Effects of Polycyclic Aromatic Hydrocarbons, Metals and Polycyclic Aromatic Hydrocarbon/Metal Mixtures on Rat Corpus Luteal Cells and Placental Cell Line, JEG-3

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that can be modified to oxygenated PAH (oxyPAHs) derivatives. It is well known that oxyPAHs tend to be much more reactive than their parent compounds. Toxicity can be attributed to direct interaction with target molecules or generation of reactive oxygen species (ROS). Metals are another class of contaminant found ubiquitously throughout the environment. Some metals are toxic at levels below the 1:1 ratio predicted by the biotic ligand model and are thought to manifest toxicity through ROS generation. Often metals and PAHs occur as co-contaminants in industrialized environments, yet little is known about their potential co-toxicity or mechanisms of action in mammalian reproductive function.

Previously, we described that a PAH, 9, 10-phenanthrenequinone (PHEQ), inhibited LH-stimulated progesterone secretion in dispersed rat corpus luteal (CL) cells (Nykamp et al., 2001). Viability was decreased in CL cells exposed to PHEQ and 1,2-dihydroxy-anthraquinone (1,2-dhATQ), but not their parent compounds phenanthrene (PHE) or anthracene (ANT). Similarly, LH-stimulated progesterone production in CL cells was inhibited by PHEQ and 1,2-dhATQ, but not PHE. Further investigation revealed that PHEQ, but not PHE, ANT nor 1,2-dhATQ generated ROS in CL cells. Viability experiments were repeated using the choriocarcinoma cell line JEG-3 with similar results.

Various metals were assessed for their toxicity to both CL and JEG-3 cells. The endpoints used to measure viability were metabolic activity and membrane integrity. In general, metabolic activity was a more sensitive indicator of toxicity than membrane integrity. The order of toxicity for metals in CL cells was $Hg^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+}$ for metabolic activity and $Hg^{2+} \approx Zn^{2+} > Cd^{2+} > Cu^{2+} > Ni^{2+}$ for membrane integrity. Only Hg^{2+} and Cu^{2+} were tested in JEG-3 cells. While Cu^{2+} was non-toxic, EC50s for Hg^{2+} metabolic activity and membrane integrity were 20 μM and 23 μM, respectively.

Experiments were designed to study the mixtures of metals and PAHs on viability, ROS production, and LH-stimulated progesterone production in CL cells. Mixtures of each metal with either PHEQ or 1,2-dhATQ were incubated with CL cells and their effect on metabolic activity and membrane integrity assessed. Generally, most metal/oxyPAH mixtures displayed only additive toxicity. However, mixtures of Cu^{2+} and PHEQ showed synergistic toxicity to both metabolic activity and membrane integrity. Mixture studies in JEG-3 cells used only combinations of Cu^{2+} or Hg^{2+} with PHEQ or 1,2-dhATQ. Similar results to metabolic activity and membrane integrity in CL cells were observed. Mixtures of Cu^{2+} and PHEQ or 1,2-dhATQ were tested in CL cells for their effect on LH-stimulated progesterone secretion and ROS production. Additive effects were observed in both LH-stimulated progesterone secretion and ROS production for Cu^{2+}/1,2-dhATQ mixtures while synergistic effects for both parameters were seen with Cu^{2+}/PHEQ.

Efforts to determine the site of action for mixtures of Cu^{2+}/PHEQ involved adding the cholesterol analogue, 22-OH cholesterol (22-OHC) to CL cells in the absence of LH. Cytochrome P450 side-chain cleavage (CYP450scc) enzyme operates constitutively and the addition of 22-OHC to CL cells resulting in a 5-fold increase in progesterone

production without added LH. Kinetic assays with 22-OHC show that while progesterone secretion was inhibited with PHEQ addition alone, a further significant reduction with both Cu^{2+} and PHEQ was not observed. The use of forskolin, an activator of adenylate cyclase, did not show any significant enhancement of progesterone secretion with the addition of Cu^{2+}/PHEQ compared to PHEQ alone. The potential targets of Cu^{2+}/PHEQ mixture include any step in the steroidogenic cascade from activation of protein kinase A onward with the proteins of the mitochondria, cytochrome P450 side chain cleavage enzyme and steroidogenic acute regulatory protein, being the most likely.

Differential display polymerase chain reaction (ddPCR) was a molecular approach taken to determine the effect of PHEQ on JEG-3 gene expression. The genes whose expression appeared to be up-regulated with PHEQ exposure were serine protease inhibitor, Alu repeat sequence, heterogeneous ribonuclear ribonucleoprotein C (hnRNP C), eukaryotic translation initiation factor 3 (eIF3), nucleoporin-like protein, eukaryotic translation elongation factor 1α1 (eEF1 α 1), autophagy-linked FYVE domain (Alfy), spectrin, and proteasome. Apparent down-regulated genes in JEG-3 cells after PHEQ exposure included poly(ADP-ribose) polymerase 10 (PARP10), polyglutamine binding protein-1 (PQBP-1), heterogeneous ribonuclear ribonucleoprotein C (hnRNP C), eukaryotic translation initiation factor 5A (eIF5A), and keratin.

In both cell types, oxyPAHs were more toxic than their parent compounds. Metals showed greater toxicity to metabolic activity than to membrane integrity. Of the combinations tested, only PHEQ and Cu^{2+} exhibited synergistic toxicity. ROS generation was the likely mechanism behind PHEQ/Cu^{2+} toxicity. Both cell types used represent critical roles in human reproductive health. The proper production of progesterone, a critical hormone for the maintenance of pregnancy in mammals, represents a unique endpoint for the assessment of toxicity. These results illustrate the need to study modified oxyPAHs, metals and metal/oxyPAH mixtures for their potential impact on human reproductive health.

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My family, especially "the mothers", has provided more support than I can say, even when no one really knew what I was doing. My husband, Mark, has been more than amazing. He has patiently listened to my wild ideas for experiments, my rantings of what went wrong in the lab and has been my most supportive critic/cheerleader. This would have never happened without you and I love you for everything. I can't forget my son, Ian, who is the joy of my life.

Finally, I am thankful for the opportunity to have known Jack Carlson. He had faith in me and gave me a chance to prove that I was meant to do this. He also showed me the wonderment and joy that can be had in doing what you love. His mentorship and friendship were wonderous gifts and he is missed.

Dedication

To my son Ian, my own experiment in reproduction.

This is his contribution….

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List of Abbreviations

PAHs	Polycyclic aromatic hydrocarbons
oxyPAHs	Oxygenated PAHs
PHE	Phenanthrene
PHEQ	9,10-phenanthrenequinone
ANT	Anthracene
1,2-dhATQ	1,2-dihydroxyanthraquinone
ROS	Reactive oxygen species
CL	Corpus luteum
EDCs	Endocrine disrupting chemicals
LH	Luteinizing hormone
LH-R	LH receptor
ER	Estrogen receptor
AhR	Aryl hydrocarbon receptor
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
StAR	Steroidogenic acute regulatory protein
P450scc	Cytochrome P450 side chain cleavage enzyme
3β-HSD	3β-hydroxysteroid dehydrogenase
AAS	Atomic absorbtion spectroscopy
22-OHC	22-hydroxycholesterol
hCG	Human chorionic gonadotropin
PMSG	Pregnant mares' serum gonadotropin
MEM	Minimal essential medium
H_2DCFDA	2',7'-dihydrodichlorofluorescein diacetate
PBSG	Phosphate buffered saline plus glucose
CFDA-AM	5-carboxyfluoscein diacetate
BC	Bathocuproine
NC	Neocuproine
GSH	Glutathione
MA	Metabolic activity
MI	Membrane integrity
ddPCR	Differential display polymerase chain reaction

cDNA	Complementary DNA
NCBI	National Centre for Biotechnology Information
BLAST	Basic local alignment search tool
hnRNP-C	Heterogeneous ribonuclear ribonucleoprotein C
eEF1a1	Eukaryotic translation elongation factor 1a1
Alfy	Autophagy-linked FYVE domain
PARP10	Poly(ADP-ribose) polymerase family 10
eIF5A	Eukaryotic translation initiation factor 5A
PQBP-1	Polyglutamic binding protein 1
eIF3	Eukaryotic translation initiation factor 3
SRP	Signal recognition particle

Chapter 1 – Introduction

Environmental toxicology has for years been concerned with the fate of chemicals in the environment and how they affect organisms. With the ever increasing levels of chemical contaminants impacting all forms of life on the planet, the need to determine the effect of these toxicants is becoming more urgent. Every day the amount of chemicals released into the environment grows, in particular polycyclic aromatic hydrocarbons (PAHs) due to increased fossil fuel combustion. Knowing the hazard these chemicals represent is not enough. Often contaminants are modified through various means such as UV light and these by-products often have very different toxicities than their parent compound. To make matters worse, chemicals are rarely found in isolation and mixtures of chemicals often react differently than when separate. There are many endpoints addressed in the study of toxicology, but of concern in this study is the reproductive system.

Reproductive toxicology has garnered much attention in recent years. What was once considered the ravings of environmental activists is now gathering credible scientific support. The scope of reproductive toxicology is massive. Even when only considering humans, the field encompasses the impact of adverse chemicals on the embryo in the uterus through to the senior in the nursing home. The ability of environmental contaminants to simulate endogenous hormones was one of the first areas of concern to receive widespread attention. The last decade has seen research focused on potential contaminant-hormone receptor interactions with particular attention paid to the thyroid hormones and androgen and estrogen receptors. Numerous contaminants have been found as weak agonists for these receptors; however,

researchers are now deciphering other mechanisms of action against the endocrine system. The International Programme on Chemical Safety (IPCS), a branch of the World Health Organization (WHO), recently defined an endocrine disrupter as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism of its progeny or (sub)populations" (IPCS, 2002).

1.1 Toxicology

In ancient times, man faced many perils in the environment. He no doubt ate plants that made him sick and were bitten by animals whose venom could kill. Eventually, man recognized these poisonous elements and either avoided them or used them. Poisons have had a long history of use as agents of suicide, execution, assassination and even medicine. In their use, the science of toxicology was born. By the time of the Renaissance, Paracelsus (1493-1541) determined the central tenet of toxicology "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy". His recognition of the dose-response relationship earned him the moniker "the father of toxicology". Many still relegated toxicology to the realm of magic but true scientific experimentation was soon to come. The late 18th century Spanish physician, Matthieu Joseph Bonaventure Orfila began a systematic correlation between the chemical and biological properties of poisons. His text, *Traité des Poisons Tirés des Regnes Minéral Végétal et Animal, ou Toxicologie Générale Considérée Sous les Rapports de la Pathologie et de Médicine Légale* (1814), was a landmark in scientific experimentation and evaluation of toxic exposures. From

these beginnings, toxicology has evolved into a quantitative discipline that attempts to determine the quantity of a substance required to elicit a deleterious effect on a target and the mechanisms behind those effects.

Toxicology is an amalgam of disciplines that merges many of the basic sciences including chemistry, biology, ecology, physics and statistics (Oehme, 1979). To address the wide variety of concerns, modern toxicology has developed into three specialized branches: clinical, environmental, and forensic (Boelsterli, 2003). Clinical toxicology deals with pharmacology and the effects of chemicals purposely administered to the body for a desired effect (Boelsterli, 2003). Forensic toxicology is involved in the legal aspect of harmful chemicals or poisons and is the fodder for many a TV crime show (Boelsterli, 2003). The focus of this thesis is environmental toxicology. This branch of toxicology is concerned with chemicals that are encountered through air, water, sediment and soil pollution.

The Industrial Revolutions of the late 18^{th} and 19^{th} centuries produced unprecedented amounts of environmental contaminants of anthropogenic origin. Mining, steel production, steam locomotives and finally automobiles generated hydrocarbon wastes as never before seen (Berg and Hudson, 1992). Factories, textile and pulp and paper mills released unknown quantities of chemicals into the environment. Medical conditions such as black lung and phossy jaw were early indicators of the deleterious effects of chemicals (Gochfeld, 2005).

The early 1960's saw public interest in the environment ignited by Rachel Carson's *Silent Spring* (Carson, 1962). Her outspoken work regarding the negligence of agricultural scientists' and chemical companies' widespread abuse of pesticides was a

landmark in environmental policy and is credited with beginning the environmental movement. The formation of the US Environmental Protection Agency (EPA) in 1970 by Richard Nixon was spurred on by various environmental disasters that focused public attention on the environment.

"The more clearly we can focus our attention on
the wonders and realities of the universe about us,
the less taste we shall have for destruction."
-- Rachel Carson © 1954

More recently, Theo Colborn's *Our Stolen Future* (Colborn et al., 1996) sounded the alarm on contaminants that could mimic endogenous hormones resulting in altered health, fertility and development. These compounds have been termed endocrine disrupting chemicals (EDCs) and a great deal of research has been focused on these compounds lately. The FDA met in 1998 to address EDCs and how to go about testing chemicals to determine their effect on the endocrine system. The Endocrine Disruptor Screening and Testing Advisory Council (EDSTAC) determined that all chemicals under the FDA jurisdiction, approximately 70 000, needed to be screened for all hormone-like activity including estrogenic, androgenic and thyroid hormones (EDSTAC, 1998). This program has become the largest undertaking in toxicology requiring both in vivo and in vitro testing using 8 different species spanning a number of taxa. Endpoints include viability and reproduction with the emphasis on discovering the mechanism of action for each toxicant.

1.2 Mechanistic Toxicology

Mechanistic toxicology describes the route by which toxicants exert their effect on biological targets (Boelsterli, 2003). The aim is to define the underlying molecular incidents that culminate in the physical manifestation of toxicant exposure. A number of fundamental questions must be answered to determine the mechanism of toxicity: (1) how does the toxicant enter the organism or cell, (2) what kind of interaction occurs between the toxicant and target molecule(s), (3) what downstream events are precipitated from toxicant/target interactions, (4) how does the organism or cell respond to the insult. Some mechanisms for toxicants are well known at one level, but completely unknown at another. There are a number of reasons for knowing the molecular targets of toxicants chief among them is the improvement of risk assessment. Monitoring through appropriate biomarkers is another benefit of knowing toxicant targets as well as defining accurate threshold values for the desired species.

Characteristics of toxicant/organism interactions can be divided generally into 4 steps: toxicokinetics, toxicodynamics, effector mechanisms, and the biological response (Figure 1.1 – Boelsterli, 2003). Toxicokinetics refers to the uptake, distribution, transformation, and elimination of the compound from the cell or organism in question. If a toxicant undergoes transformation reactions such as Phase I or Phase II reactions, the metabolites often have different toxicokinetic properties from the parent compound. Toxicodynamics relates the interaction of the toxicant to any biological structures. These could include receptors, organelles, membranes, or DNA. While in most cases where the dynamics of toxicant interactions are known, only a few steps in the cascade have been uncovered but are necessary for determining the overall mechanism of

action. One such class of compounds, 2-chloro-s-triazines, were tested for their endocrine disrupting ability, however, testing was limited to estrogen receptor (ER) binding capacity and receptor-mediated responses (Connor et al., 1996, Tennant et al., 1994a , Tennant et al., 1994b) . Upon further investigation, these pesticides were found to induce the steroidogenic cytochrome P450 aromatase (CYP19) enzyme which catalyzes the transformation of testosterone to estrogen (Sanderson et al., 2000). The downstream targets of estrogen activation in both males and females are numerous and resulted in presentations of various diseases. However, some toxicants, such as Cu^{2+} have multiple targets and yet are ultimately manifested as the same organismal disease. For instance, Wilson's disease is a rare autosomal recessive disorder with a mutation in ATPase 7B that inhibits elimination of Cu^{2+} from the liver resulting liver damage and death. The many targets of Cu^{2+} toxicity in Wilson's disease include induction of proteolysis and autophagy and inhibition of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Pourahmad and O'Brien, 2000 , Sheline and Choi, 2004). In the case of Wilson's disease, Cu^{2+} interacts with multiple initial targets and downstream effector mechanisms, however the biological response is the same, liver necrosis. Knowing the mechanism of action allows for more accurate disease modelling, therapeutic drug design and treatment regimen.

1.3 Molecular Methods in Toxicology (Toxicogenomics)

Gene expression profiling has become a powerful tool for toxicology (Lee et al., 2005, (Merrick and Madenspacher, 2005). The US EPA declared that genomic analyses will have a significant impact for scientific research and risk assessment

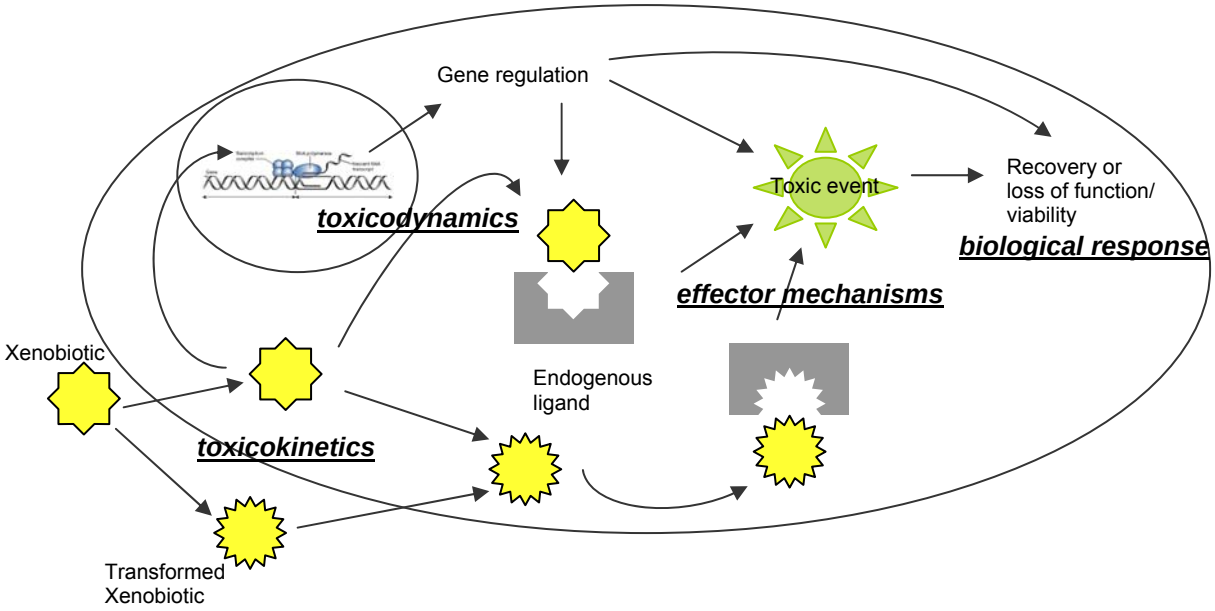


Figure 1.1 Diagrammatic representation of mechanistic toxicology. The general outcome of exposure to a toxicant can be divided into 4 basic sections: toxicokinetics – uptake, distribution and elimination, toxico-dynamics – primary interaction with target (random or specific), effector mechanisms – downstream interactions, and biological response – defence, tolerance, repair or apoptosis. Adapted from *Mechanistic Toxicology*, UA Boesterli, 2003.

