to be less adverse than 0.25 µg/mL.

With the exception of this observation, a smoother dose-response curve is observed for this experiment than for the previous data.

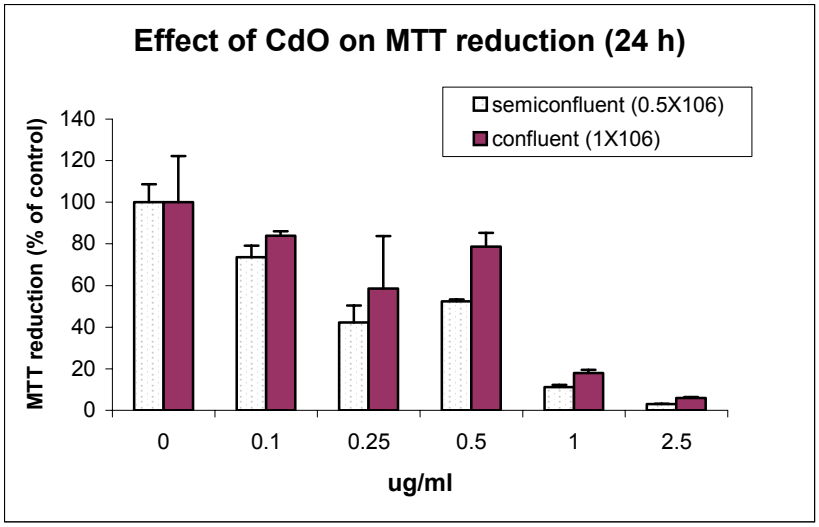

Figure 4-2. Effect of CdO, with doses ranging from 0.1 – 2.5 µg /mL, on hepatocytes following a 24 hour exposure ending on 28 Oct 04.

For the third experiment, the semiconfluent cells show more resistance to the toxin than the confluent cells (Figure 4-3). The same observations at 0.5 µg/mL presented in the previous sets of data is once again made here. With the exception of that observation, a smooth dose-response curve is observed.

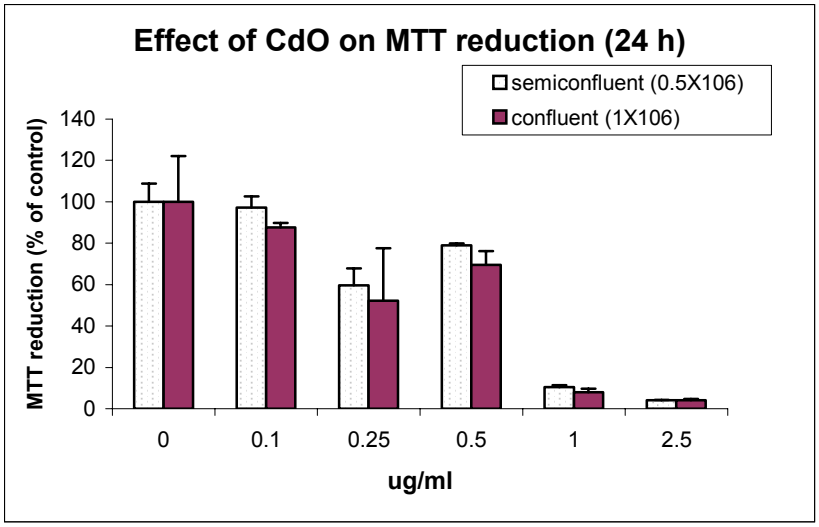

Figure 4-3. Effect of CdO, with doses ranging from 0.1 – 2.5 µg /mL, on hepatocytes following a 24 hour exposure ending on 9 Nov 04.

4.2.1.2 Low Dose Range

For this experiment, 24-hour low dose experimental results are shown in Figure 4-4. A large drop in resistance is seen in both cell densities between 0.001 ug/mL and 0.01 µg/mL. At 0.001 µg/mL, the cells seem less adversely affected than at 0.01 µg/mL. On average, the confluent cells show more resistance to the toxicant than the semiconfluent.

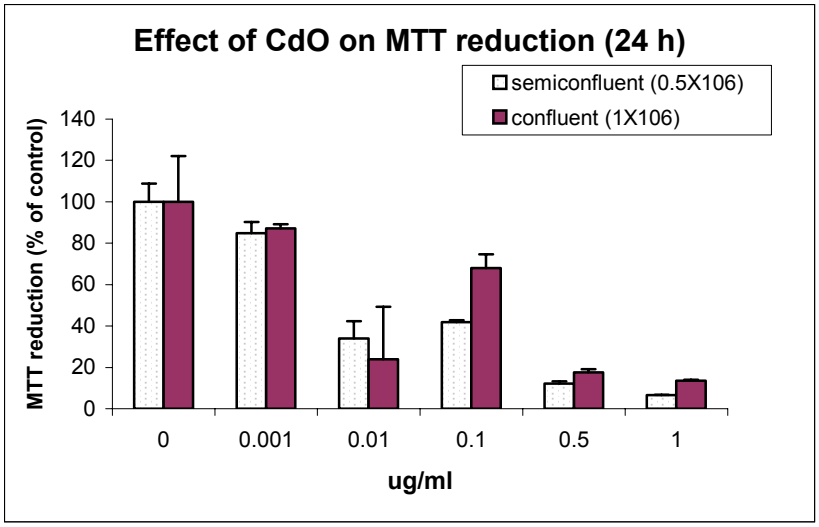

Figure 4-4. Effect of CdO, with doses ranging from 0.001 – 1 µg /mL, on hepatocytes following a 24 hour exposure ending on 16 Dec 04.

4.2.1.3 Combination of 24 Hour Experiments

Figure 4-5 shows the combined data for 24-hour experiments. A dramatic reaction of both cell densities at the duplicate dose of 1.0 µg/mL CdO, and 2.5 µg/mL (compared to the other doses) was observed. The experiments revealed the most consistency with these doses than with any of the others. There appears to be an anomaly at 0.1 µg/mL, in that the confluent cells showed a slight positive activity. Also, the effects at 0.01 µg/mL are more adverse than at many of the major concentrations. As observed in all of the previous data, the dose of 0.5 µg/mL is less toxic to the cells than 0.25 µg/mL.

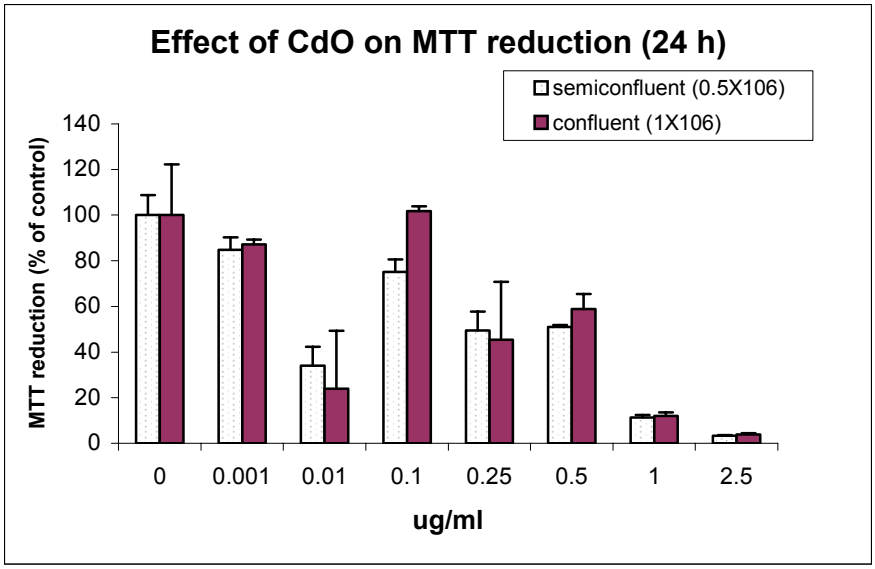

Figure 4-5. Combination of all 24 hour experiments conducted on CdO exposure, with doses ranging from 0.001 – 2.5 µg /mL.

4.2.2 Toxicity over 12 Hours

The following data reflect the effects of CdO exposure on rat hepatocyte cells over a period of 12 hours. The data was generated following completion of the MTT assay. The data is presented in order from the higher dose range (0.1 – 2.5) µg/mL to the lower dose range (0.001 – 1) µg/mL.

4.2.2.1 High Dose Range

The 12-hour exposure data from the first high dose experiment is shown in Figure 4-6. As in the 24 hour experiments, the cells show more resistance to 0.5 µg/mL CdO than they do at 0.25 µg/mL. Otherwise, a typical dose-response curve is observed. On average, the confluent cells show more resistance to the toxin than the semiconfluent cells.

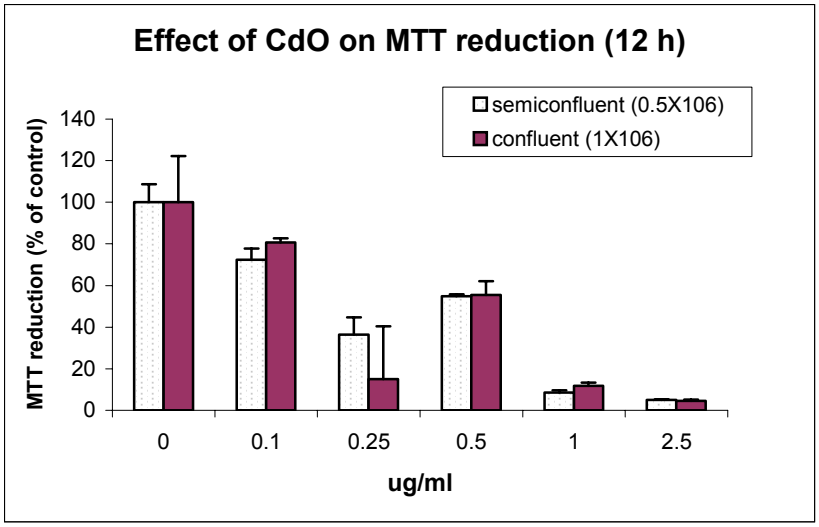

Figure 4-6. Effect of CdO, with doses ranging from 0.1 – 2.5 µg /mL, on hepatocytes following a 12 hour exposure ending on 15 Dec 04.

The results of a second 12-hour high dose experiment is shown in Figure 4-7. The confluent cells now show more resistance to the dose of 0.25 µg/mL than at 0.1 µg/mL. For the semiconfluent cells, the effect observed at 0.5 µg/mL shown in all of the previous experiments is also shown here, in that the cells are more resistant at 0.5 µg/mL than at 0.25 µg/mL. On average, the confluent cells show more resistance to the CdO than the semiconfluent.

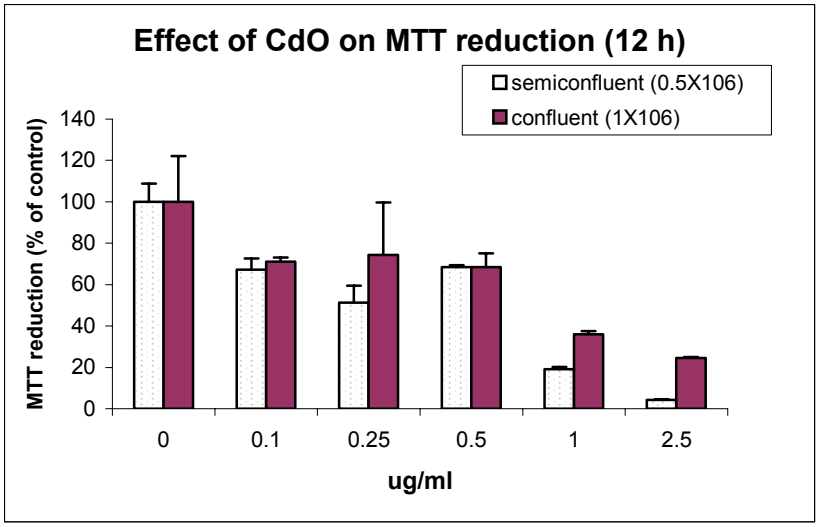

Figure 4-7. Effect of CdO, with doses ranging from 0.1 – 2.5 µg /mL, on hepatocytes following a 12 hour exposure ending on 17 Dec 04.

Another 12-hour low dose experiment is shown in Figure 4-8. As in the previous one, the confluent cells once again show more resistance at 0.25 µg/mL than at 0.1 µg/mL. The confluent cells also show more resistance at 0.5 µg/mL than at 0.1 µg/mL, but less resistance at 0.5 µg/mL than at 0.25 µg/mL. For the semiconfluent cells, the previous observations at 0.5 µg/mL holds here. On average, the confluent cells show more resistance to CdO than the semiconfluent.