(Interim Policy of Genomics, US EPA, July 26, 2002), The ability to study responses of a single gene to toxicant exposure has been available for many years but the advent of "global" gene expression techniques such as differential display reverse transcription polymerase chain reaction (ddPCR), DNA microarray, and quantitative PCR technologies have revolutionized the field.

The strategy behind these approaches has been to compare gene expression patterns between two or more samples with one control and one or more exposed samples (Snell et al., 2003). Both technologies are able to monitor the active gene expression of a cell or organism through changes in mRNA levels. The production of complementary DNA (cDNA) from mRNA provides a stable copy of the transcript suitable for experimental analysis. These technologies have advantages and disadvantages.

Microarrays allow for the simultaneous identification of thousands of genes at a time but the quantity of data generated has led to difficulties in decoding results (Liang, 2006). Microarrays are also limited in the organisms that can be studied. A wider variety of expensive commercially available microarray chips are becoming available, however, choice is still limited at this time (Akhtar et al., 2005). To overcome this limitation, custom chips can be designed but that requires both resources and time (Liang, 2006). ddPCR is much cheaper to run and requires no special equipment. All of the reactions involved in ddPCR are based on general molecular techniques currently employed (Stein and Liang, 2002a). However, deciphering banding patterns produced on a polyacrylamide gel requires experience, especially to properly determine up or

down-regulation of bands. Both technologies suffer from a high rate of false positives, but many papers have been published on ways to minimize this problem.

ddPCR was first described by Liang and Pardee (Liang and Pardee, 1992) as a means to visualize and compare mRNA expression between two samples. The technique begins with reverse transcription of RNA to its complementary DNA (cDNA) using oligo(dT) primers to anneal to the polyadenylated (polyA) tails of mRNA. The cDNA fragments are amplified by PCR and then separated by polyacrylamide gel electrophoresis. The resulting banding patterns between samples can be compared and differentially expressed DNA fragments visualized. Bands of interest can be excised from the gel and the DNA extracted. Further amplification could then be accomplished by cloning in bacteria (Diener et al., 2004, (Akhtar et al., 2005, (Lees, 2005). After retrieving the DNA from the bacteria, the fragment can be cloned and sequenced and the results compared with the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST). Ideally, differential expression of sequences would then be confirmed by Northern blotting or quantitative PCR (qPCR) (Stein and Liang, 2002b).

The advent of the GenBank genome database and BLAST search tools has also revolutionized genomics (Altschul et al., 1997). Researchers can identify correlations between treatment and expression of known genes as well as identifying novel genes through comparisons of nucleotide-nucleotide sequences, translated nucleotide-protein sequences and many other variations. From the information given, the researcher can determine the quality of the alignment. One such measure, the bit score, calculates the alignment of identical and/or similar residues as well as accounting for any gaps that

may occur in the sequence (Altschul et al., 1997). The other measure of alignment, the E-value, is similar to the P-value in statistics (Altschul et al., 1997). Factors such as pair wise alignment, the size of database used and the scoring system are used to calculate the E-value. As with the P-value, the lower the E-value, the more significant the alignment (Altschul et al., 1997).

For toxicologists, the key to using these technologies began with identifying differentially regulated genes in relevant model organisms to known environmental contaminants. Particular attention has been paid to toxicants such as metals, dioxin, and benzo[a]pyrene (Chang et al., 2004, Abe et al., 2006, Fong et al., 2005). However, little data has been collected thus far regarding the effect of environmental polycyclic aromatic hydrocarbons (PAHs) on global gene expression.

The ultimate goal of toxicogenomics is to anchor the gene expression profile with a standard toxicological endpoint, such as cytotoxicity, apoptosis or tissue pathology (Merrick and Madenspacher, 2005). In this way, the gene signatures that precede an obvious toxic outcome could be used as diagnostic tools. Additionally, this could signal a new way to classify toxicants in that chemicals could be grouped not on chemical structure, such as polycyclic aromatic hydrocarbons, but based on common gene expression response.

1.4 Sources and Chemical Properties of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds found ubiquitously in the environment. While found naturally in the environment as products of forest fires, volcanic eruptions and oil deposits, most PAHs have anthropogenic origins

including incomplete fossil fuel combustion, refuse incineration, coal tar, asphalt and smelting furnaces (Neff, 1985, Eisler, 1987, Amodio-Cocchieri et al., 1993, Xing et al., 2006, Ciganek et al., 2004). Two commonly found PAHs are phenanthrene (PHE) and anthracene (ANT). Studies have determined that approximately 54% of aromatic PAHs in diesel exhaust are comprised of PHE (Shimada et al., 2004b).

PAHs are conjugated ring structures of a highly hydrophobic nature with a tendency to associate with particulate matter in the atmosphere (Nikolaou et al., 1984, Oda et al., 1998, Macdonald et al., 2000), sediments in the water column (Law and Biscaya, 1994, Macdonald et al., 2000, Lampi et al., 2001) and soil (Macdonald et al., 2000, Chen et al., 2004). This hydrophobicity also results in the accumulation of PAHs in lipids of various organisms such as phytoplankton (Marwood et al., 1999), fish (Meador et al., 1995, White and Triplett, 2002) and even humans (Younglai et al., 2002).

PAHs can be activated either biotically or abiotically. Biotic transformations are mediated through processes like phase I and phase II enzymes located primarily in the liver (Shimada and Nakamura, 1987, Slawson et al., 1996). PAHs can be modified by abiotic means as well. Due to their extensive π-orbital systems, PAHs can absorb light in the visible and UV spectra (Huang et al., 1993, Diamond et al., 2000). The absorption of light can modify the effects of these compounds through 2 different mechanisms; photomodification and photosensitization. Photosensitization of PAHs leads to the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) (Foote, 1991).

Photomodification can result in production of oxygenated PAHs (oxyPAHs) such as PAH quinones (Mallakin et al., 2000). These oxyPAHs are much more reactive than

their parent compounds (Oris and Giesy, 1985, Huang et al., 1993, Huang et al., 1995, McConkey et al., 1997, Huang et al., 1997, Brack et al., 2003, Lampi et al., 2006). Additionally, these oxyPAHs become more water soluble yet still remain somewhat hydrophobic allowing them to accumulate as well as cross over biological membranes (Huang et al., 1993, Ren et al., 1994, McConkey et al., 1997). Quinones can undergo 1 or 2 electron reductions. One electron reductions are catalyzed by enzymes such as NADPH:cytochrome P450 oxidoreductase. These reductases have a broad specificity (Jarabak et al., 1997). Two electron reductions can be catalyzed by dihydrodiol dehydrogenases (DD) like 3α- and 17β-hydroxysteroid dehydrogenase both of which occur in the human placenta and show a limited specificity for *o*-quinones (Jarabak et al., 1996, Jarabak et al., 1997). Quinones have the ability to redox cycle with production of superoxide anion radicals ($O_2^{\bullet-}$) and *o*-semiquinone radicals (Jarabak et al., 1996, Jarabak et al., 1997, Flowers-Geary et al., 1996). The reactions can be illustrated by the following equations:

$$Q \rightarrow Q^{\bullet-} \quad (1)$$

$$Q^{\bullet-} + O_2 \leftrightarrows Q + O_2^{\bullet-} \quad (2)$$

$$Q^{\bullet-} + H_2O_2 \leftrightarrows QH^- + H^+ + O_2 \quad (3)$$

$$QH^- + O_2 \leftrightarrows Q^{\bullet-} + O_2^{\bullet-} + H^+ \quad (4)$$

The first equation illustrates the 2 electron reduction of the quinone to produce a semiquinone radical catalyzed by DD (Jarabak et al., 1997). Semiquinones can react with O_2 to produce $O_2^{\bullet-}$ and regenerate the quinone. Alternatively, the semiquinone could react with H_2O_2 producing a hydroquinone that could go on to react with O_2

generating more semiquinones and $O_2^{\bullet-}$. This futile redox cycling would continue independent of the enzymatic reaction required initially (Jarabak et al., 1997).

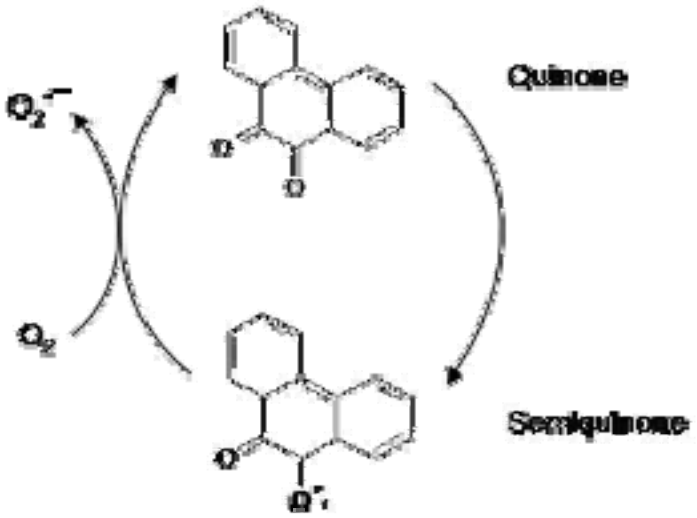


Figure 1.2 Redox cycling of PHEQ from quinone to semiquinone and regeneration via superoxide radical formation.

1.5 Toxicity of PAHs

The prevalence of PAHs in the environment is one of the main reasons for concern in assessing risk of PAHs to organisms. Concentrations of PHE have been found with the lowest levels occuring in water (2.1 ng/L – (Chenier et al., 1993) compared to 179.2 $\mu g/m^3$ in air (Binková et al., 2003). A wide variety of environmental levels of ANT have been published ranging from 4.7 ng/L in Tokyo Bay seawater (Kurihara et al., 2005) to 48 $\mu g/g$ in St. Lawrence River sediment (Arcaro et al., 1999) to 33 mg/kg in soil (Fouchécourt et al., 1999) and 11.5 $\mu g/m^3$ in air (Binková et al., 2003).

Bioavailability is another concern for risk assessment; without exposure, there is no risk. Studies investigating the exposure hazard for various organisms have found that PAHs can be readily taken up by both plants and animals. Various studies have determined the concentrations of PAHs in biota. Of the many PAHs catalogued, both ANT and PHE are typically found (Table 1.1). Kadry et al (Kadry et al., 1995) found that of two administration routes, PHE accumulated more in rats treated orally versus dermally. In aquatic systems, fish can readily take up various PAHs including PHE and anthracene (ANT) through association with contaminated sediment (Djomo et al., 1995). Although PAHs typically do not have high water solubility, photomodification can increase their solubility as shown by the aquatic plant, *Lemna gibba* which can readily take up PHE and ANT photoproducts more so than the parent compounds (Duxbury et al., 1997). PAHs have been studied for their genotoxicity through direct interaction with DNA (Flowers-Geary et al., 1996) or by receptor-mediated gene regulation. The receptors most studied are the aryl hydrocarbon receptor (AhR)

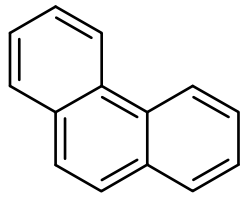
Table 1.1 Summary of PAH concentrations in various organisms and tissues.

Species	Tissue	ANT	PHE	Reference
H. sapiens	Blood (ppb)	3.6	9.0	Singh et al., 2007
	Urine (ng/L)	8.0	48.0	Campo et al., 2006
C. magister	Liver (μg/L)	< 0.001	0.037	Chenier et al., 1993
E. lucius	Wet weight (μg/L)	0.001	0.040	Chenier et al., 1993
B. vulgaris	Dry weight (ng/g)	3.2	35	Nadal et al., 2004

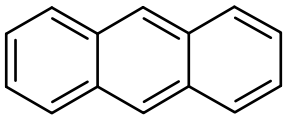
and the estrogen receptor (ER) in rats and mice (Charles et al., 2000, Borman et al., 2000, Revel et al., 2001, Machala et al., 2001a, Matikainen et al., 2001, van Lipzig et al., 2004). There are a number of PAHs that do not activate the AhR such as PHE and ANT

(Till et al., 1999, Bols et al., 1999). Included in this group are the oxyPAHs, many of which are redox active.

Most of our current understanding of PAH-mediated toxicity has been derived from experimentation with unmodified PAHs. Early testing using the Ames protocol deemed that PHE and ANT were neither mutagenic nor carcinogenic (McCann and Ames, 1976). However, various studies have found that oxyPAHs including 9,10-phenanthrene-quinone (PHEQ) and 1,2-dihydroxyanthraquinone (1,2-dhATQ) are more toxic than their parent compounds (Bücker et al., 1979, Mallakin et al., 1999). Toxicity has been demonstrated in invertebrates (Weinstein and Polk, 2001, Xie et al., 2006) and vertebrates (Landrum et al., 1984, Choi and Oris, 2000a, Choi and Oris, 2000b) as well as growth inhibition in plants (Mallakin et al., 1999, Babu et al., 2001, Paskova et al., 2006).



PHE



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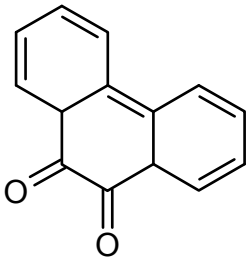
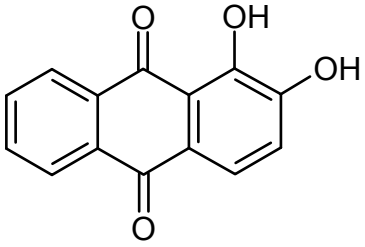


PHEQ



1,2-dhATQ

Figure 1.3 Structures of PAHs used in this study. Abbreviations as follows: PHE, phenanthrene; ANT, anthracene; PHEQ, 9,10-phenanthrenequinone; 1,2-dhATQ, 1,2-dihydroxyanthraquinone.

In terms of human health concerns, some diseases of unknown etiology may be attributable to PAH metabolites. One case in point may be air pollution where volatile PAHs are easily accessible to UV light for photomodification. Both indoor and outdoor air contains high levels of PAHs (Wang et al., 2005, Kameda et al., 2005) as does diesel exhaust (de Kok et al., 2005). PAHs in air pollution have been implicated in a number of toxic insults including respiratory allergic responses (Tsien et al., 1997, Polosa et al., 2002, Dong et al., 2005), lung infection (Castranova et al., 2001), and lung cancer (Aam and Fonnum, 2007, Bao et al., 2007) as well as genotoxicity in *in vitro* testing (Binková et al., 2003, de Kok et al., 2006). This evidence shows that with the availability and toxicity of photomodified PAHs, it is imperative that oxyPAHs such as PHEQ and 1,2-dhATQ undergo further assessment to determine the full extent of toxicity as well as their mechanisms of action.

1.6 Sources and toxicity of metals in the environment

Metals are another class of contaminants ubiquitously found in both aquatic and terrestrial environments. Introduction to the environment has occurred anthropogenically through industrial manufacturing, mining, fossil fuel combustion, and hazardous waste sites (Jacobson and Turner, 1980). The result is increased metal concentrations in air (Karar et al., 2006), water and soil (Madrid et al., 2006, Wong et al., 2006). Metals are water soluble, non-biodegradable and can accumulate in biological organisms. Many metals have been examined for both lethal and sub-lethal effects. Some divalent metals, such as Cu^{2+} and Zn^{2+}, have biological functions and are known as trace metals or micronutrients (Valentine and Gralla, 1997, Takahashi et al., 2002,

Eide, 2003, Bao et al., 2003). However; many more metals have no known biological function including Cd^{2+}, Ni^{2+} and Hg^{2+} (Jacobson and Turner, 1980, Zalups, 2000, Prasad et al., 2006). In general, all metals are toxic. Even metals that are essential trace elements for organisms can be toxic (Pourahmad et al., 2001, Fuentealba et al., 2000, Handy, 2003, Hamatake et al., 2000, Asano et al., 2004). As the concentration of metals an organism is exposed to varies, physiological and/or genetic adaptation is essential. This situation explains the myriad of protective mechanisms developed by organisms to tightly regulate metal concentration and distribution (Chen et al., 1996, Harrison et al., 1999, Bossuyt and Janssen, 2003).

Cu^{2+} and Zn^{2+} can alter plasma membrane fluidity by linking to phosphate moieties of phospholipids or interacting with thiol or carbonyl groups of membrane proteins (Kumar and Prasad, 2000, Kaur et al., 2006). Metal exposure can occur during uptake, usually through cell membrane transporter systems used for micronutrient absorption. It is at this point where metals can exert their first toxic effect by displacing essential nutrients. For example, Cu^{2+} or Cd^{2+} can displace Ca^{2+} in Ca^{2+} ion pumps thus decreasing the overall uptake of Ca^{2+} into the cell (Manzl et al., 2004, Clemens et al, 1998). Additionally, both Cu^{2+} and Zn^{2+} can be directly toxic through replacement of natural ligands in metal-containing proteins (Piao et al., 2003). This displacement of the endogenous metal ligand for an exogenous one has formed the basis of the biotic ligand model (BLM) (Di Toro et al., 2001). In general, the BLM is a mechanism that explains toxicity occurring at high concentrations of metals since displacement would occur on a 1:1 ratio (Di Toro et al., 2001, Paquin et al., 2002). However, stoichiometric limitations do not explain toxicity observed at low concentrations.

Some metals also have the ability to redox cycle with the potential to produce ROS (Lynn et al., 1997, Lund et al., 1991, Galhardi et al., 2004, Manzl et al., 2004, Pathak and Khandelwal, 2006). For the benefit of an organism, redox cycling is a strategy exploited by many enzymes such as NADPH:oxidoreductase for electron transfer and bioenergetics. However, by-products include hydroxyl (OH^-) and $O_2^{\bullet-}$ radicals generated in a Fenton-like reaction which can then in turn react with proteins, lipids, and DNA (Halliwell and Gutteridge, 1989, Harrison et al., 1999). Cu^{2+} in particular is known to inhibit growth in plants (Yruela et al., 1996, Babu et al., 2001), cause gill damage in fish (Karan et al., 1998), produce lipid and nucleic acid damage (Halliwell and Gutteridge, 1989), and induce apoptosis (Gyulkhandanyan et al., 2003). Much of the observed toxicity may be due to ROS production (Pourahmad and O'Brien, 2000, Babu et al., 2001). In situations where the organism is already stressed, exposure to metals can increase susceptibility to toxic insult (Galhardi et al., 2004). For instance, lipid peroxidation (Galhardi et al., 2004) and DNA adduct formation (Jung and Surh, 2001) are some of the results of metal-mediated ROS toxicity. Additional damage has been observed in mitochondrial metabolism, cytokine secretion, and steroid synthesis (Bao et al., 2003, Piao et al., 2003, Riley et al., 2003).

Ni^{2+} is another metal of concern. Studies detailing Ni^{2+} concentrations in humans have found appreciable amounts in blood, plasma, serum, urine (Templeton et al., 1994), and semen (Danadevi et al., 2003). Exposure to Ni^{2+} has been shown to have deleterious effects in plants (Li and Xiong, 2004), invertebrates (Pane et al., 2004), and mammals (Haber et al., 2000a, Haber et al., 2000b). Consequences of Ni^{2+} exposure in

mammals include nasal and lung cancer (Haber et al., 2000a, Haber et al., 2000b), and nephrotoxicity (Prasad et al., 2006).

Another non-metabolic metal, Cd^{2+} has been well documented as an environmental pollutant. Sources of Cd^{2+} occur mainly through use in dyes, solder, electroplating and manufacture of Ni-Cd batteries (Jacobson and Turner, 1980). Exposure routes for Cd^{2+} include water, air and food intake (Hellström et al., 2007). Once exposed, Cd^{2+} can exert its toxic effect through binding to thiol moieties of proteins (Rai et al., 2003), lipid peroxidation (Howlett and Avery, 1997), DNA damage (Hartwig et al., 2002) and ROS generation (Adamis et al., 2004).

Inorganic Hg^{2+} is one of the most ubiquitous and toxic metals in the environment (Aston and Fowler, 1985, He et al., 2005, Florea and Büsselberg, 2006, Jiang et al., 2006). Studies have demonstrated nephrotoxicity, tumor formation, immunotoxicity and neurodegeneration in mammals. Various mechanisms may account for Hg^{2+} toxicity. Hg^{2+} is known to bind protein thiols (Gassó et al., 2001, Zalups and Lash, 2006), and produce ROS (Lund et al., 1993, Schurz et al., 2000).

The common theme of metal-induced toxicity shows that the mechanism is two-fold; high concentration toxicity described by the biotic ligand model (BLM) and low concentration toxicity. Briefly, the BLM suggests that exogenous metals can either (1) bind thiol or alcohol groups, usually inactivating them or (2) replace endogenous metals in the active sites of important biological compounds such as enzymes and other proteins resulting in inactivation. However, this can only occur in a 1:1 ratio and requires a high concentration to inactivate enough biomolecules for toxicity to take place. A different mechanism is responsible for toxicity observed at lower

concentrations which may include ROS generation. This would suggest that low level metal toxicity would depend on the organism, specifically the protective mechanisms that the organism possess and that risk assessment would require this information to accurately predict risk.

Table 1.2 Summary of metal levels in various tissues of mammals and fish.

Species	Tissue	Cd	Cu	Hg	Ni	Zn	Reference
Elk *(Cervus elaphus)*	Liver (μg/g)	2.44	108.81	---	0.71	74.77	Parker and Hamr, 2001
	Kidney	24.64	9.21	---	1.23	164.47	
H. sapiens	Liver (ppm)	1.08	6.52	---	---	65.31	Saltzman et al., 1990
	Kidney	15.62	2.22	---	---	49.10	
	Placenta (nmol/kg)	50	15	---	---	170	Osman et al., 2000
	Blood (μg/L)	1.72	---	0.78	---	---	Butler Walker et al., 2006
	Plasma	---	2160	---	---	581	
	Urine						
Northern fur seal *(Callorhinus ursinus)*	Liver (μg/g)	14	23	59	---	82	Ikemoto et al., 2004
Dall's porpoise *(Phocoenoides dalli)*	Liver (μg/g)	2.9	5.0	3.8	---	32	
Yellow perch *(Perca flavescens)*	Kidney (μg/g)	---	17.38	---	3.74	1862.2	Levesque et al., 2003

1.7 Mixture studies of PAHs and metals

The study of mixture toxicity has only been started in the last few years. It was first necessary to gather information on individual chemicals before attempting to determine the toxicity of mixtures. However, most contaminants are not found alone, but more often in combination with other compounds: often in complex mixtures (Kamalakkannan et al., 2005, Brown and Peake, 2006, Gravato et al., 2006, Wichmann et al., 2006, Dai et al., 2007). Metals are commonly found together with PAHs. Because PAHs can be photomodified to form oxyPAHs, the probability of oxyPAH:metal mixtures is very high. Indeed, oxyPAHs have been found in environments that are contaminated with metals

(Lampi et al., 2001). Both oxyPAHs and metals have demonstrated the ability to produce ROS individually (Nykamp et al., 2001, Garcia-Fernández et al., 2002).

PAH quinones have the ability to undergo a one or two electron reduction to produce a semi-quinone radical, which in turn can pass the unpaired electron to electrophilic species such as molecular oxygen to produce the superoxide radical and other related ROS (Jarabak et al., 1997, Bolton et al., 2000). Recent work suggests that oxyPAHs and metals may act together to promote toxicity in a catalytic manner involving reactive oxygen species (ROS) production targeting DNA and the mitochondria (Babu et al., 2001, Das et al., 1997) (Figure 1.3).

1.8 Reproductive toxicology

In the ten years since the Endocrine Disrupter Screening and Testing Advisory Council (EDSTAC) proposals were published, two limitations on the data have been cited: only a small subset of chemicals have been tested in vivo, and most testing has revolved around receptor agonism/antagonism (Daston et al., 2003). However, the EDSTAC recommendations are broader stating that all known mechanisms of endocrine disrupting action need to be explored including enzyme inhibition/induction in hormone production (EDSTAC, 1998). Both limitations stated above will be addressed in this thesis.

Recently, more data has been published regarding other mechanisms of endocrine disruption beyond receptor binding. For instance, one of the main enzymes of the steroidogenic pathway responsible for the translocation of cholesterol from the outer to the inner mitochondrial membrane, steroidogenesis acute regulatory (StAR) protein,

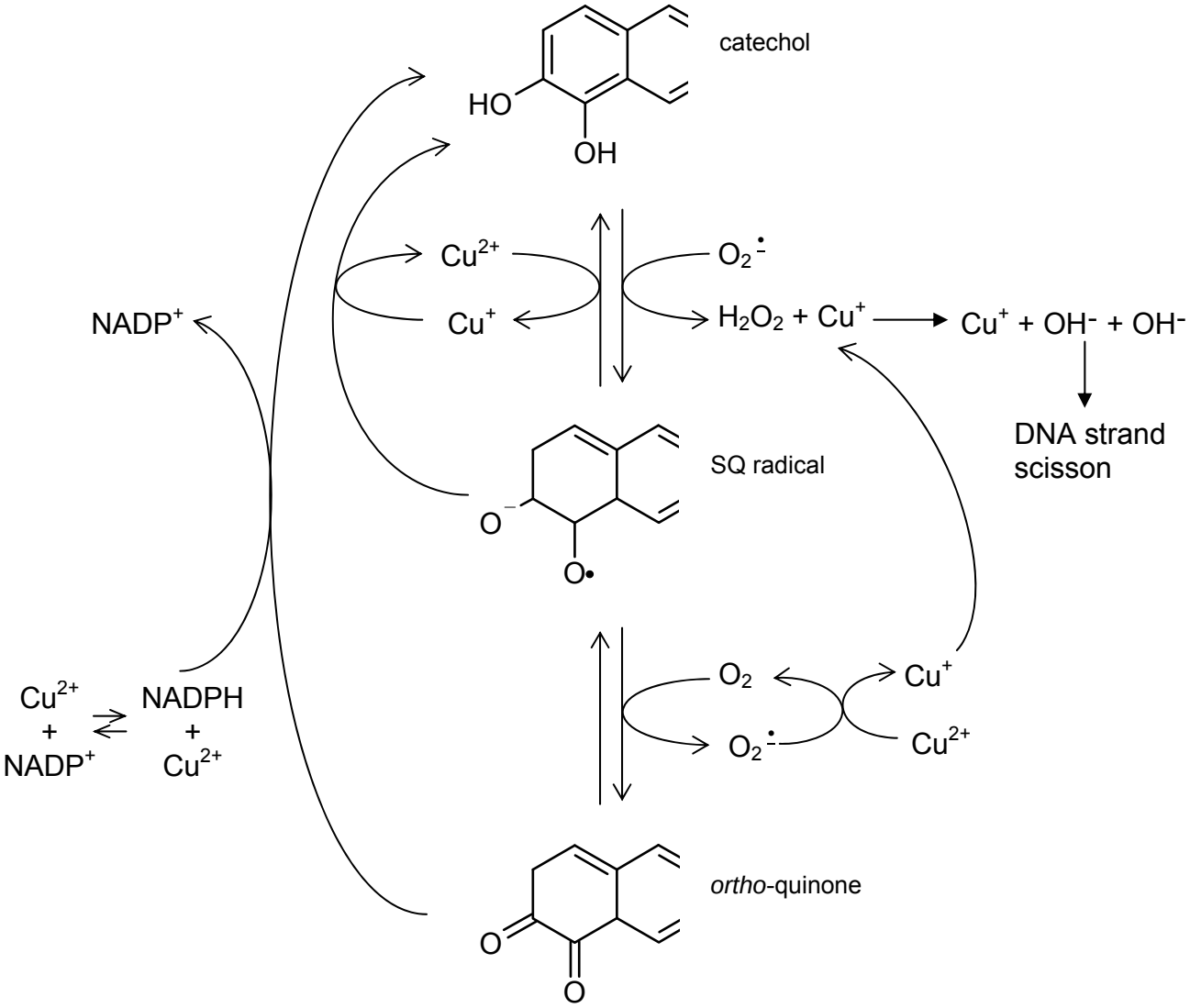


Figure 1.4 Schematic representation of PAH o-quinone/metal redox cycling. Diagram depicts interaction of quinone in the presence of Cu^{2+} and NADPH with generation of reactive oxygen species. Adapted from Flowers et al., 1997.

is susceptible to toxic assault. Various chemicals including lindane, econazole, miconazole, glyphosphate, and dimethoate interfere with either StAR protein expression or post-transcriptional modification (Walsh and Stocco, 2000, Walsh et al., 2000a, Walsh et al., 2000b, Walsh et al., 2000c).

Many reviews on EDCs have been published in the last few years; however, conflicting results have been reported (Sharara et al., 1998, Tyler et al., 1998, Golden et al., 1998, Daston et al., 2003, Harvey and Everett, 2003). Much of the confusion appears to be linking in vitro results to in vivo experiments (Hutchinson, 2002). For example, aquatic toxicologists using marine invertebrates have found that some estrogen and ecdysteroid agonists are highly toxic to the marine copepod *Tisbe battagliai*, but in vitro testing shows no reactivity of these compounds to the ecdysteroid receptor (reviewed by Hutchinson, 2002).

PAHs have been examined for sub-lethal effects in reproductive tissues of various animals and humans. Early work using mouse embryonic cells demonstrated the ability of various PAHs to induce irreversible morphological changes upon incubation with various PAHs (Kamei, 1980). Work done with model toxicants, such as benzo[a]pyrene (BaP), has shown that both fertility and primordial oocyte number can decline with exposure (Mattison et al., 1983). Further studies have revealed that many different adult and fetal cell types are sensitive to PAHs including maternal mammary glands (Moore and Gould, 1984, Werts and Gould, 1986), adult spermatogenic cells (Georgellis et al., 1989), fetal liver and lung (Hatch et al., 1990), maternal and fetal white blood cells (Whyatt et al., 2000), and adult lung tissues (Tao et al., 2003). While the literature on BaP exposure is extensive, few other PAHs, including oxyPAHs, have been tested in

regard to mammalian reproductive sensitivity. One study found that 7 PAHs of varying molecular weight were present in the placentas of all women tested with no relation to differing environmental conditions (Gladen et al., 2000). While no associations were observed between placental PAH concentration and birth weight of the infant, this study shows the prevalence of PAHs in relation to the reproductive system.

Reproductive effects of metals have been demonstrated as perinatal toxicity in rat pups (Smith et al., 1993), blastocyst development (Paksy et al., 1999), oxidative DNA lesions in neonatal rats (Zhou et al., 2001), and mammalian adult sperm production and quality (Danadevi et al., 2003). Of further concern are studies that have detailed the presence and possible accumulation of Ni^{2+} in placental tissue (Reichrtova et al., 1998) and a component of human breast milk, colostrum (Turan et al., 2001).

The effect of Cd^{2+} has been studied in steroidogenic cells of various animals including rat (Paksy et al., 1990, Paksy et al., 1992), mouse (Mgbonyebi et al., 1993, Mgbonyebi et al., 1994a, Mgbonyebi et al., 1994b, Mgbonyebi et al., 1998a, Mgbonyebi et al., 1998b), pig (Henson and Chedrese, 2004), fish (Leblond and Hontela, 1999) and human (Kawai et al., 2002). Early studies on rats exposed orally to Cd^{2+} showed that not only could Cd^{2+} accumulate rapidly in ovarian tissue, but also depress serum progesterone levels in a time and dose dependent manner (Paksy et al., 1990). *In vitro* studies have demonstrated the ability of Cd^{2+} to interfere with basal steroidogenesis (Mgbonyebi et al., 1994a), hormone stimulated steroidogenesis (Paksy et al., 1992, Mgbonyebi et al., 1994b) or steroidogenic enzyme function (Kawai et al., 2002).

A third non-metabolic metal of interest, Hg^{2+}, is able to cross the placenta from mother to fetus (Bjornberg et al., 2005) and has been shown to accumulate in the

placenta (Shimada et al., 2004a). Further investigation revealed that prenatal exposure to Hg^{2+} caused a persistent, inhibitory effect on cytokine production in thymocytes, lymph cells, and splenocytes in female mice (Silva et al., 2005b).

1.9 Models for studying reproductive toxicology

The female mammalian reproductive system has been extensively studied in both medical and environmental toxicology (Crellin et al., 1999, Matikainen et al., 2002, Miller et al., 2004, Bretveld et al., 2006). The interest in this system is two-fold in that both mother and offspring may be affected through exposure to toxicants. Although this system has many potential targets of toxic exposure, the ovary remains of central interest due to its essential roles in steroid hormone production, follicular maturation and as an ova repository (Richards, 2001).

The rat corpus luteum (CL) is one of the central models studied in ovarian function (Richards, 2001). The maturation of the oocyte occurs within an ovarian follicle under control of follicle stimulating hormone (FSH) (Hadley, 1996). The primary follicle is comprised of granulosa and theca cells. Under FSH control, granulosa cells secrete estrogen. When the circulating levels of estrogen reach a critical point, a surge of luteinizing hormone (LH) is released from the anterior pituitary. LH causes ovulation of the oocyte which is released into the fallopian tubes. The rupture of the follicle may be achieved through the breakdown of extracellular collagen by proteases (Robker et al., 2000). Also under LH influence, luteinization of granulosa and theca cells occurs transforming these cells into luteal cells. These cells cease to grow and become highly steroidogenic expressing various steroidogenic enzymes resulting in the production and

secretion of progesterone (Richards, 2001). Progesterone is a key hormone that is essential for maintaining pregnancy in mammals. LH stimulates progesterone production through a signal transduction pathway which is initiated with the binding of LH to its transmembrane receptor and subsequent activation of the G-protein complex in luteal cells. The pathway involves activation of adenylate cyclase, protein kinase A, the steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD). Any one of these enzymes represents a potential target for toxicant interaction.

The mammalian placenta is responsible for the exchange of nutrients, the elimination of wastes, and the production of hormones used for the maintenance and development of the fetus (Hadley, 1996). The regulation of placental progesterone production is of great interest with regard to preserving pregnancy, onset of labour, timing of delivery and fetal growth (Gellersen and Brosens, 2003, Bulletti and de Ziegler, 2006). The placenta is composed of both maternal and fetal elements (Fazleabas and Strakova, 2002). After an oocyte is fertilized, the cleaving embryo, blastocyst, differentiates into two groups of cells; a peripheral outer cell mass and a central inner cell mass (Fazleabas and Strakova, 2002). The outer cell mass or trophoblast constitutes the main source of the placenta while the inner cell mass develops into the fetus. As the blastocyst implants itself into the endometrial lining of the uterus, the epithelial cells of the endometrium are transformed into decidual cells which accumulate lipid and glycogen (Fazleabas and Strakova, 2002). Higher lipid concentrations in the decidual cells are the source for steroidogenesis (Malassine and Cronier, 2002). The decidual

cell layer thickens and becomes highly vascularized to provide adequate circulation of blood to the developing fetus.

JEG-3 cells are a placental choriocarcinoma cell line of trophoblastic origin (Lala et al., 2002). Although transformed, these cells retain many of the characteristics necessary for placental function (Burnside et al., 1985, Graham and Lala, 1992, Huang and Miller, 2004). Studies using this cell line have established the regulation of various steroidogenic enzymes and co-factors involved in placental steroidogenesis (Ritvos, 1988, Ringler et al., 1989, Ritvos and Voutilainen, 1992a, Ritvos and Voutilainen, 1992b, Tremblay and Beaudoin, 1993, Black et al., 1993, Beaudoin et al., 1997, Henderson et al., 2007) making them a suitable model for studying placental function.

With the increased testing recommended by the EDSTAC proposal, large numbers of animals would be needed for testing with systems such as the rat CL (EDSTAC, 1998). An in vitro system like JEG-3 cells provides an attractive alternative to whole animal testing. Various contaminants have been tested in JEG-3 cells including pesticides (Vinggaard et al., 2000), metals (Hardy et al., 2001, Eneman et al., 2000), and PAHs (Zhang et al., 1995, Zhang and Shiverick, 1997). JEG-3 cells were used to document the effect of contaminants on metallothionein and apoptosis induction, aromatase activity, epidermal growth factor levels, and human chorionic gonadotropin (hCG) secretion (McAleer and Tuan, 2001a, McAleer and Tuan, 2001b, Olivares et al., 2002, Nativelle-Serpentini et al., 2003, Vinggaard et al., 2000, Zhang et al., 1995 , Letcher et al., 2000, Allèra et al., 2004). Thus, JEG-3 cells will be used in the current study to determine the cytotoxicity of PAH, oxyPAH, metal, and (oxy)PAH/metal

mixtures. Additionally, the convenience and reproducibility of JEG-3 cells makes them ideal to study the effects of PHEQ on gene expression.