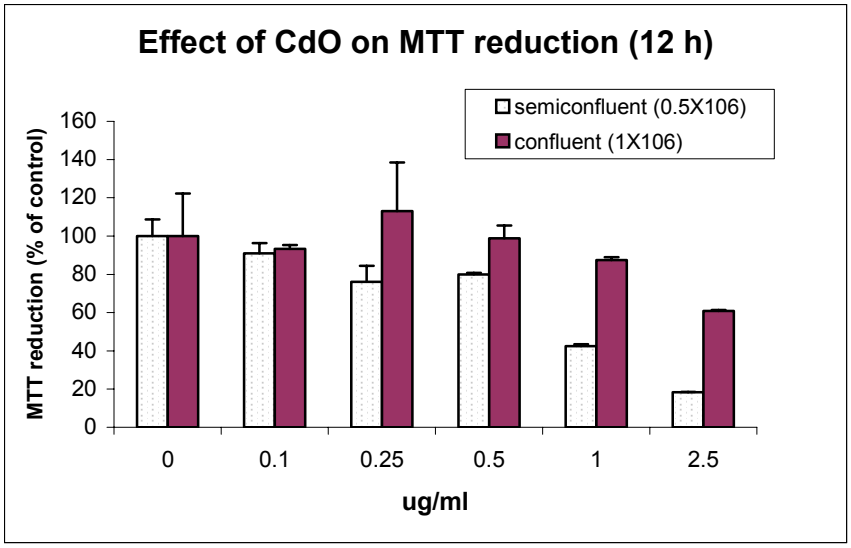

Figure 4-8. Effect of CdO, with doses ranging from 0.1 – 2.5 µg /mL, on hepatocytes following a 12 hour exposure ending on 4 Jan 05.

4.2.2.3 Low Dose Range

Figure 4-9 shows the results of a 12-hour low dose experiment. The confluent cells show more resistance at 0.1 µg/mL than at 0.01 and 0.001 µg/mL. For the semiconfluent cells, the only observation disrupting the dose-response curve is more resistance at 0.1 µg/mL than at 0.001 and 0.01 µg/mL. On average, the semiconfluent cells show more resistance than confluent cells.

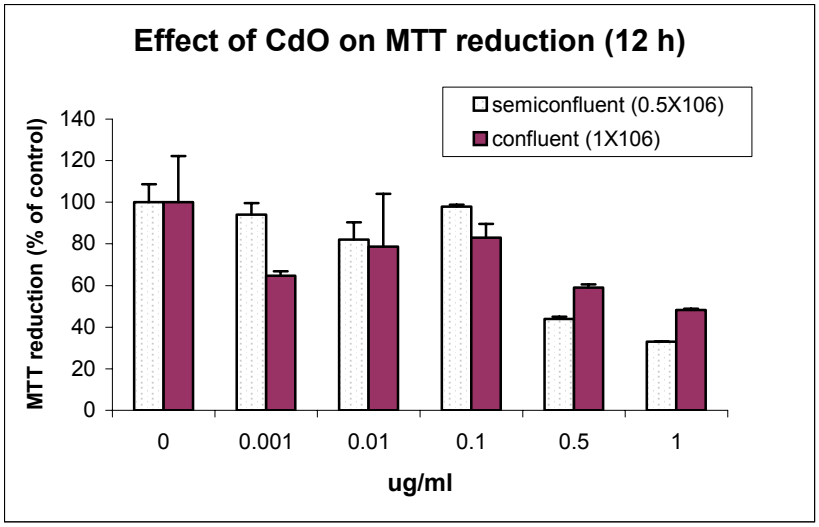

Figure 4-9. Effect of CdO, with doses ranging from 0.001 – 1 µg /mL, on hepatocytes following a 12 hour exposure ending on 17 Dec 04.

Figure 4-10 shows the second 12-hour experiment with a low dose. The confluent cells show more resistance at 0.1 µg/mL than at 0.001 and 0.01 µg/mL. The same observation is made for 1 µg/mL versus 0.5 µg/mL. For the semiconfluent cells, the most resistance to the toxin is observed at 0.01 µg/mL. In addition, 0.1 µg/mL is seen to be slightly less toxic than 0.001 µg/mL. On average, the confluent cells show more overall resistance than the semiconfluent cells.

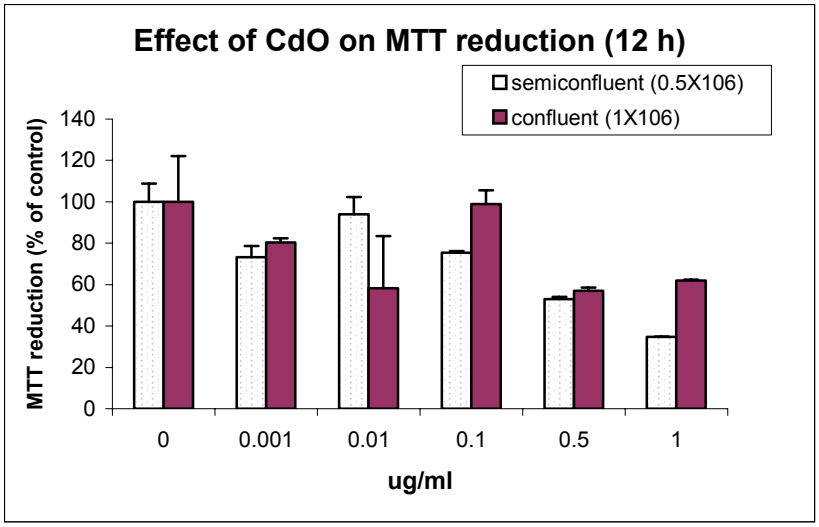

Figure 4-10. Effect of CdO, with doses ranging from 0.001 – 1 µg /mL, on hepatocytes following a 12 hour exposure ending on 12 Jan 05.

Figure 4-11 shows the results of the final 12-hour low dose exposure. The confluent cells show less resistance to CdO at 0.001 µg/mL than at 0.01 and 0.1 µg/mL. However, the semiconfluent cells show a steady decline in resistance as the doses get higher. On average, the confluent cells show more overall resistance than the semiconfluent cells.

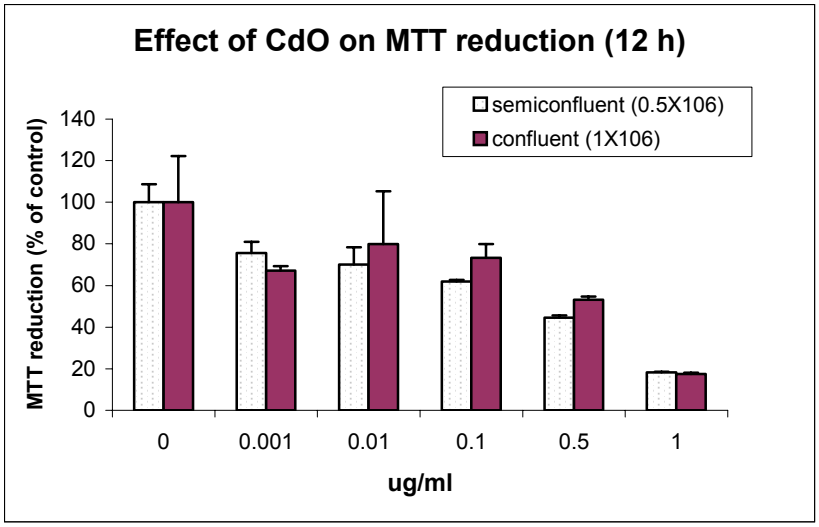

Figure 4-11. Effect of CdO, with doses ranging from 0.001 – 1 µg /mL, on hepatocytes following a 12 hour exposure ending on 17 Jan 05.