1.10 Objectives

Given that little is known about the effect of oxyPAHs, metals and their mixtures on mammalian female reproduction, it is of interest to determine their cytotoxicity, targets of toxic action, interference with hormone production, and gene expression. For instance, there are no known studies detailing if any toxicants affect LH-stimulated progesterone production. Furthermore, information on toxicity of oxyPAHs as endocrine disrupting chemicals (EDC) is sparse. Most literature regarding PAHs as EDCs involves activation through the aryl hydrocarbon receptor (Machala et al., 2001a, Machala et al., 2001b, Matikainen et al., 2001, Matikainen et al., 2002).

Recently, attention has focused on the enzymes involved in steroidogenesis. Monteiro et al (Monteiro et al., 2000) found that various PAHs including PHE could inhibit cytochrome P450-17,20l, 17β-hydroxysteroid dehydrogenase (17β-HSD), and cytochrome P450-aromatase in fish. However, no other information regarding PAH-steroidogenic enzyme toxicology was found and none at all involving oxyPAHs. There is more information concerning metals as EDCs (Piasek and Laskey, 1994, Leblond and Hontela, 1999, Kawai et al., 2002, Handy, 2003) but not metal/(oxy)PAH mixtures. Similarly, studies regarding the influence of metals (Bartosiewicz et al., 2001, Simpson et al., 2004) and PAHs (Ng et al., 1998, Alkio et al., 2005, Kim et al., 2007) on gene expression exist, there is little information on the effect of oxyPAHs alone or combined with metals. Taken together, a paucity of data exists concerning PAH, oxyPAH, metal

and oxyPAH/metal mixtures in reproductive toxicology specifically cytotoxicity, endocrine disruption, and gene expression and needs to be addressed.

The primary purpose of this study was to explore the potential of alternate mechanisms to receptor-mediated endocrine disruption using chemicals that do not bind to receptors. Four investigations were undertaken to explore the cytotoxicity of polycyclic aromatic hydrocarbons, metals and mixtures of the two. These studies used both the rat CL and JEG-3 cells as models of endocrine importance and can be summarised as follows:

(1) Determination of the cytotoxicity of individual PAHs. Compounds investigated included: PHE, PHEQ, ANT, and 1,2-dhATQ

(2) Determining cytotoxicity of the metals Cu^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+}, and Hg^{2+}

(3) A mixture study to determine metal/PAH mixture cytotoxicity. This included:

- Cu^{2+} combined with PHE or PHEQ
- Cu^{2+} combined with ANT or 1,2-dhATQ
- Hg^{2+} combined with PHE or PHEQ
- Hg^{2+} combined with ANT or 1,2-dhATQ

(4) Assessment of the effect of PHEQ and Cu^{2+}, alone and in combination on progesterone secretion

Endpoints used for these investigations were based on cytotoxicity and/or measurements of LH stimulated progesterone.

A fifth investigation was undertaken with the JEG-3 cell line to explore differences in gene expression of JEG-3 cells with PHEQ treatment. For this investigation, differential display PCR (ddPCR) was employed to identify changes in gene expression.

Chapter 2 – Effect of oxygenated PAHs and Cu(II) on Rat Corpus Luteal Cell Viability, LH-Stimulated Steroidogenesis and Reactive Oxygen Species Production

2.1 Introduction

Phenanthrene (PHE) is a common polycyclic aromatic hydrocarbon (PAH) that is found in the environment occurring both naturally and synthetically through processes such as incomplete fossil fuel combustion (Kadry et al., 1995). Recently, it has been shown that toxicity of PHE can increase greatly upon exposure to light through photooxidation to produce quinones with 9, 10-phenanthrenequinone (PHEQ) as the major component (McConkey et al., 1997). Intact PAHs have been studied extensively for years in mammalian systems, but in general most of our current understanding is centered on PAHs that mediate their actions through the aryl hydrocarbon (AhR) and estrogen (ER) receptors. Benzo[a]pyrene (B[a]P) and dimethylbenzanthracene (DMBA) are examples of PAHs that use the AhR and/or the ER to mediate various forms of toxicity including ovotoxicity (Charles et al., 2000, Revel et al., 2001, Machala et al., 2001a, Matikainen et al., 2001, Borman et al., 2000). Much less is known about the toxic mechanisms of PAHs that do not activate the AhR such as PHE (Till et al., 1999, Bols et al., 1999). Included in this group are oxygenated PAHs (oxyPAHs), many of which are redox active.

Metals are another important class of chemical contaminant. Cu^{2+} is an essential metal in biological systems and is used as a cofactor in key enzymes with important biological functions such as ATP synthesis. While the cycling of copper from its Cu(II) to Cu(I) oxidation states are necessary for single-electron transfer reactions in copper-

containing enzymes, it is this potential that can lead to copper toxicity (Harrison et al., 1999). Cu^{2+} can produce hydroxyl radicals via the Fenton reaction as well as redox cycle using ascorbic acid, glutathione, and cysteine to accelerate radical formation (Kachur et al., 1999, Ohta et al., 2000). Cu^{2+} in particular is known to inhibit growth in plants (Yruela et al., 1996), cause gill damage in fish (Karan et al., 1998), produce lipid and nucleic acid damage (Halliwell and Gutteridge, 1989), and induce apoptosis (Gyulkhandanyan et al., 2003). Much of the observed toxicity in hepatocytes of both mammals and fish may be due to ROS production (Pourahmad and O'Brien, 2000, Manzl et al., 2004).

Both metals and PAHs are generated in large quantities through industrial processes and are found throughout terrestrial and aquatic environments (Kishikawa et al., 2004, Fent, 2004). Often these chemicals exist as co-contaminants thereby creating the potential for greater risk than may be exhibited by either compound on its own (Babu et al., 2001). Additionally, photoproducts of PAHs are capable of producing ROS and this characteristic may be the origin of PAH induced toxicity observed in many animal and plant systems (Marwood et al., 1999). Quinones are found both naturally and synthetically and have the ability to undergo a one or two electron reduction to produce a semi-quinone radical, which in turn can pass the unpaired electron to electrophilic species such as molecular oxygen to produce the superoxide radical and other related ROS (Jarabak et al., 1997, Bolton et al., 2000). Recent work suggests that oxyPAHs and metals may act together to promote toxicity in a catalytic manner involving reactive oxygen species (ROS) production targeting DNA and the mitochondria (Babu et al., 2001, Das et al., 1997).

The female mammalian reproductive system has been extensively studied in both medical and environmental toxicology (Kamei, 1980, Sharara et al., 1998, Borman et al., 2000, Miller et al., 2004, Bretveld et al., 2006). The interest in this system is two-fold in that both mother and offspring may be affected through exposure to toxicants. Although this system has many potential targets of toxic exposure, the ovary remains of central interest due to its essential roles in steroid hormone production, follicular maturation and ova repository (Otto et al., 1992). The rat corpus luteum (CL) is one of the central models studied in ovarian function (Pepperell et al., 2003, Leng et al., 2000, Kodaman and Behrman, 1999). The CL is a temporary endocrine organ formed from the ovarian follicle after ovulation that produces progesterone in response to stimulation by luteinizing hormone (LH) released from the anterior pituitary gland. Progesterone is a key hormone that is essential for maintaining pregnancy in mammals (Richards, 2001). LH acts through a signal transduction pathway which is initiated by the binding of LH to its transmembrane receptor and subsequent activation of the G-protein complex in luteal cells. The pathway involves activation of adenylate cyclase, protein kinase A, the steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD) (Figure 2.1) (Richards, 2001). Due to the nature and number of enzymes involved in this pathway, any one of these enzymes may be considered as potential targets for toxicant action.

The primary cell suspension culture has been used for study of CL function including viability, steroidogenic pathways, and apoptosis; also known as regression (Wu et al., 1993, Sawada and Carlson, 1994, Sawada and Carlson, 1996). The primary media used in CL suspension cultures is a complex medium; without which steroidogenesis is

not supported (Carlson et al., 1986). However, due to many components found in complex media, the use of this media can interfere with experiments designed to study reactive oxygen species (ROS) and metal uptake (Brubacher et al., 2003). Therefore, simpler media must be used that allows research into these endpoints while maintaining cell viability.

Previously, it was demonstrated that 9,10-phenanthrenequinone (PHEQ), the photoproduct of phenanthrene (PHE), could increase ROS production and inhibit LH-stimulated progesterone secretion in the rat CL (Nykamp et al., 2001). With implications of oxyPAHs being novel endocrine disrupters, another PAH/oxyPAH pair will be tested to compare with PHE/PHEQ. Also to investigate the potential of oxyPAH/metal mixtures to alter ROS levels and LH-stimulated progesterone secretion, a known redox active metal, Cu^{2+}, will be tested in the CL cell system. Therefore, this chapter examines the ability of two oxyPAH quinones, PHEQ and 1,2-dihydroxyanthraquinone (1,2-dhATQ), and Cu^{2+} mixtures to alter progesterone secretion, cell viability, and ROS production in dispersed cells from the rat ovarian CL. This study will also attempt to find the site of action for the toxicants using a pseudo-substrate, 22-hydroxycholesterol (22-OHC) and the adenylate cyclase activator, forskolin.

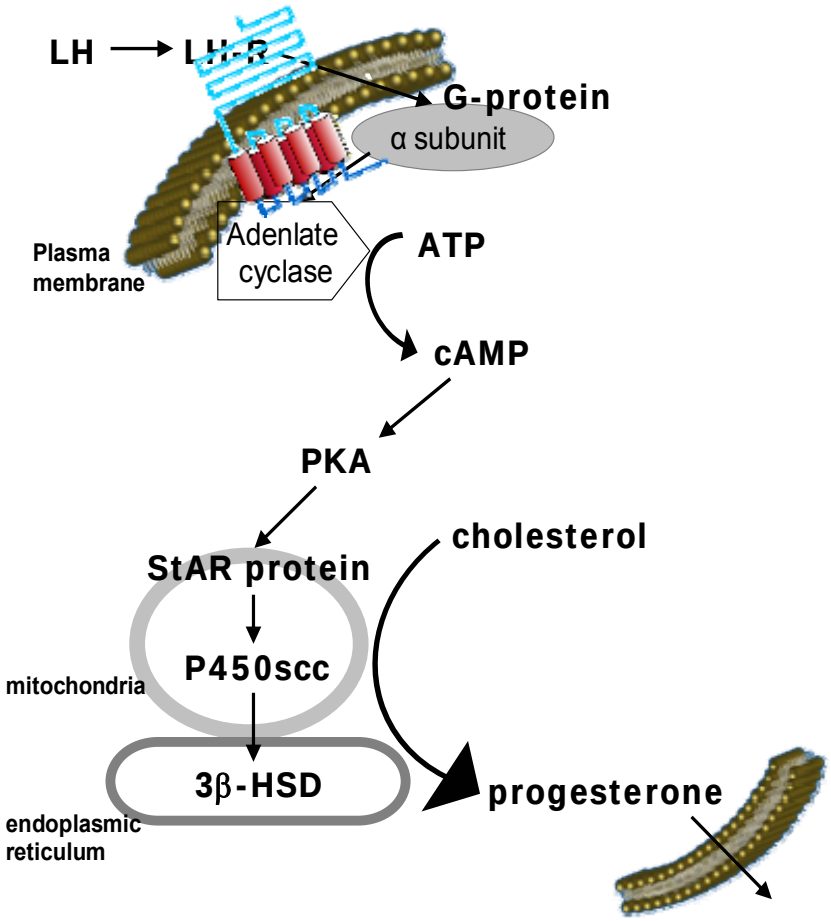


Figure 2.1 The LH signal transduction pathway in CL cells. LH binds to its transmembrane receptor which alters the conformation of the associated G-protein. The subunit of the G-protein activates adenylate cyclase. This enzyme cleaves ATP to form cAMP, an intracellular second messenger. cAMP activates protein kinase A (PKA) which is thought to activate a transport protein that delivers intracellular cholesterol to the mitochondrial membrane. The StAR protein translocates cholesterol from the outer to the inner mitochondrial membrane. P450scc converts cholesterol to pregnenolone, the precursor of all steroid hormones. Pregnenolone then moves into the endoplasmic reticulum where it is cleaved to progesterone by 3β-HSD. Progesterone is able to pass through the cellular membrane into the blood where it is transported to its various targets.

2.2 Materials and Methods

2.2.1 Animals

A breeding colony of Wistar rats was maintained by the Department of Biology, University of Waterloo. The care and use of the animals was approved by the Canadian Council on Animal Care. For this study, 25 to 30 day old immature female Wistar rats were superovulated with a subcutaneous (sc) injection of 50 IU of pregnant mare serum gonadotropin (PMSG – Intervet, Whitby, ON) followed 65 hours later with a sc injection of 50 IU of human chorionic gonadotropin (hCG).

2.2.2 Reagents

Most reagents were purchased from Sigma (St. Louis, MO) including Minimal Essential Medium Eagle (Joklik modified, MEM), collagenase IV, hCG, PHE, PHEQ, anthracene (ANT), 1,2-dhATQ and copper sulfate ($CuSO_4$). Earle's Balanced Salt Solution (EBSS) was obtained from Life Technologies (Burlington, ON). PMSG was purchased from Intervet (Whitby, ON) and DNase I from Roche (Laval, QC). 2', 7'-dichlorodihydro-fluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes (Eugene, OR). Ovine LH was acquired from the National Hormone and Pituitary Program. LH was dissolved in 0.2 M sodium phosphate buffer. Stock solutions of PHE, PHEQ, ANT, and 1,2-dhATQ were prepared in dimethyl sulfoxide (DMSO) as described previously (Nykamp et al., 2001). Stock solutions of $CuSO_4$ were prepared in distilled, deionized H_2O.

2.2.3 Preparation of corpora lutea cells

Ovaries were removed from superovulated rats 4 days after hCG injection. Cells were dispersed in a manner similar to the procedure described previously (Carlson et al., 1995). Ovaries were dissected from surrounding tissue, minced and suspended in EBSS. Corpora lutea were dispersed using 0.2 % collagenase and 0.02 % DNase I at 37°C for 30 minutes in a shaking water bath. The atmosphere was enriched with 95 % air and 5 % CO_2 to minimize changes in pH and to simulate in vivo conditions. After dispersion, the cells were filtered through Nitex (75 µm) and then centrifuged at 200 x g for 6 minutes. Sedimented cells were suspended and washed in MEM before recentrifugation as above. The buffer was decanted and fresh MEM was added to resuspend cells. The cells were pre-incubated for 60 minutes in MEM with added L-glutamine at 37°C in the 95 % air and 5 % CO_2 enriched atmosphere. The cells were then centrifuged as before and resuspended in fresh MEM before an aliquot was removed and examined for viability using Trypan Blue and counted on a hemocytometer. Average viability remained approximately 75 % and sample size was based on the number of viable cells. The cells were then monitored for progesterone secretion, ROS production and viability after various treatments.

2.2.4 Progesterone secretion

The basic experimental design consisted of incubating 1 ml of dispersed CL cells (5 x 10^5 viable cells/ml) in MEM with added LH (1.0 µg/5 x 10^5 viable cells – Carlson et al., 1995) in 12 x 75 mm glass tubes at 37°C in an atmosphere of 95% air and 5% CO_2. At pre-determined time intervals, the test tubes were centrifuged at 1000 x g for 6 minutes,

the media removed and frozen for future progesterone analysis by radioimmunoassay (RIA) using the procedure described previously (Carlson and Gole, 1978). To determine the effect of PHEQ, PHE, ANT, 1,2-dhATQ or copper (singly or in combination) on progesterone secretion, CL cells were incubated with various doses of toxicant in the presence of added LH. For this study, each experiment was replicated 3 or more times and dispersed cells for each replicate were pooled from ovaries of 4 or more rats. None of the PAHs were found to affect the binding of the anti-progesterone antibody to progesterone in the assay.

2.2.5 Detection of Reactive Oxygen Species (ROS)

The production of ROS by CL cells was monitored using the fluorescent probe H_2DCFDA. This probe diffuses into cells and is cleaved by cellular esterases to yield H_2DCF, which can be oxidized by ROS to the fluorescent product DCF (Brubacher et al., 2003). The media used for the fluorescence assays was a phosphate-buffered saline containing 5 mg/mL glucose (PBSG – pH 7.4) due to the high background fluorescence with MEM, the media used in the progesterone experiments. This background fluorescence was minimal using PBSG and was subsequently used for all fluorescence assays. The first set of experiments examined the ability of the PAHs alone to generate ROS. The cell suspensions were plated at 2.5 x 10^5/microwell followed by the addition of various concentrations of PAHs and 50 nM H_2DCFDA. ROS formation was monitored for 4 hours using a fluorimetric plate reader (Cytofluor, Series 4000, PerSeptive Biosystems) with excitation and emission filters of 485 nm and 530 nm respectively.

When testing the ability of Cu^{2+} to produce ROS, we found that the probe fluoresced in the presence of Cu^{2+}. In order to minimize the contact of Cu^{2+} with H_2DCFDA, cells (5 x 10^5/mL) were incubated with PAH and/or Cu^{2+} for 20 minutes in microcentrifuge tubes at 37°C then centrifuged, the media removed and the cells resuspended in fresh PBSG. The cells were plated at the same cell density as before followed by 50 nM H_2DCFDA. ROS production was monitored over 4 hours as stated above.

2.2.6 Viability Assessment

CL cell viability was assessed using the Trypan Blue dye exclusion test. For samples incubated in MEM, duplicate aliquots were removed from each test tube at the end of the 4 hour incubation period and mixed with Trypan Blue for approximately 2 minutes. The ability of the CL cells to exclude the dye was assessed using a hemocytometer. The viability of treated cell samples was divided by the viability of the untreated control samples and then multiplied by 100. Samples tested in PBSG were treated the same as in the ROS assay prior to Trypan Blue viability assessment.

2.2.7 Copper accumulation by CL cells

To assess the levels of copper taken up by CL cells, atomic absorption spectroscopy (AAS) analysis was performed using a graphite furnace AAS (SpectrAA-600, Varian, Lake Forest, CA) according to Borgmann et al (Borgmann et al., 1998) with a few modifications. After incubation, CL cell samples in microcentrifuge tubes were centrifuged at approximately 1000 x *g* for 6 minutes in order to pellet the cells without

lysing. Media was removed and the cells were dried overnight in a 50°C oven. They were digested by adding concentrated HNO_3 acid (UltraPure grade, VWR/Canlab) and incubating at room temperature for 3 days. Sterile Milli-Q water was added before the samples were diluted for analysis on AAS.

2.2.8 Statistics

Significance was tested by two-way ANOVA followed by Fisher's LSD post hoc test for significance. Data that did not fit into the ANOVA was analyzed by Student's paired *t* test.