4.2.2.3 Combination of 12 Hour Experiments

Figure 4-12 shows the combined data for 12-hour experiments. Here, the confluent cells show less resistance at 0.001 µg/mL than at 0.01 – 0.5 µg/mL. Also, 0.5 µg/mL appears to be less toxic than 0.25 µg/mL. For the semiconfluent cells, 0.001 µg/mL appears to be more toxic than 0.01 and 0.1 µg/mL. Also, 2.5 µg/mL is observed to have less adverse affects for this cell density than at 1 µg/mL.

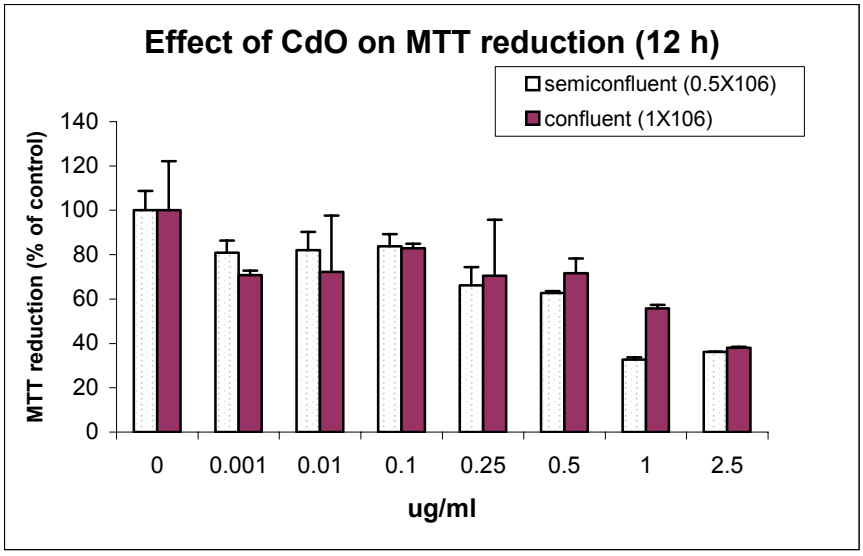

Figure 4-12. Combination of all 12 hour experiments conducted on CdO exposure, with doses ranging from 0.001 – 2.5 µg /mL.

4.2.3 Toxicity over 6 Hours

The following data reflect the effects of CdO exposure on rat hepatocyte cells over a period of 6 hours. The data was generated following completion of the MTT assay. The data is presented in order from the higher dose range (0.1 – 2.5) µg/mL to the lower dose range (0.001 – 1) µg/mL.

4.2.3.1 High Dose Range

Figure 4-13 shows results from a 6-hour exposure with the high dose. The confluent cells show more resistance at 0.5 µg/mL than at 0.25 µg/mL. Otherwise, a smooth dose-response curve is present. For the semiconfluent cells, 0.25 µg/mL CdO appears to have a slightly less adverse affect than 0.1

µg/mL, otherwise, a smooth dose-response curve is present. On average, the semiconfluent cells show more resistance than the confluent cells.

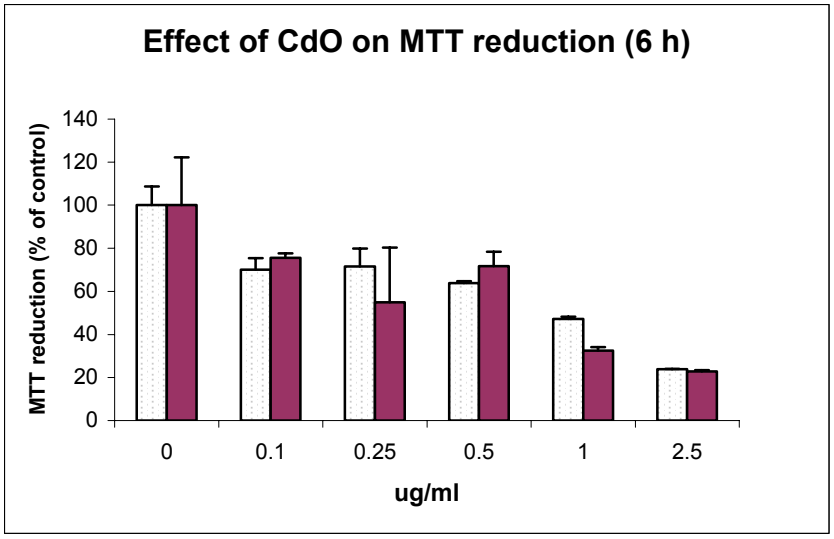

Figure 4-13. Effect of CdO, with doses ranging from 0.1 – 2.5 µg /mL, on hepatocytes following a 6 hour exposure ending on 24 Jan 05.

Figure 4-14 shows the results of a second 6-hour high dose experiment. The same observation made at 0.5 µg/mL in the previous experiment is also made here for both cell densities. This is the only observation disrupting the dose-response curve. On average, the semiconfluent cells show more resistance to the toxicant than the confluent cells.

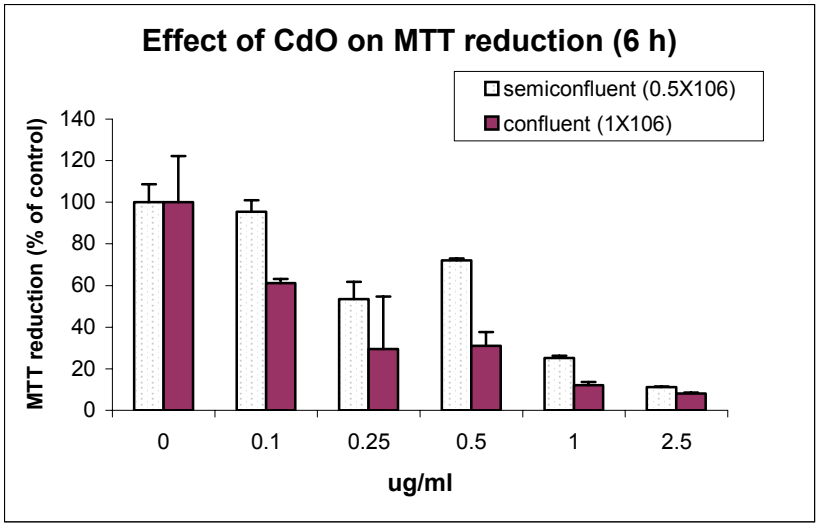

Figure 4-14. Effect of CdO, with doses ranging from 0.1 – 2.5 µg /mL, on hepatocytes following a 12 hour exposure ending on 26 Jan 05.

4.2.3.2 Low Dose Range

Figure 4-15 shows the results of a 6-hour low dose experiment. The confluent cells showed less resistance at 0.01 µg/mL than at 0.001 and 0.1 µg/mL. The semiconfluent cells show some positive activity at 0.01 and 0.1 µg/mL compared to the control cells. On average, the semiconfluent cells show more resistance than the confluent cells.

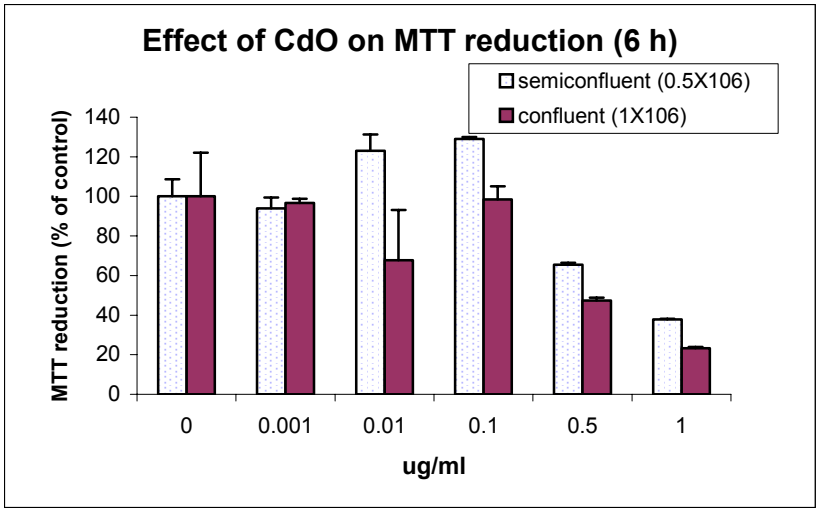

Figure 4-15. Effect of CdO, with doses ranging from 0.001 – 1 µg /mL, on hepatocytes following a 6 hour exposure ending on 21 Jan 05.

Figure 4-16 shows the results of a second 6-hour low dose experiment. The confluent cells show positive activity for 0.001 and 0.1 µg/mL, with more growth at 0.1 µg/mL. However, 0.01 µg/mL shows a more adverse affect than either of the aforementioned doses. The semiconfluent cells show a rise in resistance to the CdO from 0.001 µg/mL to 0.1 µg/mL.

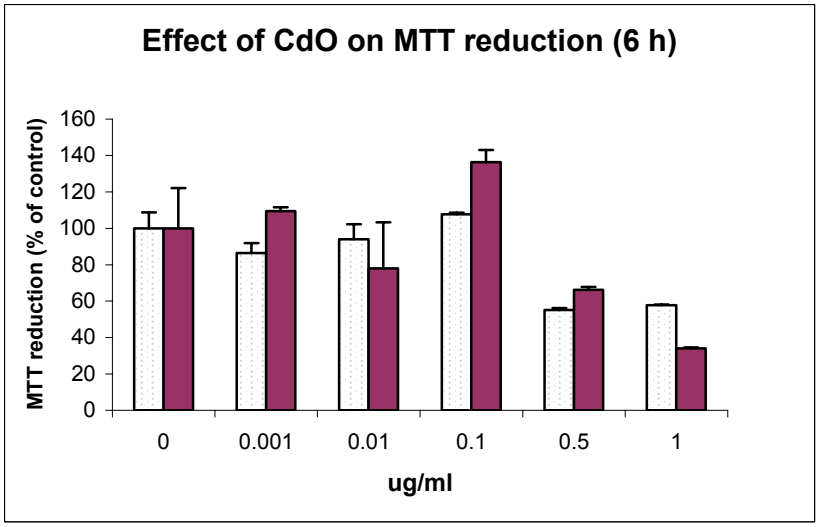

Figure 4-16. Effect of CdO, with doses ranging from 0.001 – 1 µg /mL, on hepatocytes following a 6 hour exposure ending on 24 Jan 05.

Figure 4-17 shows the final 6-hour exposure with the low dose range. The confluent cells show a rise in resistance to the CdO from 0.001 – 0.1 µg/mL with positive activity observed at 0.1 µg/mL. The semiconfluent cells show slightly more resistance at 0.01 µg/mL than at 0.001 µg/mL. The same affect is observed at 0.1 µg/mL versus 0.001 µg/mL. On average, the confluent cells show more overall resistance to the toxin than the semiconfluent cells.

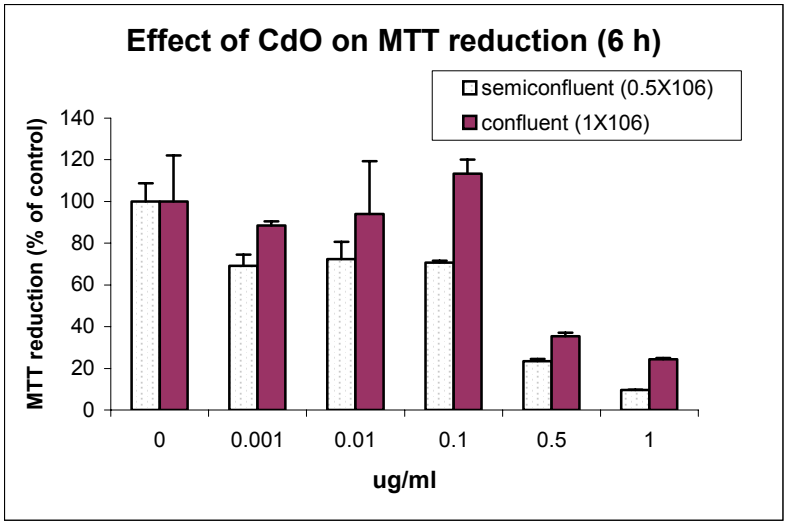

Figure 4-17. Effect of CdO, with doses ranging from 0.001 – 1 µg /mL, on hepatocytes following a 6 hour exposure ending on 26 Jan 05.