2.3 Results

2.3.1 Copper accumulation by CL cells

In both media to be used in the upcoming experiments, AAS analysis revealed that the amount of Cu^{2+} present in the luteal cells incubated in PBSG was from 2 fold, for 10 μM Cu^{2+} added, to 10 fold, for 100 μM Cu^{2+} added, higher compared to cells incubated in MEM (Figure 2.2). Also tested was the hypothesis that PHEQ may influence Cu^{2+} uptake. No significant difference was observed between samples treated with Cu^{2+} alone and the addition of PHEQ to Cu^{2+} treated cells (Figure 2.2).

2.3.2 Inhibition of LH-Stimulated Progesterone Secretion by PAHs and Copper

The pseudopregnant rat model responds to LH stimulation maximally at day 4 after hCG injection (Carlson et al., 1995) . In the present study, it was observed that a 4-hour exposure to 1.0 μg of LH/5 x 10^5 cells/ml caused a 4 – 5 fold increase in progesterone secretion in MEM. Increasing the amount of LH added to the cell suspension did not significantly stimulate additional progesterone secretion and cell viability was adversely affected as concentrations of added LH increased (data not shown).

Previously it was demonstrated that by incubating dispersed luteal cells for 2 hours with PHEQ an inhibition of LH-stimulated progesterone production occurred in a concentration-dependent manner (Nykamp et al., 2001). The experiment was repeated with PHEQ in the present study except that it was incubated with 0.1, 1.0 and 10.0 μM for 4 hours (Figure 2.3). The effect of incubating luteal cells with Cu^{2+} alone was tested as well. External concentration of Cu^{2+} up to 100 mM did not have a significant effect on

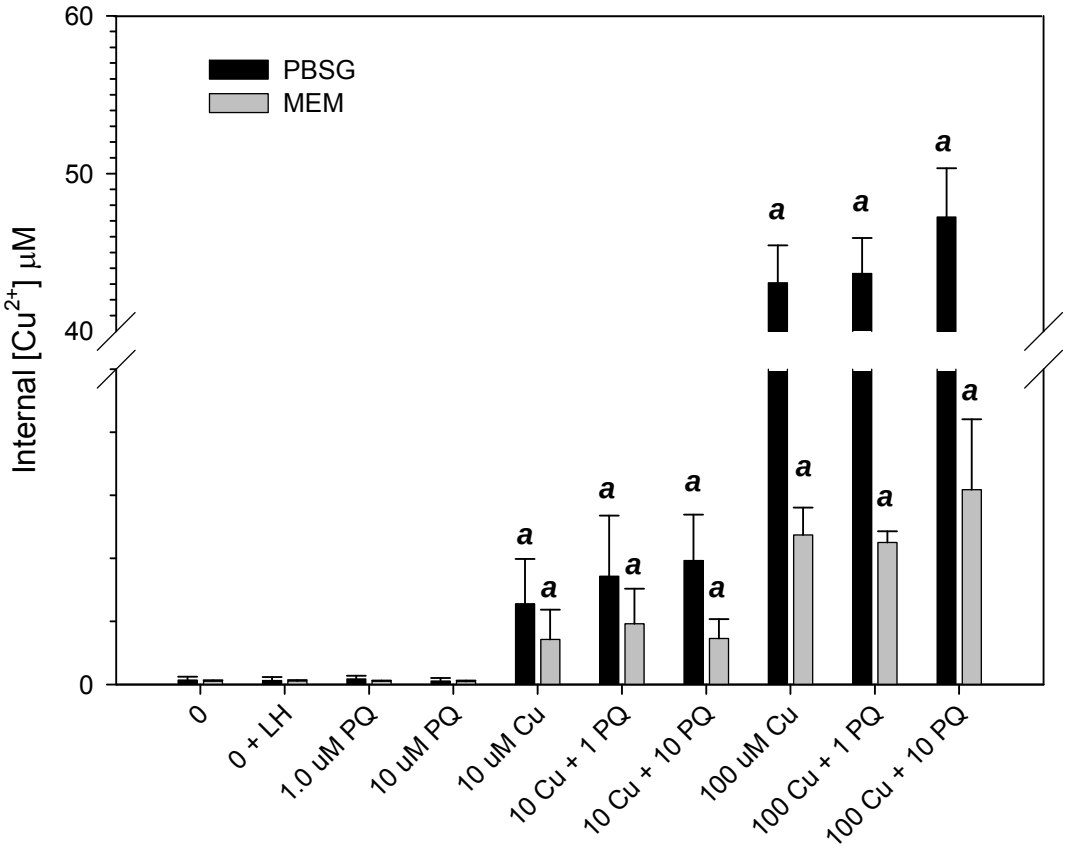


Figure 2.2 Effect of media on Cu^{2+} accumulation in CL cells. For exposure in MEM, CL cells were treated with varying doses of Cu^{2+} for 4 hours. In PBSG, CL cells were exposed for 20 minutes with Cu^{2+} before being centrifuged, the media containing toxicants removed, and the cells resuspended in fresh media and incubated for an additional 4 hours. ***a*** Significantly different from control. Results analyzed using Student's paired t-test ($p < 0.05$).

progesterone secretion. However, when CL cells were co-incubated with Cu^{2+} (100 μM) and PHEQ, there was a greater inhibition of progesterone production than with exposure to PHEQ alone (Figure 2.3). Interestingly, 100 μM Cu^{2+} appeared to enhance PHEQ inhibition of progesterone secretion the greatest at lower doses of PHEQ (0.1 and 1.0 μM) but not with the highest dose (10 μM) compared to PHEQ addition alone. The amount of LH-stimulated progesterone secreted at 10 μM PHEQ + 100 μM Cu^{2+} approached the basal level. Therefore, this represents the lowest amount of progesterone secreted by CL cells and would explain why no further inhibition of LH-stimulated progesterone production was observed. Calculations to determine the possibility of a synergistic greater than additive inhibition used the following formula:

$E_{AB} = E_A + E_B - E_AE_B$ (Sűhnel, 1996).

where E = % effect

While it appeared that 0.1 μM PHEQ + 100 μM Cu^{2+} had a synergistic inhibition, statistically this was not the case. However, the addition of 1.0 μM PHEQ + 100 μM Cu^{2+} to CL cells did statistically cause a synergistic inhibition in LH-stimulated progesterone secretion. The presence of 10 μM Cu^{2+} did not appear to enhance PHEQ toxicity over that observed with PHEQ alone. The parent compound of PHEQ, phenanthrene (PHE), did not significantly alter progesterone secretion alone or with Cu^{2+} at any concentration of PHE tested (data not shown).

To determine the speed with which the contaminants act, kinetic studies on LH-stimulated progesterone secretion were performed. It is well-known that LH activates the signal transduction pathway that leads to progesterone production within minutes (Duleba et al., 1999). This study confirmed that LH significantly stimulated progesterone

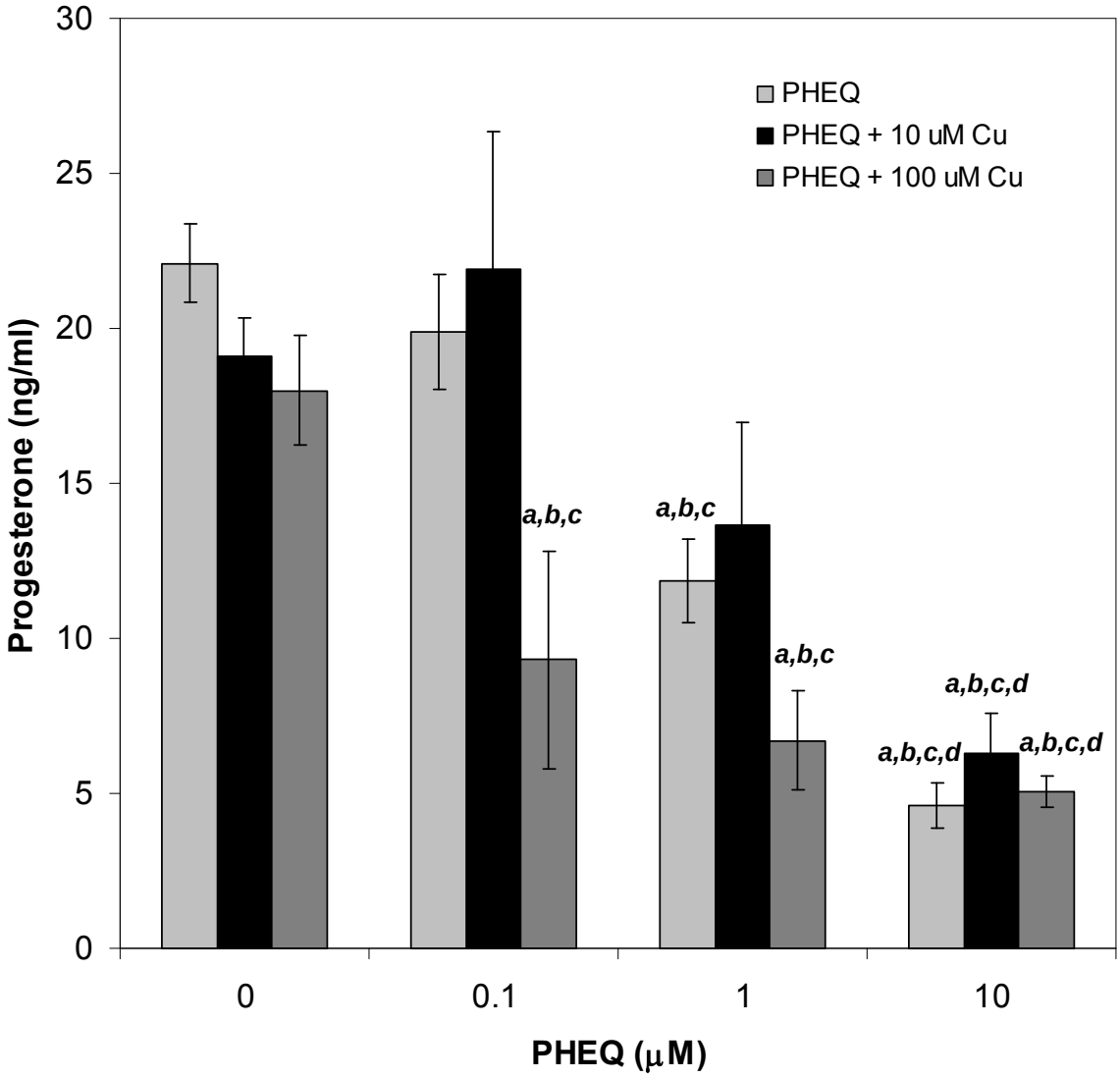


Figure 2.3 Inhibition of LH-stimulated (1.0 µg LH/10^5 cells) progesterone production in CL cells with exposure to PHEQ with and without 100 µM Cu^{2+}. PHEQ alone significantly inhibited progesterone secretion in a dose dependent manner. The addition of Cu^{2+} alone did not have a significant effect on progesterone secretion. However, in the presence of Cu^{2+}, PHEQ elicited a greater inhibitory effect on progesterone production when compared with PHEQ alone. ***a*** Significantly different from 0 µM PHEQ with or without Cu^{2+} added. ***b*** Significantly different from 0.1 µM PHEQ without Cu^{2+} added. ***c*** Significantly different from 0.1 µM PHEQ + 10 µM Cu^{2+} added. ***d*** Significantly different from 1.0 µM PHEQ without Cu^{2+} added. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

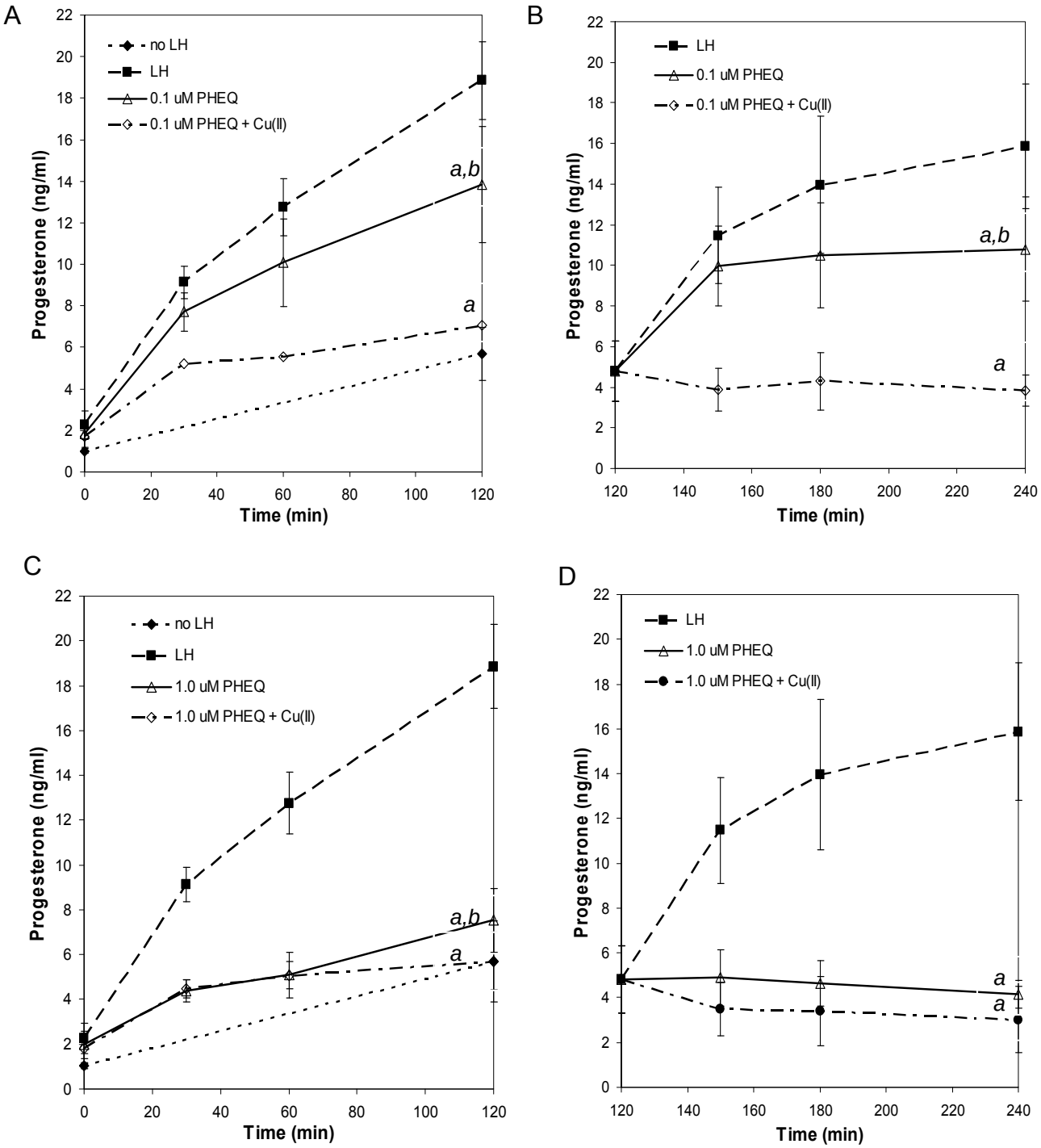

A
no LH
LH
0.1 uM PHEQ
0.1 uM PHEQ + Cu(II)
a,b
a
Progesterone (ng/ml)
Time (min)
B
LH
0.1 uM PHEQ
0.1 uM PHEQ + Cu(II)
a,b
a
Progesterone (ng/ml)
Time (min)
C
no LH
LH
1.0 uM PHEQ
1.0 uM PHEQ + Cu(II)
a,b
a
Progesterone (ng/ml)
Time (min)
D
LH
1.0 uM PHEQ
1.0 uM PHEQ + Cu(II)
a
a
Progesterone (ng/ml)
Time (min)

Figure 2.4 Kinetic analysis of 0.1 or 1.0 µM PHEQ with and without 100 µM Cu^{2+} on LH-stimulated progesterone production in CL cells. The addition of chemicals and 1.0 µg LH at time 0 (A and C) and pre-incubation of the chemicals for 2 hours prior to LH stimulation (B and D) illustrate the rapidity with which the contaminants act upon LH-stimulated progesterone secretion. CL cells treated with LH alone show maximal stimulation compared to basal progesterone levels over the various time periods. Cells treated with 0.1 or 1.0 µM PHEQ rapidly inhibited LH-stimulated progesterone secretion. The incubation of 0.1 or 1.0 µM PHEQ + 100 µM Cu^{2+} also caused a rapid and significant inhibition. Pre-incubation with the contaminants does not appear to enhance toxicity. [a] Significantly different from LH treatment at corresponding time point. [b] Significantly different from LH treatment at initial addition. Results analyzed using Student's paired t-test ($p < 0.05$).

within 30 minutes with no further significant stimulation after 120 minutes (Figures 2.4A and C). The addition of 0.1 and 1.0 μM PHEQ significantly inhibited LH stimulation. Both treatments caused significant inhibition by 120 minutes, but only 1.0 μM PHEQ significantly inhibited within 30 minutes of addition. Combining 100 μM Cu^{2+} and 0.1 or 1.0 μM PHEQ also rapidly inhibited LH-stimulated progesterone production. However, only the combination of 0.1 μM PHEQ + 100 μM Cu^{2+} resulted in significantly inhibiting progesterone secretion further than PHEQ alone (Figure 2.4A). Interestingly, with 1.0 μM PHEQ +/- 100 μM Cu^{2+} LH-stimulated progesterone levels were decreased to the point of basal (non-LH stimulated) progesterone production. Given that LH appeared to rapidly stimulate progesterone production, CL cells were also pre-incubated with PHEQ or PHEQ + 100 μM Cu^{2+} for 2 hours prior to LH stimulation to determine if extra time to diffuse into CL cells would enhance inhibition of LH (Figures 2.4B and D). In both experiments, this did not significantly inhibit LH-stimulated progesterone secretion beyond addition of chemicals at time 0.