4.2.3.2 Combination of 6 Hour Experiments

Figure 4-18 shows the combined data for 6-hour experiments. Here we see the confluent cells have almost no reaction to 0.001 and 0.1 µg/mL CdO. However, a decline in resistance is observed at 0.01 µg/mL. Also, 0.5 µg/mL is observed to have a less adverse affect than 0.25 µg/mL. The semiconfluent cells show less resistance at 0.01 µg/mL than at 0.001 and 0.1 µg/mL. Also, 0.1 µg/mL shows a less adverse affect on this cell density than 0.001 µg/mL.

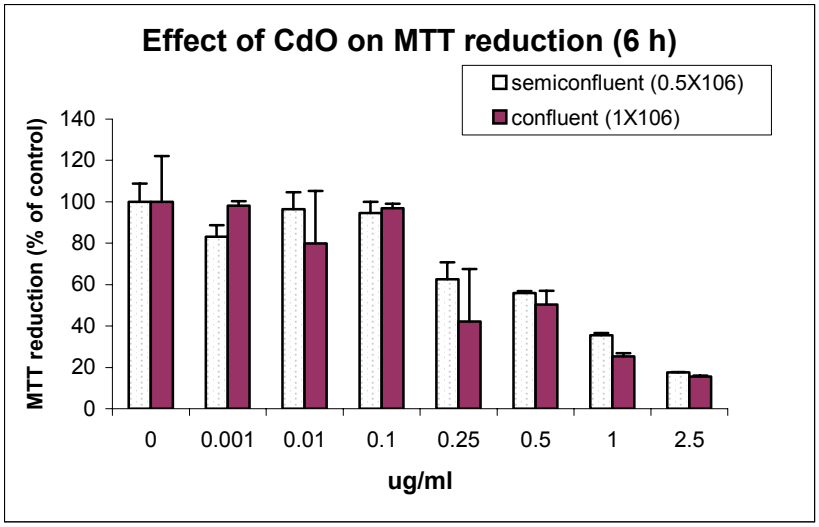

Figure 4-18. Combination of all 6 hour experiments conducted on CdO exposure, with doses ranging from 0.001 – 2.5 µg /mL.

4.3 Discussion

Figures 4-5, 4-12, and 4-18 depict the three combined sets of data (24, 12, 6 hours) previously shown in the chapter, and are presented again here, together, for comparison as Figures 4-19, 4-20, and 4-21.

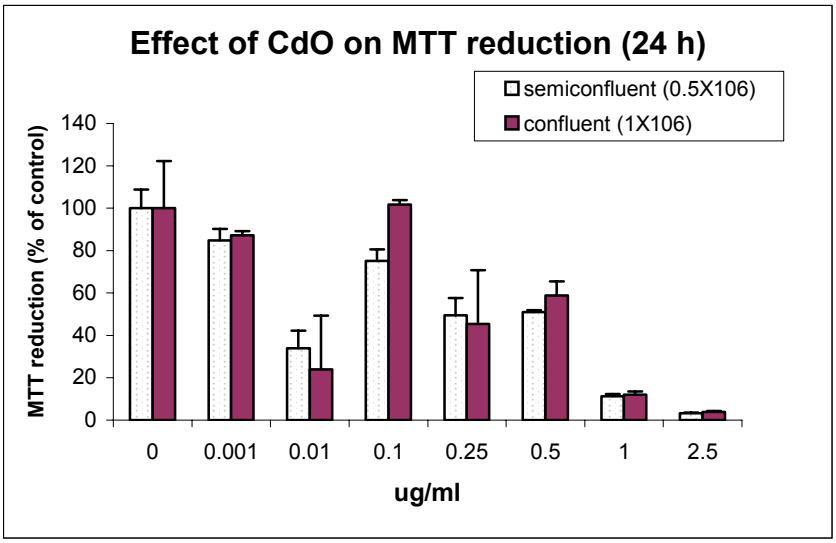

Figure 4-19. Combination of all 24 hour experiments conducted on CdO exposure, with doses ranging from 0.001 – 2.5 µg /mL.

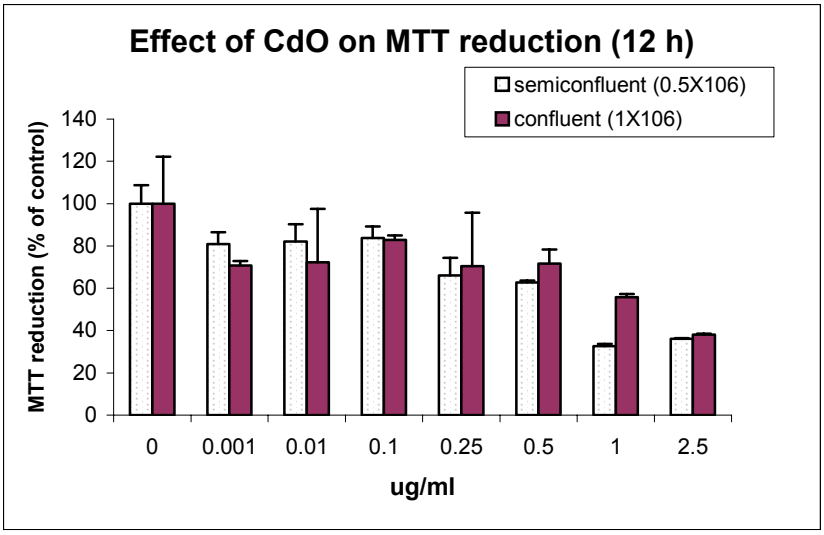

Figure 4-20. Combination of all 12 hour experiments conducted on CdO exposure, with doses ranging from 0.001 – 2.5 µg /mL.

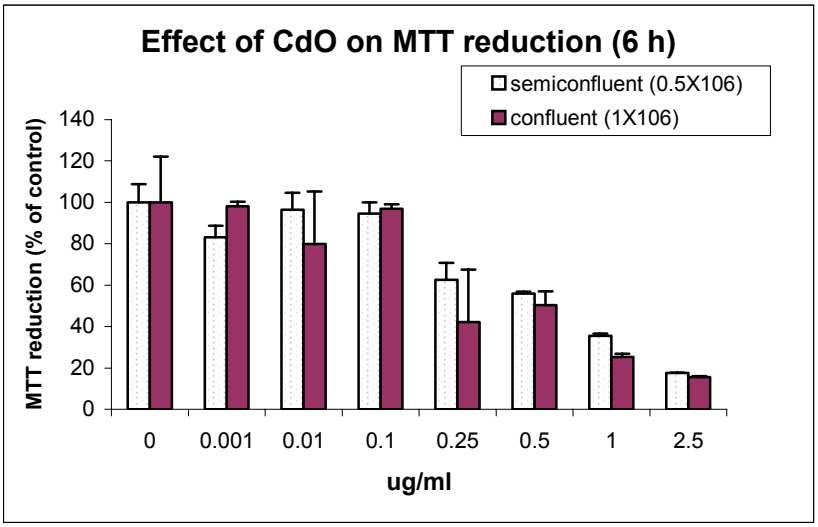

Figure 4-21. Combination of all 6 hour experiments conducted on CdO exposure, with doses ranging from 0.001 – 2.5 µg /mL.