To investigate potential targets of PHEQ and Cu^{2+} action, two substrate analogues were substituted into the CL steroidogenic pathway in the absence of added LH. Forskolin, an adenylate cyclase activator, was used with and without chemicals added. In the presence of both PHEQ and Cu^{2+}, progesterone secretion was still significantly inhibited compared to forskolin stimulation alone (Figure 2.5). The cholesterol analogue 22-hydroxycholesterol (22-OHC) is capable of crossing cellular and mitochondrial membranes. Since cytochrome P450 side-chain cleavage enzyme (P450scc) is constitutively active, the addition of 22-OHC allows increasing progesterone synthesis in a concentration-dependent manner (Sawada and Carlson, 1994). In this experiment,

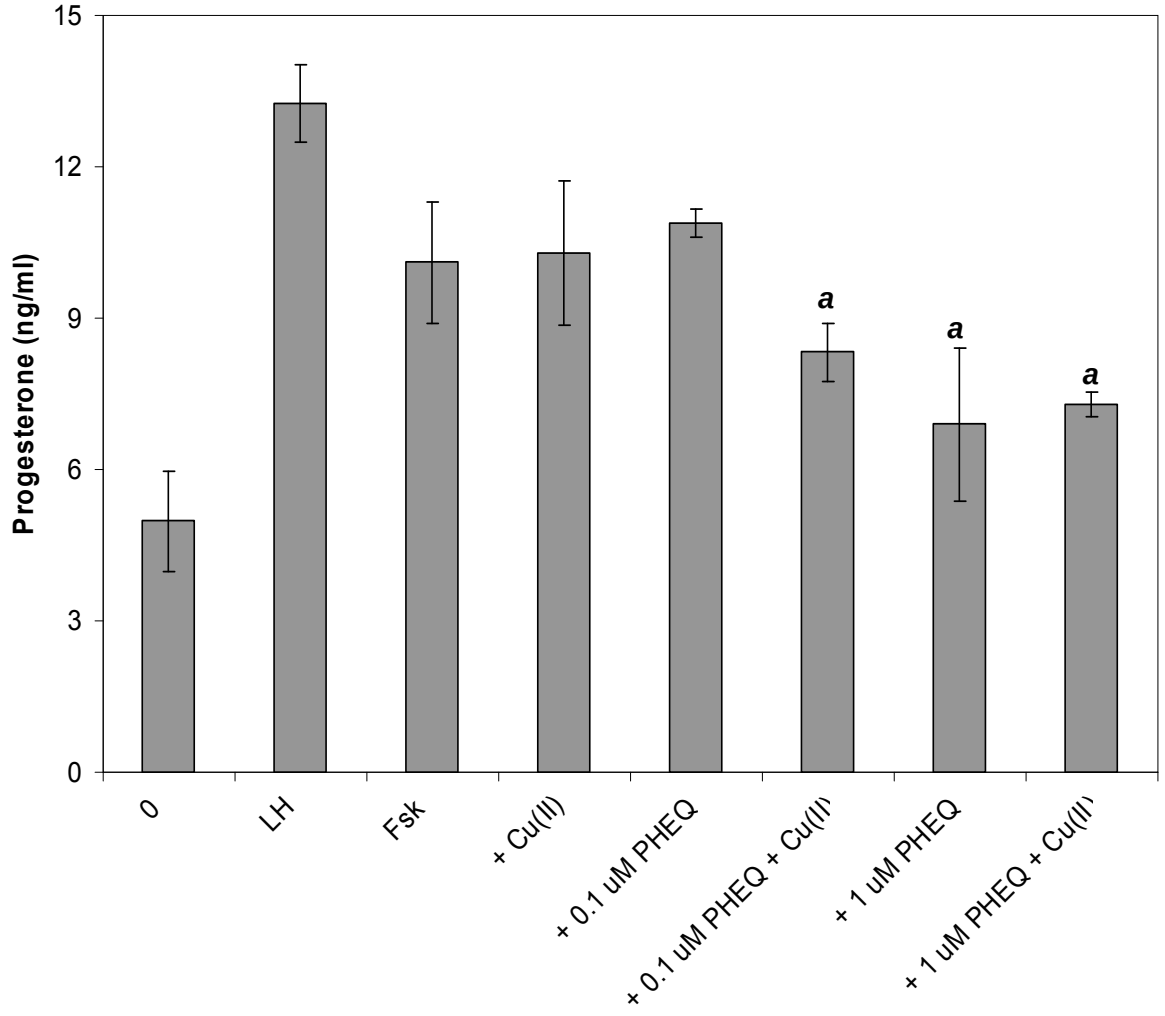


Figure 2.5 Effect of PHEQ and Cu^{2+} on forskolin stimulated progesterone production in CL cells. The addition of 50 μM forskolin doubled progesterone secretion from basal levels without added LH. Treatment of CL cells with forskolin and 100 μM Cu^{2+} or 0.1 μM PHEQ did not significantly inhibit forskolin stimulated progesterone production. Incubating cells with 0.1 μM PHEQ and 100 μM Cu^{2+} or 1.0 μM PHEQ with or without 100 μM Cu^{2+} did significantly inhibit progesterone secretion in forskolin treated cells. [a]Significantly different from forskolin treatment. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

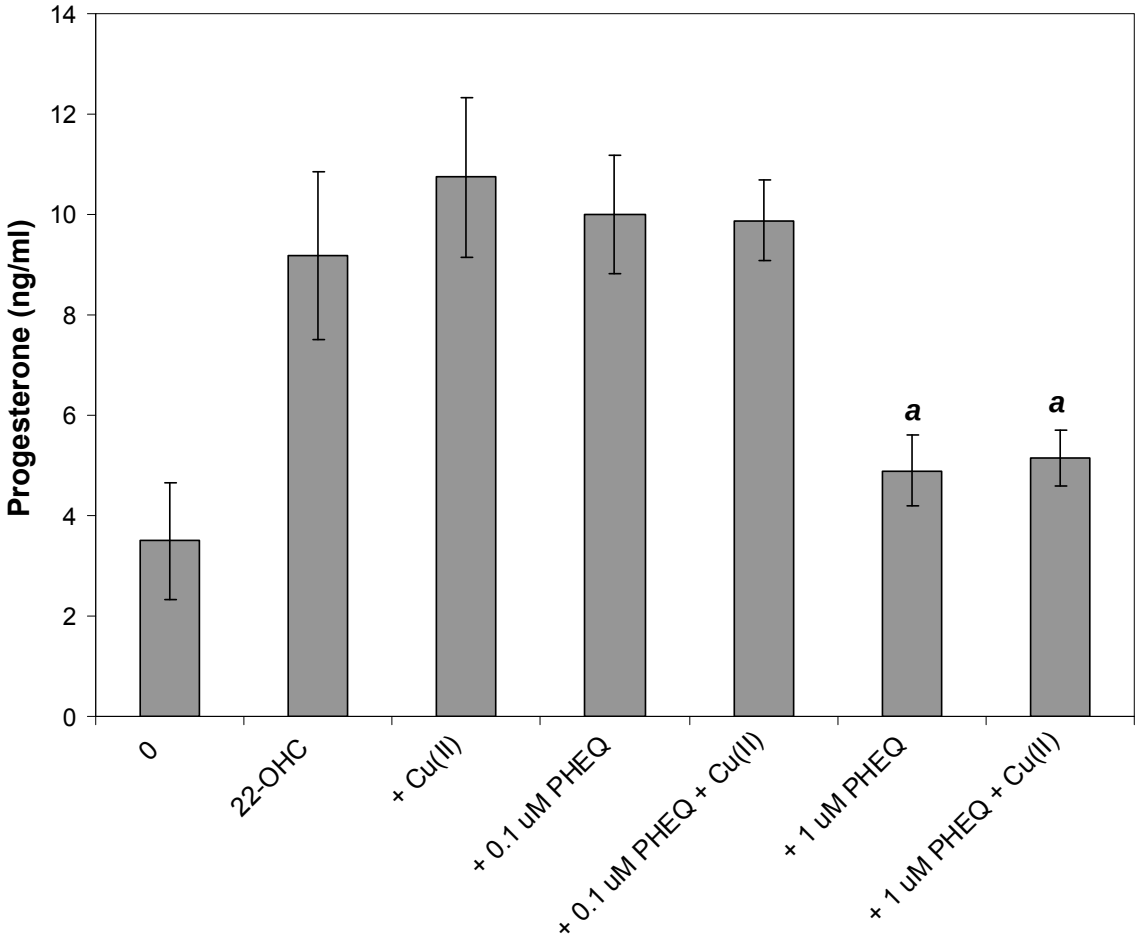


Figure 2.6 Effect of PHEQ and Cu^{2+} on 22-hydroxycholesterol (22-OHC) stimulated progesterone secretion in CL cells. The addition of 1.0 µg 22-OHC doubled progesterone secretion from basal levels without added LH after 4 hour incubation. Treatment of CL cells with 22-OHC and 100 µM Cu^{2+} or 0.1 µM PHEQ did not significantly inhibit 22-OHC stimulated progesterone production. Incubating cells with 1.0 µM PHEQ with or without 100 µM Cu^{2+} did significantly inhibit progesterone secretion in 22-OHC treated cells. *Significantly different from 22-OHC treatment. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

adding 22-OHC also significantly elevated progesterone production over basal levels. Exposing CL cells treated with 22-OHC to PHEQ with and without Cu^{2+} significantly inhibited this increase in progesterone (Figure 2.6).

The initial experiments conducted with PHEQ and Cu^{2+} were repeated with another contaminant, 1,2-dihydroxyanthranquinone (1,2-dhATQ). The addition of 1,2-dhATQ to LH stimulated CL cells caused a dose-dependent inhibition of progesterone secretion (Figure 2.7). However, co-incubating the cells with Cu^{2+} and 1,2-dhATQ did not show any more significant inhibition than with the treatment of 1,2-dhATQ alone. The parent compound of 1,2-dhATQ, anthracene (ANT) was not tested.

2.3.3 Effect of PAHs and Copper on Luteal Cell Viability

Due to differences in Cu^{2+} uptake depending on the media used, experiments detailing the effect of PHEQ and Cu^{2+} on CL cell viability were conducted in both MEM and PBSG. Exposure of CL cells to PHEQ (1.0 – 10 μM) caused a significant decrease in viability (Figure 2.8). Only the highest dose of Cu^{2+} (100 μM) alone to CL cells in MEM caused a slight, but significant decline in cell viability (Figure 2.8). The co-incubation of 100 μM Cu^{2+} with increasing doses of PHEQ in MEM caused a further dramatic decrease in CL cell viability compared to exposure of PHEQ alone (Figure 2.8). The possibility of greater than additive toxicity was calculated using the same formula as before. In all cases of PHEQ + 100 μM Cu^{2+}, the toxicity observed was greater than additive (Table 2.1). Viability experiments conducted in PBSG used lower concentrations of Cu^{2+} due to increased Cu^{2+} uptake into CL cells (Figure 2.2). It was found that Cu^{2+} significantly decreased CL cell viability with as little as 1.0 μM (Figure

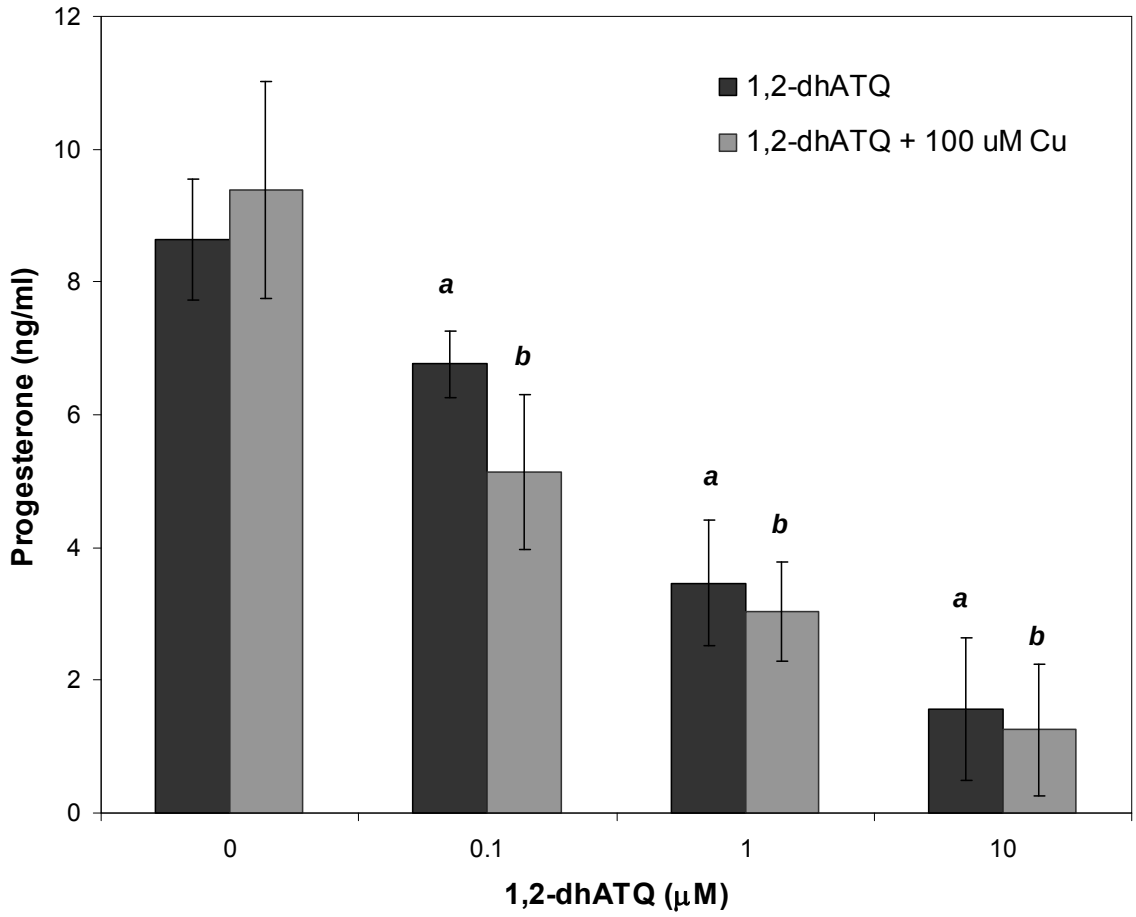


Figure 2.7 Effect of 1,2-dhATQ and Cu^{2+} on LH-stimulated (1.0 µg LH/10^5 cells) progesterone production. 1,2-dhATQ alone significantly inhibited progesterone secretion in a dose dependent manner. The addition of Cu^{2+} alone did not have a significant effect on progesterone secretion. Furthermore, the combination of Cu^{2+} with 1,2-dhATQ did not have any significant inhibitory effect on progesterone production when compared with 1,2-dhATQ alone. ***a*** Significantly different from LH-stimulated progesterone production. ***b*** Significantly different from LH + 100 µM Cu^{2+} progesterone secretion. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

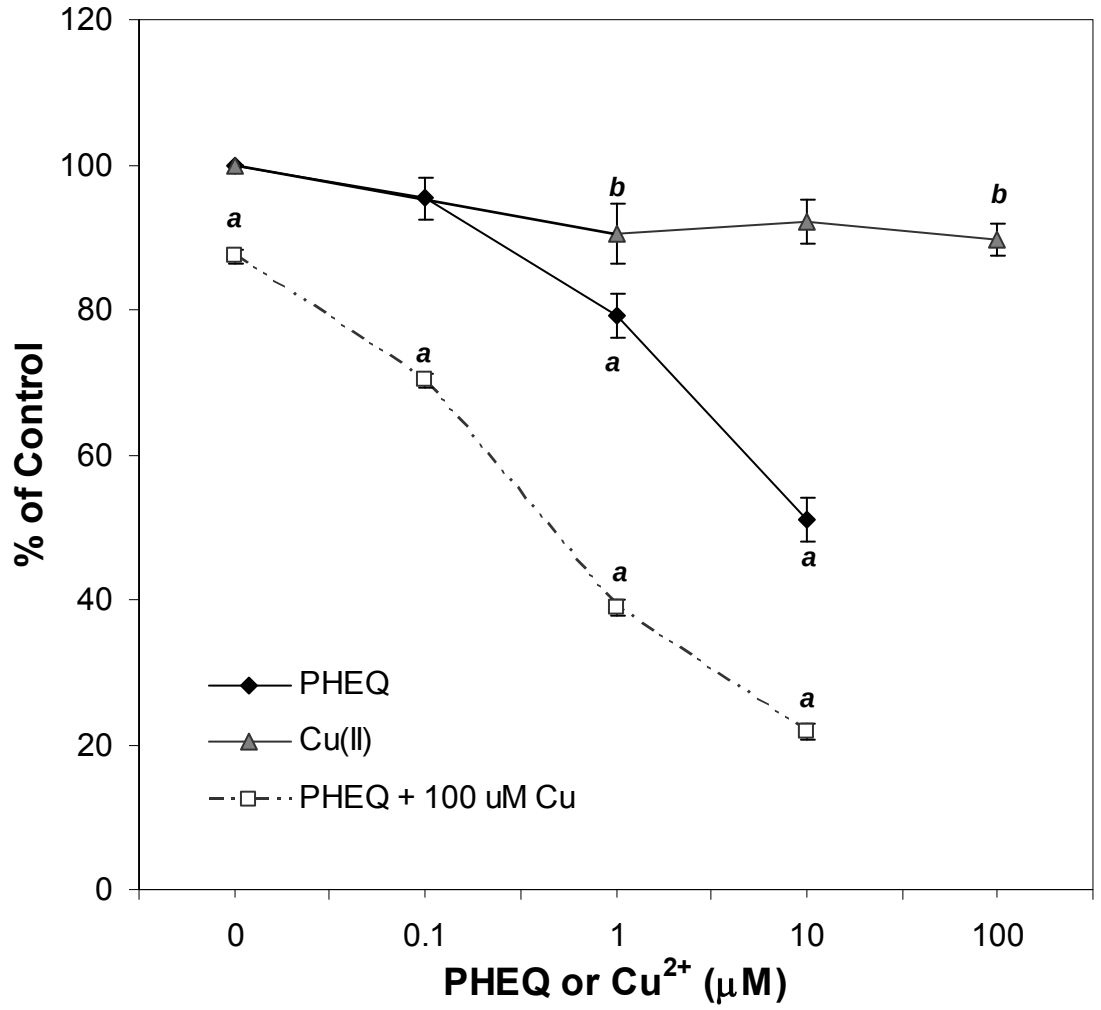


Figure 2.8 Effect of PHEQ and Cu^{2+} on CL cell viability. All incubations were conducted over 4 hours in MEM. The addition of Cu^{2+} alone caused a small but significant decrease in CL cell viability at 100 µM while exposure to PHEQ resulted in a significant decrease at 1.0 µM. The addition of 100 µM Cu^{2+} to PHEQ caused a dramatic decrease in CL viability from control. [a] Significantly different from all other data points. [b] Significantly different from 0. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

2.9). PHEQ also caused a significant decrease in viability in PBSG (Figure 2.9) but at concentrations comparable to those used in the MEM viability experiments (Figure 2.9). Combining Cu^{2+} with 0.1 μM or 1.0 μM PHEQ did not show a greater than additive toxicity as was seen in viability experiments conducted in MEM (Figure 2.9).

The ability of 1,2-dhATQ and Cu^{2+} to effect CL cell viability were conducted in MEM as was done with PHEQ and Cu^{2+}. Exposure of CL cells to 1,2-dhATQ (0.1 – 10 μM) caused a significant decrease in viability (Figure 2.10). The co-incubation of 100 μM Cu^{2+} with increasing doses of 1,2-dhATQ caused a further reduction in CL cell viability compared to exposure of 1,2-dhATQ alone (Figure 2.10).

Treatment	MEM (100 μM Cu^{2+})		
	Predicted Toxicity	Actual Toxicity	p (<0.05)
0.1 μM PHEQ + Cu^{2+}	83.7 ± 3.9	70.3 ± 3.5	0.013
1.0 μM PHEQ + Cu^{2+}	69.2 ± 3.5	38.9 ± 4.8	0.000
10 μM PHEQ + Cu^{2+}	44.4 ± 3.5	21.8 ± 2.8	0.001
	PBSG (0.1 μM PHEQ)		
1.0 μM Cu^{2+} + PHEQ	64.3 ± 9.2	78.6 ± 9.0	0.073
3.0 μM Cu^{2+} + PHEQ	58.5 ± 8.9	65.3 ± 9.7	0.360
6.0 μM Cu^{2+} + PHEQ	48.5 ± 3.3	67.6 ± 7.6	0.098
10 μM Cu^{2+} + PHEQ	42.0 ± 4.8	48.6 ± 2.7	0.073

Table 2.1 Calculation of synergism in toxicity between Cu2+, PHEQ and mixtures of the two contaminants. The difference between predicted and actual toxicity in viability experiments conducted in MEM shows a consistent and statistically significant synergistic greater than additive effect with PHEQ and Cu^{2+} mixtures. When tested in PBSG, the greater than additive toxicity is abolished.

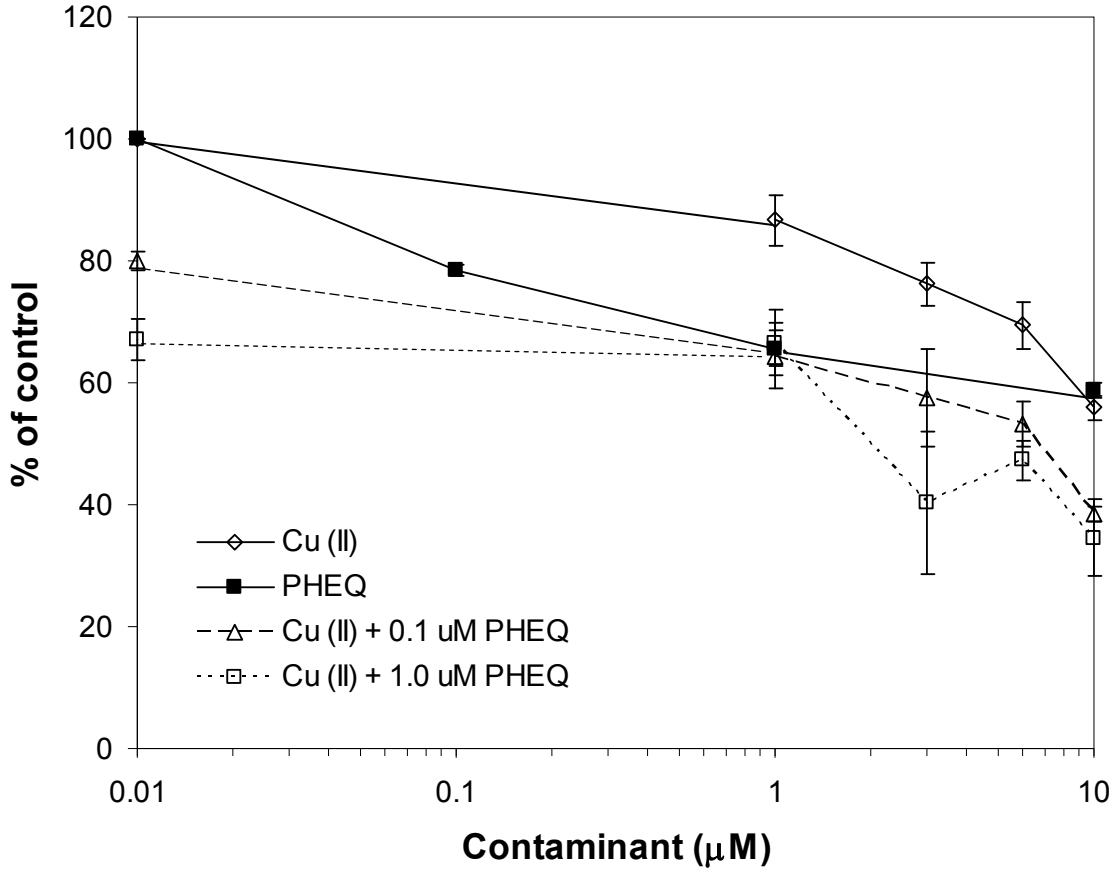


Figure 2.9 Effect of PHEQ with and without Cu^{2+} on CL viability in PBSG. Cu^{2+} alone caused a significant decrease in CL cell viability at 1.0 µM. Exposure to PHEQ also resulted in a significant decrease at 0.01 µM. The addition of 0.1 µM PHEQ to Cu^{2+} to caused a further reduction in CL viability from control as did increasing PHEQ to 1.0 µM. ***a*** Significantly different from 0 PHEQ. ***b*** Significantly different from 0 Cu^{2+}. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

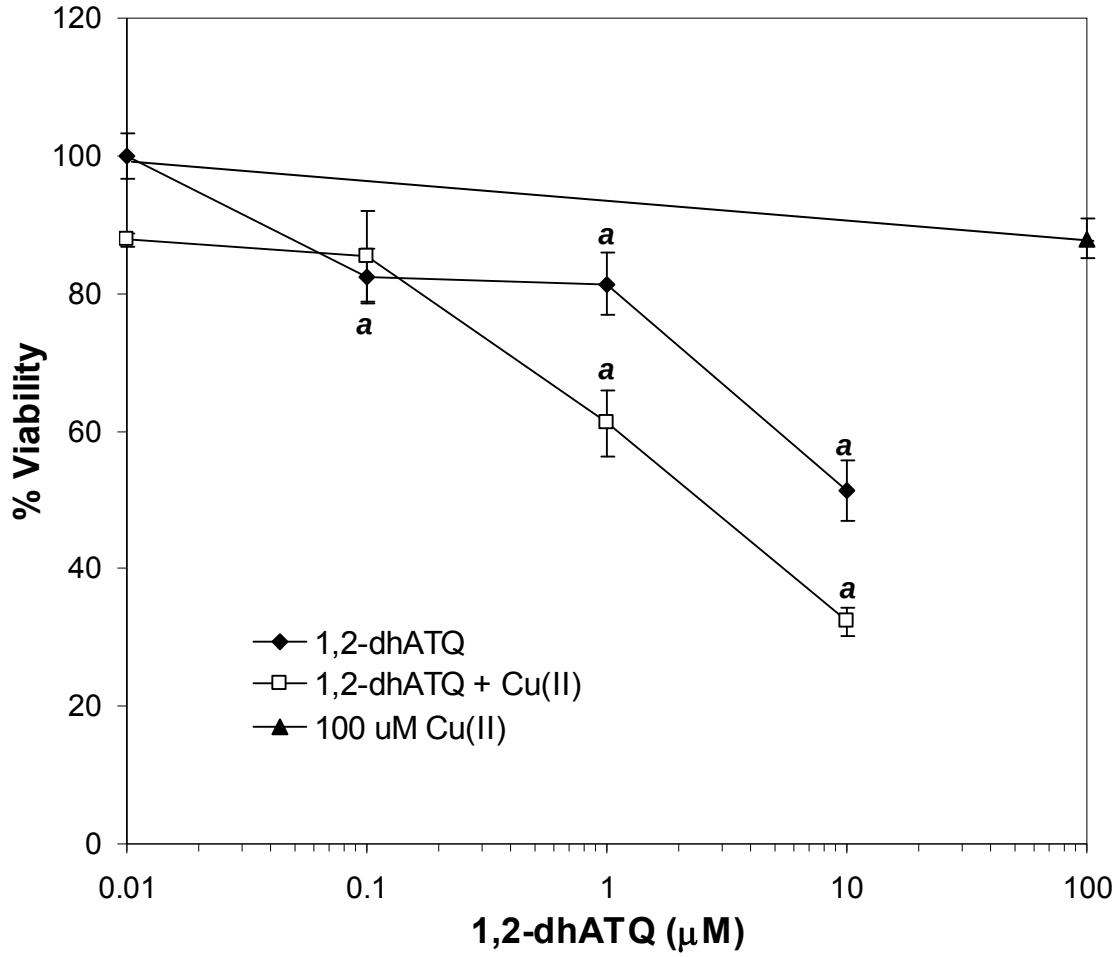


Figure 2.10 Effect of 1,2-dhATQ and Cu^{2+} on CL viability. All incubations were conducted over 4 hours in MEM. The addition of Cu^{2+} alone caused a small but significant decrease in CL cell viability at 100 μM while exposure to 1,2-dhATQ resulted in a significant decrease at 10 μM. The addition of 100 μM Cu^{2+} to 1,2-dhATQ caused an additional decrease in CL viability from control. *a* Significantly different from 0 1,2-dhATQ. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

2.3.4 ROS Production in Luteal Cells after PAH and Copper Exposure

All fluorescence assays were conducted with CL cells in PBSG since MEM interfered with fluorescence measurements. Generally, the probe 2',7'-dichlorodihydro-fluorescein diacetate (H_2DCFDA) penetrates the cell membrane where it is de-acetylated by cellular esterases in the cytoplasm to form H_2DCF. At this point, ROS can react with the non-fluorescent H_2DCF to produce the highly fluorescent DCF. This probe is regarded as a general ROS indicator since multiple ROS can react with H_2DCF (Brubacher et al., 2003). It was demonstrated previously that PHEQ is capable of stimulating > 800 % increases in ROS (Nykamp et al., 2001). These experiments were repeated in order to directly compare the ability of PHEQ and 1,2-dhATQ to generate ROS. Of these two chemicals, only PHEQ showed a significant dose-dependent increase in ROS over the 4 hour incubation period (Figure 2.11). These experiments were conducted by adding the desired concentration of the PAH to the cells just prior to introducing the probe. Cumulative ROS production was observed over 4 hours (Figure 2.11).

Further experiments were conducted to determine if the addition of Cu^{2+} enhanced the production of ROS. Unfortunately, controls consisting of Cu^{2+} and H_2DCFDA in PBSG without cells present exposed the non-specific reaction of Cu^{2+} with the probe. To minimize this reaction, CL cells were incubated with the toxicants in PBSG for 20 minutes at 37°C and then removed through gentle centrifugation prior to introducing the probe. This alteration in the method reduced non-specific fluorescence; however, overall fluorescence values were lower than previously observed. General trends within the sets of experiments for PHEQ and 1,2-dhATQ alone (Figure 2.11) and PAH + Cu^{2+} (Figure 2.12) may be compared but not between. Exposing CL cells to Cu^{2+} alone

resulted in significant ROS production. However, the combined treatment of Cu^{2+} and PHEQ caused a much more significant increase in ROS than either chemical alone (Figure 2.12). Treatment of CL cells with 1,2-dhATQ with added Cu^{2+} did not generate ROS over the 4 hour incubation period (data not shown).

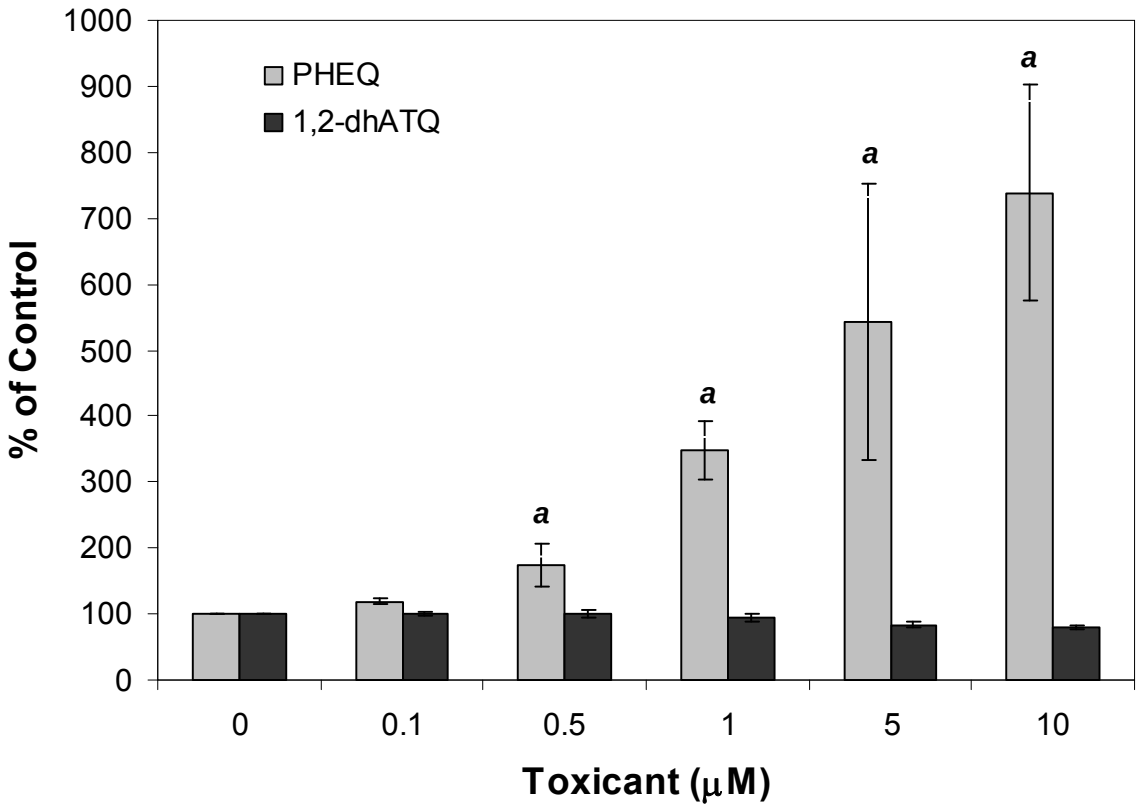


Figure 2.11 Effect of PHEQ and 1,2-dhATQ on ROS production in CL cells as measured by H_2DCFDA. All incubations were conducted over 4 hours in PBSG without washing treatments out of cells prior to addition of H_2DCFDA. Addition of PHEQ caused maximum 1000 fold increase in ROS compared to control over the incubation period. In the same time interval, no significant increase in ROS compared to control was observed after exposure to 1,2-dhATQ. [a] Significantly different from control. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

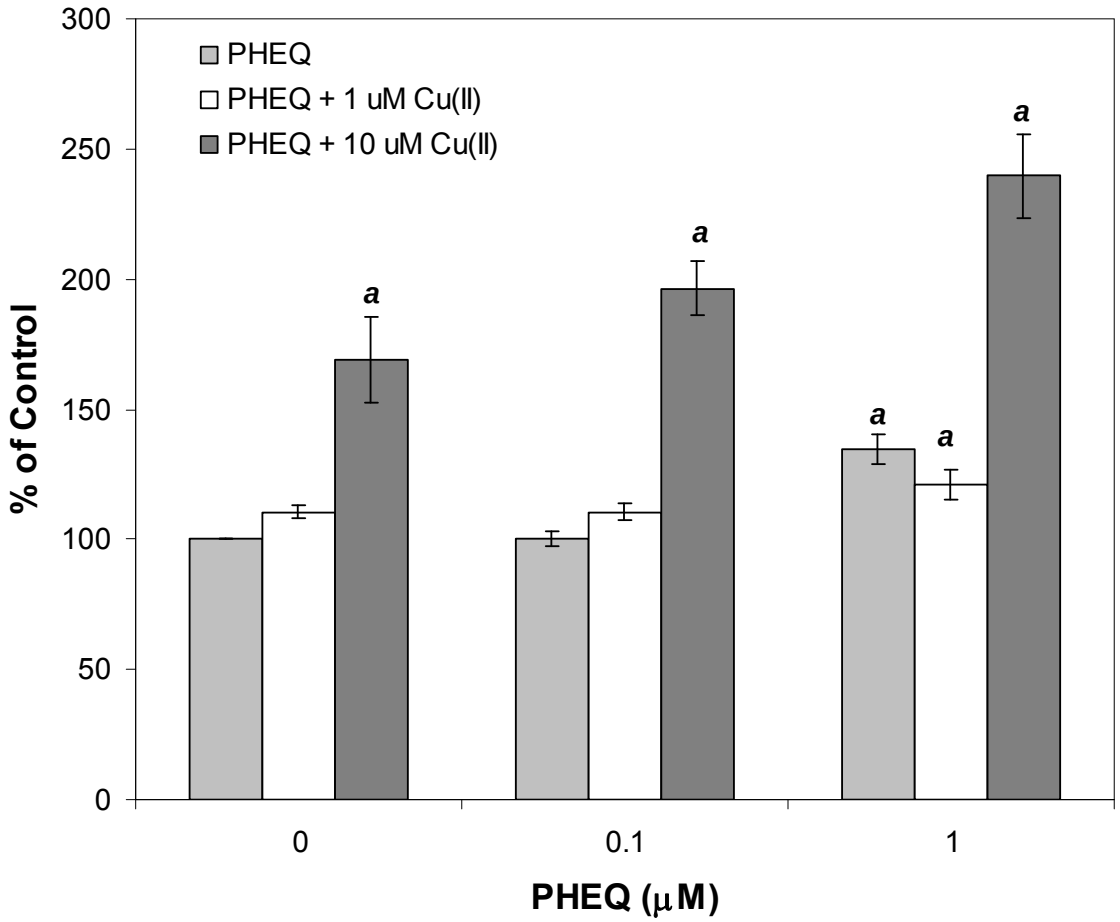


Figure 2.12 Effect of PHEQ and Cu^{2+} on ROS production as measured by DCF fluorescence. A significant increase in DCF fluorescence occurred with addition of 1.0 μM PHEQ alone. The incubation of CL cells with 10 μM Cu^{2+} alone also caused a significant increase in fluorescence. Co-incubation of Cu^{2+} with PHEQ significantly enhances fluorescence at each dose. *a* Significantly different from control. Results analyzed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

2.4 Discussion

The ability of oxyPAHs alone and in combination with Cu^{2+} to inhibit LH-stimulated progesterone production as well as viability in rat CL cells was assessed. Both oxyPAHs tested were able to inhibit progesterone secretion in CL cells under LH stimulation. Addition of Cu^{2+} alone did not affect either progesterone secretion or cell viability, but when combined with PHEQ, synergistic toxicity was observed. This was not found in mixtures of 1,2-dhATQ and Cu^{2+}. AAS analysis showed that PHEQ had no effect on Cu^{2+} uptake in CL cells. The ability for each chemical to produce ROS was tested as well. Only PHEQ alone and PHEQ with Cu^{2+} produced significant amounts of ROS with synergistic ROS generation found in PHEQ/Cu^{2+} mixtures. Addition of pseudo-substrate 22-OHC and cAMP stimulator forskolin helped to narrow the potential targets of toxic action to 7 sites. These sites pictured in Figure 2.12 include the plasma membrane and protein kinase A. Potential mitochondrial targets include StAR protein, P450scc and the mitochondrial membrane. Also of importance are the endoplasmic reticulum membrane and the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD).