Tables 4-1 and 4-2 show the combination of all results from each experiment (for confluent cells and semiconfluent cells, respectively) with the numerical percent of control cells.

Table 4-1. Combination of all experiments for confluent cells with numerical percent of control cells.

	Percent of Control Cells		
Dose (µg/mL CdO)	**6-hour**	**12-hour**	**24-hour**
0	100	100	100
0.001	98.19	70.77	87.14
0.01	79.92	72.27	24.02
0.1	96.96	82.92	101.75
0.25	42.21	70.47	45.40
0.5	50.38	71.62	58.86
1.0	25.30	55.78	12.00
2.5	15.56	38.03	3.94

Table 4-2. Combination of all experiments for semi-confluent cells with numerical percent of control cells.

	Percent of Control Cells		
Dose (µg/mL CdO)	**6-hour**	**12-hour**	**24-hour**
0	100	100	100
0.001	83.15	80.95	84.82
0.01	96.41	82.03	34.02
0.1	94.56	83.80	75.16
0.25	62.56	66.12	49.41
0.5	56.01	62.69	50.95
1.0	35.54	32.73	11.36
2.5	17.57	36.15	3.38

The first observation to make would be that the CdO was, on average, more toxic to the cells after 24 hours than at any other end point. However, comparing the 12 hour and 6 hour data, the difference is not so obvious. While the lower doses (between 0.001 and 0.1 µg/mL) show less toxicity at 6 hours than at 12 hours, as expected, the higher doses show the reverse. For example, in comparing the two end points at 2.5 µg/mL, it would seem the cells show a more adverse reaction at the end of 6 hours than at 12 hours. The same observation is made for 0.25 – 1 µg/mL. Also, out of the three combined sets of data, it appears the 6 -hour experiments show the smoothest dose-response curve. In other words,

as the doses get higher, the cells generally experience more fatalities, with a couple of exceptions at the lower doses. On average, for the entire study, the confluent cells showed more resistance to the cadmium oxide than the semiconfluent. However, the difference in resistance capability was very close. At no time during the entire experiment was a 100% fatality of the cells observed, though in some cases, the percent of control cells was less than 2%.

Approximately twice as many experiments as what is shown in this chapter were carried out or attempted to be carried out. The other half of the experiments not shown were excluded either because of data that was obviously too much of an outlier, or various technical problems in the lab prevented the experiments from reaching completion.

Comparing the results of this study to other *in vitro* studies is somewhat difficult. While there have been such studies performed and published, the field is still young enough to make finding suitable literature for comparison difficult. The literature cited in chapter two revealed studies accomplished for the express purpose of identifying hazard characteristics of various chemicals. However, these studies focused on different cell lines, other than hepatocytes, involved different chemicals and endpoints, and were not conducted in the same manner or with the same assays as was used in this thesis study.

In previous sections of this chapter, observations were made in each of the individual sets of data, which would require explanations. That is, explanations as to why the data may not have turned out the way one would expect, mainly, the

numerous variations and inconsistencies observed between individual experiments. Below are some arguments to help explain these observations.

1. The spectrograph (described in chapter three) template may not have been set correctly, thus leading the researcher to paste data from the template to the Excel program incorrectly. For example, on many of the graphs, 0.5 ug/mL did not seem as toxic as 0.25 ug/mL. This could be a possible explanation.
2. During almost every stage of an experiment, pipetting techniques were employed. Some pipetting is accomplished with an electric pipetter, some with manual pipettes. Consequently, pipetting could vary slightly from one experiment to the next.
3. During cell culture and trypsonization, the cells were observed to grow often at dramatically different rates from day to day, including growth after seeding in the exposure plates. For example, after seeding in the 6-well plates, cells could have grown faster over a 24 hour period on one day, than on the next day. (Seeding takes place 24 hours prior to exposure.) This being the case, it's possible there was a slight difference in the cell count on different days during exposure, causing slightly different reactions.
4. The method for conducting the MTT assay was described in chapter three. During the course of the research, the assay would sometimes react more slowly than other times, thus varying the length of time the cells were incubated with the MTT. For example, some cells

incubated twice as long as usual but the MTT had still not had the usual effect.

5. On a daily basis, bottles of media, flasks with cells, CdO solutions, and plates with cells would be carried and handled by hand in a nonsterile environment for several seconds (through the lab) before and after being placed under the hood. In this situation, certain bacteria or germs could have passed to the media or cells. Bacteria contamination is possible during exposure as well. While there is no strong evidence to support contamination in the data, the possibility should not be ruled out.

4.4 Summary

The data description and analysis chapter has discussed the results of hepatocyte cell exposure to cadmium oxide using *in vitro* experimentation. The study attempted to show the toxicity of CdO at different doses and times, as well as the affect of the toxicity on two different cell densities. First, the affect of CdO on the cells over a period of 24 hours was analyzed, followed by 12 hours, and 6 hours. The chapter concludes with statements of possible reasons for variations in the data.

V. Conclusions and Recommendations

5.1 Overview

In vitro experimentation has been used to determine toxicity and environmental regulations for a number of chemicals. Several cell lines have been exposed to known or suspected toxins, some of these cells from animals, some human. In the process of studying the effects of toxins on mammalian cells, the research ultimately involved extrapolating the data to the human physiological system. That was in fact, one important reason for the research conducted in this thesis. Presenting a range of doses of a suspected human carcinogen, in this case, cadmium oxide, and the affects of exposure on rat liver cells, better equips decision makers to draw conclusions about acceptable exposure levels.

This research effort provides data for a small part of this on-going field of study. It will provide a basis for comparison in future experiments carried out by the Air Force Research Laboratory. Long after this thesis is accomplished, there will continue to be studies undertaken on cadmium oxide toxicity and its effects on the human body.

Relating or comparing the results of this research to the limited sources of literature proved difficult. Although there was a significant amount of data collected, given the amount of time available, there were some obstacles to overcome in the lab for a first-time biological and toxicological researcher.

Altogether, this work is a first step in understanding the effects of cadmium oxide on rat liver cells. As with all research efforts, the results of this study are prone to

their own uncertainty. The topic is certainly not exhaustive but should provide the reader with a feel for the studies limitations in the previous chapters.

5.2 Conclusions

The conclusions discussed will answer the following research questions originally stated in the first chapter, while the first research question was answered in the literature review:

1. What is the cellular toxicity of cadmium oxide particles?
2. What is the effect of cell density on cadmium oxide toxicity?

5.2.1 Comparing Experiments

The most obvious conclusion to draw from this study is that cadmium oxide is more toxic to liver cells after 24 hours than after 12 or 6 hours. At some of the points during the experiments, a positive growth was observed at certain doses. In other words, the cells would actually grow beyond the density of the control cells after being exposed. This observation was made most often during the 6 hour experiments, at a dose of 0.1 ug/mL. However, there were other isolated incidents of this phenomenon during the 12 hour and 24 hour experiments, at different doses. Some of these cases may be a direct result of the possibility that a small dose of cadmium oxide actually does provide a positive growth environment over a limited period of time. Another possibility, however, would be the fact that no two experiments were carried out in exactly the same manner, due to technical complications in the lab and/or lack of experience on the part of the researcher. For example, preparing and pipetting the cadmium oxide solutions, and seeding the exposure plates with proper cell densities may have differed from one experiment to the next. This would result in

the differences observed in the data. Unfortunately, the time required to duplicate enough experiments to reach a solid conclusion on this point, was outside the scope of this thesis.

5.2.2 Comparing Effect on Cell Density

Another point to make after observing the data is that the confluent cells (1,000,000 cells/mL) were more resistant to the CdO than the semiconfluent cells (500,000 cells/mL) because there existed more particles per cell in the semiconfluent situation. However, the difference between the two resistance capabilities was limited. At first glance, it would seem the two cell densities had almost equal resistance, but not at the same doses. The explanation for this may become more apparent if the researcher was able to repeat the experiments. In fact, through repetitive experiments, a very clear average of a cell density's reaction to the toxin may easily be seen. The evidence in the data presented does not strongly support the conclusion that CdO does not affect confluent cells any differently than semiconfluent cells. However, since repetition was not available, drawing conclusions as to the affects of CdO on cell densities may prove to be yet another difficulty within this research.

5.3 Recommendations

5.3.1 Research Time

From the beginning of this research, it was apparent that the greatest challenge would be accomplishing the objectives of the research within the time allotted. Since the researcher had no experience in conducting experiments of this nature, considerable time was needed for training and preparation. In addition, the Air Force Research Laboratory requires various mandatory training sessions. At least 12 months

of lab work, after the training period, would be ideal for achieving these experiments, and also provide extra time for replicate studies should the need arise.

5.3.2 Hepatocytes

Hepatocyte cells seem to grow and culture very well. There were no major or irreparable incidents in culturing the hepatocyte cell line. Studying the use of other cells was outside the scope of this thesis. However, Dr. Hussain at the Air Force Research Laboratory confirms that hepatocytes are among the easiest cell lines to culture. Sometimes, for example, macrophages (lung cells) may not grow as fast as hepatocytes. Therefore, a researcher with either a short time frame or a lack of experience would find that hepatocytes may be the cell line of choice.

5.3.3 Number of Experiments

At the completion of the study, some of the data was excluded, if it seemed to present too many outliers. For this reason, conducting more experiments than what the objective calls for would be recommended. Perhaps two experiments for every one would be suitable. This should be taken into consideration when planning the timeframe.

5.3.4 Other Related Studies

There is a long road ahead for studies on cadmium oxide and other forms of cadmium. Certainly, additional studies on the affects of the chemical on hepatocytes are called for. However, there are many other areas of the human body, which a toxic compound can affect. A useful study for professional researchers in general would be comparing the affects of cadmium oxide, using *in vitro* methods, on different cells,

and compare the results. This would go a long way in determining exposure levels to airborne particles versus exposure from soil or food.

In addition, there are many other doses and end points to explore in cadmium oxide studies. For example, long- term affects of low doses was not studied in this thesis. Other cadmium compounds, different assays, and lower cell densities should also be studied. Lastly, a positive control should be used in future experiments. For example, a toxicant with a known adverse effect should be used in the same experiment, to compare the toxicity.

5.4 Summary

While the experiments conducted in this study don't lead to definitive answers, the results certainly offer a starting point and/or comparisons for future research involving cadmium oxide toxicity. Ultimately, this research will contribute to the overall efforts to determine safer and more accurate exposure levels of cadmium oxide for the general population. Though further research is needed in the general field of *in vitro* toxicology, the method is currently gaining acceptance as a credible tool for helping scientists determine the toxicity of a wide range of chemicals.

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From – To)*
21-03-2005	**Master's Thesis**	Jun 2004 – Feb 2005

4. TITLE AND SUBTITLE

In Vitro Toxicity of Cadmium Oxide Particles in BRL 3A Rat Liver Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Shelley, Janice, M., Captain, USAF

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

AFIT/GES/ENV/05M-04

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10. SPONSOR/MONITOR'S ACRONYM(S)
AFRL/HEPB

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

For the last decade or so, *in vitro* toxicology has been studied as a method for determining human toxicity, as well as hazard identification and characterization. One purpose of *in vitro* studies is to determine the possibility of developing systems in which the toxicity of chemicals can be quantified, without heavy reliance on animal experimentation. Specific data pertaining to cadmium toxicity in humans through use of *in vitro* methods and models is sparse, at best. There is a need to complete more studies and collect more data to study particular chemicals and their effects and to complete proper evaluation and comparison to *in vitro* studies on human cells. The need for such data is the overall reason for this thesis research and experimentation.

Toxicology *in vitro* refers to a method of exposing cell tissue directly to the chemical compound under study. In this research, rat liver cells, or hepatocytes, were cultured, exposed to cadmium oxide particles, and analyzed with an MTT assay. Experiments were carried out with doses ranging from 0.001 μg/mL to 2.5 μg/mL for 6, 12, and 24 hours. Two cell densities were also studied for each experiment, 1,000,000 cells/mL and 500,000 cells/mL, in order to ascertain the effects of toxicity on cell density. The research led to the conclusion 1,000,000 cells/mL is more resistant to the toxicant than 500,000 cells/mL and the toxicant is most toxic to the cells after 24 hours.

15. SUBJECT TERMS
In vitro, cadmium, cell culture, hepatocytes

16. SECURITY CLASSIFICATION OF: U			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
REPORT	**ABSTRACT**	**c. THIS PAGE**			Ellen England, Lt Col, USAF (ENV)
U	**U**	**U**	**UU**	76	**19b. TELEPHONE NUMBER** *(Include area code)* (937) 255-3636, ext 4711; e-mail: ellen.england@afit.edu

Standard Form 298 (Rev: 8-98)
Prescribed by ANSI Std. Z39-18