2.4.1 Effect oxyPAHs on CL Cell Viability and LH-Stimulated Progesterone Production

This study demonstrated that the chemical contaminants PHEQ and 1,2-dhATQ both inhibited CL cell viability and progesterone production in the presence of added LH. For PHEQ treatments, the degree of inhibition in LH-stimulated progesterone secretion was 50 – 80% compared to 20 – 50% for CL cell viability at the same concentrations.

Conversely, the degree of inhibition for both CL cell viability and LH-stimulated progesterone secretion was similar suggesting that loss of progesterone production was likely due to loss in cell viability.

When comparing the overall loss in cell viability and inhibition of LH-stimulated progesterone secretion, the magnitude of effect between both chemicals was comparable. However, experiments showed that only PHEQ generated significant amounts of ROS as confirmed by our previous paper (Nykamp et al., 2001). While PHEQ is known as a redox cycler (Jarabak et al., 1997), it is likely that 1,2-dhATQ alone does not cause ROS production (Babu et al., 2001). Given the data from the experiments, the most plausible mechanism of PHEQ toxicity indicates ROS-mediated cellular damage. However, the decline in viability and LH-stimulated progesterone secretion upon 1,2-dhATQ exposure must be due to another mechanism since ROS production was not observed. Direct interactions with enzyme complexes have been noted previously with cytochromes appearing as the preferred target (Tripuranthakam et al., 1999, Takahashi et al., 2002). Due to the reliance of CL cells on P450scc for steroidogenesis combined with the demands on cytochromes present in the mitochondrial electron transport chain may make CL cells particularly sensitive to 1,2-dhATQ exposure.

ROS generated by various chemicals or cell processes are well-known for targeting cell membranes and proteins including those involved in steroidogenesis (Deimer et al., 2003, Dröge, 2002, Halliwell and Gutteridge, 1989, Vroegop et al., 1995). Various viability tests demonstrate membrane integrity and it is therefore plausible that ROS-mediated destruction of the cell membrane would be detected. Likewise,

steroidogenesis involves a signal transduction pathway (Figure 2.1) which uses many proteins ranging in location from the cell membrane to the cytosol and mitochondria. These proteins would be likely targets of ROS (Endo et al., 1993). Additionally, the mitochondria is known as a generator and target for ROS (Nemoto et al., 2000, Finkel, 2003). ROS-mediated damage of the signal transduction pathway or the mitochondria may result in inhibition of LH-stimulated progesterone synthesis. For both chemicals tested, mitochondria represent a particularly sensitive target whether through direct interaction with the toxicant in question or through toxicant-induced ROS-mediated damage.

2.4.2 Effect Cu^{2+} on CL Cell Viability and LH-Stimulated Progesterone Production

The metal contaminant tested was Cu^{2+}. This metal is used extensively in biological systems and the ability of Cu^{2+} to redox cycle accounts for its use in the reaction centers of enzymes. The concentration of this metal is tightly regulated in cells through use of metallothionine and Cu-chaperone proteins (Harrison et al., 1999). Copper may induce ROS production through a Haber-Weiss reaction whereby cuprous ions interact with H_2O_2 (Pourahmad and O'Brien, 2000). When tested in complex media, the effect of Cu^{2+} on either progesterone secretion or viability was minimal. However, viability was decreased to a greater extent when simpler media was used. This was likely due to two factors present in complex media that were in lower concentration or absent in the simpler media; chelators and antioxidants. The limited effect of Cu^{2+} on LH-stimulated progesterone secretion may be explained by these factors as well. While use of the complex media was necessary to accommodate progesterone production in CL cells,

AAS analysis demonstrated the extent to which the complex media chelated Cu^{2+}. While glucose is a copper chelator as well (Cerchiaro et al., 2005), the significantly greater levels of internal copper present in cells incubated in PBSG compared to those in MEM suggests that the chelating ability of glucose in PBSG is not as great as the constituents found in MEM. In addition to chelators in MEM, the presence of antioxidants also found in MEM may have masked ROS produced by Cu^{2+}.

2.4.3 Effect of combining Cu^{2+} with PHEQ or 1,2-dhATQ to CL cell viability and progesterone synthesis

The combination of PHEQ and Cu^{2+} provided a greater than additive effect in both CL cell viability and LH-stimulated progesterone production. When tested, this synergistic effect was shown to be statistically significant in some cases. However, due to high variability, some experiments did not show statistically significant synergism although this relationship was likely present. Synergism was demonstrated when lower doses of the chemicals were used in combination. Higher doses of the contaminants likely were too toxic either individually or combined to ascertain whether synergism had occurred. The fact that 100 μM Cu^{2+} was used in experiments conducted in MEM was justified when consulting the AAS data that showed only 1% of Cu^{2+} added to the media was available to the cells.

Viability experiments were also conducted using the simpler media, PBSG. In these tests, Cu^{2+} decreased viability in CL cells at a much lower concentration than was observed in assays using the complex media MEM. Cu^{2+} availability is the most reasonable explanation for this discrepancy. The presence of copper chelators in MEM

appeared to bind Cu^{2+} and lower the free [Cu^{2+}] to approximately 1 % of the original concentration, while 10 % of added Cu^{2+} was available as free [Cu^{2+}] when PBSG was used. Interestingly, the greater than additive toxicity when combining PHEQ and Cu^{2+} seen in MEM is not observed when PBSG is used. The reason for this discrepancy is not yet known, however, two possible mechanisms could account for the apparent loss of synergism. First, it is possible that without the presence of chelators to lower free [Cu^{2+}], the amount of Cu^{2+} present might have been too lethal for the cells. Second, the absence of potential antioxidants in the simpler media would have allowed higher levels of ROS to exist. A diagram representing the potential targets in the CL and how each chemical might mediate their effect is in Figure 2.12.

The experiments utilizing forskolin or 22-OHC were designed to narrow the list of potential targets of action for PHEQ and Cu^{2+}. The stimulation of progesterone production by forskolin, an adenylate cyclase inducer, was used to investigate whether the toxicants affected LH binding to its receptor, the activation of the G-protein after LH binding, or the ability of the α-subunit of the G-protein to activate adenylate cyclase. If addition of the contaminants caused no inhibition of forskolin stimulated progesterone production, the LH receptor, G-protein and adenylate cyclase would be implicated as possible targets. If inhibition occurred, the LH receptor, G protein and adenylate cyclase would be eliminated as targets. Since some inhibition occurred with the addition of PHEQ, this suggests that the target of PHEQ lies downstream of the receptor-G protein complex. Combining Cu^{2+} with PHEQ caused an enhancement of progesterone inhibition. This finding suggests that Cu^{2+} may affect the targets downstream of the LH receptor-G protein complex.

The cholesterol analogue, 22-OHC, has the ability to cross the mitochondrial membrane without assistance from the StAR protein or any other enzyme involved in translocating cholesterol to the mitochondria. It is well known that P450scc is constitutively active and will continuously convert cholesterol to pregnenolone when substrate is available (Behrman and Aten, 1991). Therefore, the ability of PHEQ and Cu^{2+} to inhibit 22-OHC stimulated progesterone production would suggest that either P450scc or 3β-HSD may be important targets for these toxicants. No inhibition would suggest a target upstream of P450scc. The addition of PHEQ did cause inhibition of progesterone production. However, combining Cu^{2+} with PHEQ did not cause any further decrease in progesterone. This finding suggests that Cu^{2+} may have targets upstream of P450scc and 3β-HSD.

Because progesterone production was inhibited by PHEQ in both experiments, the only common sites of action could be the two enzymes downstream of both forskolin and 22-OHC, P450scc and/or 3β-HSD. The chemical properties of PHEQ would allow it to move through both membranes and cytoplasm, therefore their ability to diffuse to the mitochondria is highly likely. Only in forskolin treated cells did the addition of Cu^{2+} and PHEQ result in enhanced inhibition compared to PHEQ alone and that no synergistic toxicity was observed. This suggests that there may be more than one target that the chemicals may act upon since we know from our LH-stimulated progesterone data that a greater than additive toxicity appears when these chemicals are presented together. Because we know that ROS are produced, anything that could react with ROS would become targets. This would include proteins, membranes and nucleic acids.

The co-incubation of 1,2-dhATQ with Cu^{2+} did not result in greater inhibition than 1,2-dhATQ alone as measured by LH-stimulated progesterone production. Combining Cu^{2+} with 1,2-dhATQ did result in a greater decrease in cell viability but this was not greater than additive. In contrast, addition of Cu^{2+} to PHEQ had a much more profound effect on both viability and LH-stimulated progesterone secretion. One reason may be the increase in ROS observed with the co-incubation of PHEQ with Cu^{2+}. No increase in ROS occurred after addition of Cu^{2+} to 1,2-dhATQ treatment. The mechanism behind the enhanced toxicity of PHEQ + Cu^{2+} may be due to the property of Cu^{2+} as a redox-active metal which in combination with PHEQ produced significantly greater amounts of ROS than either compound alone. With the greater than additive toxicity observed in both viability and progesterone synthesis, Cu^{2+} may be working as a catalyst in the formation of ROS. Studies with the plant *Lemna gibba* suggest that Cu^{2+} and PAH's, in this case 1,2-dhATQ, may act in a concerted manner to produce ROS in the photosynthetic electron transport chain of the chloroplast (Babu et al., 2001). However, the current data does not discount the possibility of these chemicals acting through separate mechanisms.

In conclusion, while these chemicals have the potential to directly inhibit LH-stimulated progesterone production, the ability of Cu^{2+} and PHEQ but not 1,2-dhATQ to produce ROS would explain the synergistic effect observed in LH-stimulated progesterone production and CL cell viability. It is a possibility as well that other divalent metal cations may act synergistically with PHEQ to produce ROS and will be investigated later in this thesis.

2.4.4 Toxicity and steroidogenesis

Several studies have documented the effect of ROS on the steroidogenic pathway with sensitive sites including adenylyl cyclase (Endo et al., 1993, Behrman and Aten, 1991) and more recently StAR protein expression (Deimer et al., 2003) as well as cytochrome P450 side-chain cleavage enzyme (P450scc) activity (Tsai et al., 2003). The steroidogenic pathway in the CL is initiated through binding of LH to its receptor and continues through a G-protein linked second messenger system. In addition, other steroidogenic specific elements such as StAR, P450scc, and 3β-hydroxysteroid dehydrogenase (3β-HSD) all have specific roles to play in steroid production. While studies have shown that P450scc is constitutively active (Tuckey and Atkinson, 1989, Tuckey, 1992), the StAR protein has been implicated as the rate-limiting step in this process and has been extensively reviewed (Stocco, 2001).

It is well known that the mitochondrion is a large producer of ROS during metabolic processes. While the specific location of StAR is still undetermined, it must come into contact with the outer mitochondrial membrane in order to deliver the cholesterol substrate to P450scc (Stocco, 2001, Kallen et al., 1998, Arakane et al., 1998). This suggests that StAR may be at increased risk for ROS damage compared to any other point in the steroidogenic cascade. P450scc, which is found in the inner mitochondrial membrane, may also be a target of ROS-mediated damage. The presence of antioxidants such as superoxide dismutase (SOD), catalase, and glutathione reductase in the mitochondrion confirm the need to protect the many redox-sensitive enzymes present.

Recently, researchers have demonstrated that ROS participate in many diverse aspects of normal cellular function including growth and apoptosis (Finkel, 2003). Unfortunately, it is still unclear how such varied responses are accomplished by ROS and whether or not ROS are effectors or products of the results observed. Members of the NADPH-oxidase family appear to require ROS generation for initiation of activity (Arbiser et al., 2002). Furthermore, it appears that integrin-mediated cell shape changes may use oxidants released from the mitochondria as part of its signaling pathway (Werner and Werb, 2002). Targets of these oxidants appear to be the reversible oxidation of protein-tyrosine phosphatases resulting in their regulation (Rao and Clayton, 2002, Blanchetot et al., 2002, and Meng et al., 2002).

Mounting evidence suggests that the redox state of the cell is a fine balance requiring small amounts to perform needed functions throughout the cell while uncontrolled levels can cause damage and death. While our understanding of the dangers of large, uncontrolled ROS production are well-developed, the possibility that low levels of ROS produced in the cell by toxicants might be a part of signal transduction (Pani et al., 2001), alter transcription (Bauer et al., 1999, Finkel, 2003), or initiate apoptosis (Xu and Finkel, 2002) is only beginning to take shape.

While the CL is characterized by its abundance of antioxidants, LH stimulation naturally causes a secretion of ascorbate from the cell rendering this model more susceptible to ROS attack than the non-stimulated cell Behrman and Aten, 1991). Additionally, antioxidant levels decrease as the CL ages and prepares for regression. Increases in ROS may prematurely initiate regression leading to an alteration of normal endocrine function. Other characteristics of the CL make this an interesting model for

toxicological study. Compounds that affect the CL on the biochemical and molecular level may have ramifications on the physiology of the entire organism. With its importance in the mammalian reproductive cycle, the rat CL model remains a paradigm for the study of reproductive toxicology. Additionally, this model provides a good compromise between the molecular and physiological approaches to the study of toxicology. The combination of the CL as a novel model system of study in endocrine toxicology coupled with the increasing validation of ROS as an important mechanism of toxicity presents a new direction for the study of toxicology that has the potential to shed light on many previously unknown phenomena.

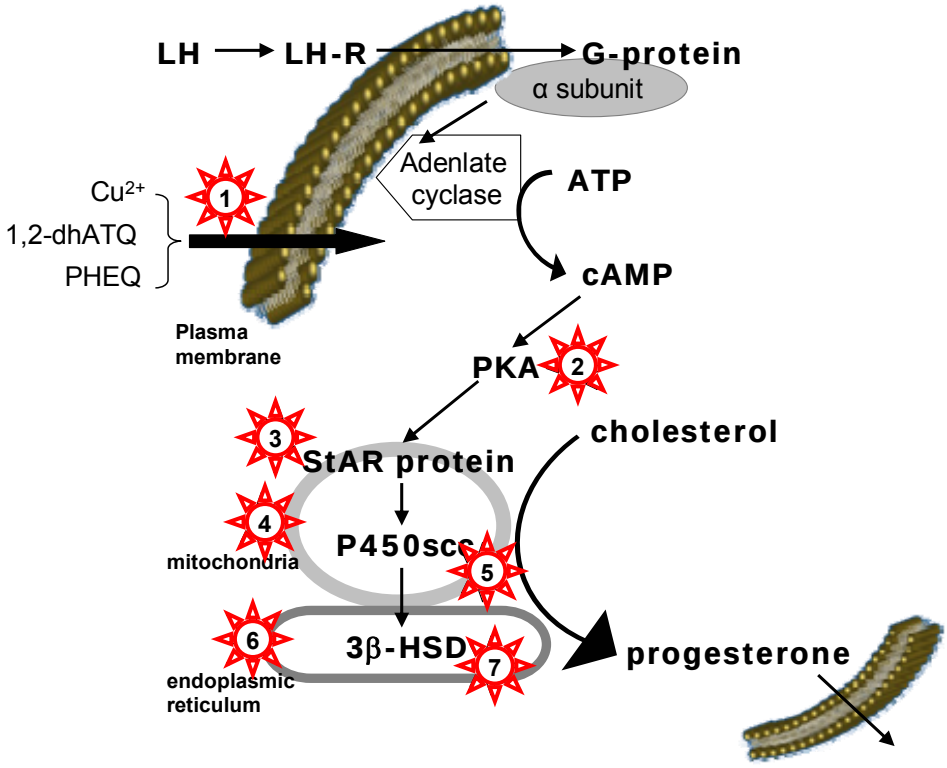


Figure 2.12 Diagramatic representation of potential targets for 1,2-dhATQ, PHEQ, and Cu^{2+}. Since both PHEQ and Cu^{2+} can produce ROS, this must also be considered as one of the facilitators of cellular damage. Target 1 refers to the plasma membrane whose fluidity may be perturbed by PHEQ or 1,2-dhATQ. ROS may also reduce membrane fluidity through lipid peroxidation. Target 2 indicates protein kinase A, an important signal transduction enzyme that could be inactivated by Cu^{2+}. Targets 3 and 4 both refer to StAR protein due to its activity as a transporter protein between the outer and inner mitochondrial membranes. PHEQ and 1,2-dhATQ could disrupt mitochondrial membrane permeability themselves or through ROS produced by either PHEQ or PHEQ with Cu^{2+}. P450scc is target 5 that could be inactivated by Cu^{2+} or ROS. Target 6 is the endoplasmic reticulum membrane which, like other membranes could have its fluidity compromised by PHEQ, 1,2-dhATQ or ROS. The enzyme 3β-HSD which converts pregnenolone to progesterone is target 7. This enzyme could be inactivated by Cu^{2+} or ROS.

Chapter 3 – Toxicity of Polycyclic Aromatic Hydrocarbons and Metal Mixtures to Rat Corpus Luteal Cells

3.1 Introduction

Polycyclic aromatic hydrocarbons have been studied for many years in regard to their acute and chronic toxicity in the form of lethality, mutagenicity and carcinogenicity (Bücker et al., 1979, Plant et al., 1983, Landrum et al., 1984, Mumford et al., 1995, Schirmer et al., 1998a, Schirmer et al., 1998b). The conjugated π-bonding orbitals in PAHs allow for absorption of UV light that can result in modifications of these compounds to oxygenated PAHs (oxyPAHs) and PAH quinones (Landrum et al., 1984, Mallakin et al., 1999, Mallakin et al., 2000, Brack et al., 2003). These oxyPAHs and quinones are much more reactive than their parent compounds (Oris and Giesy, 1985, Huang et al., 1993, McConkey et al., 1997). Quinones, especially *o*-quinones, have the ability to redox cycle either through producing superoxide anion radicals ($O_2^{\bullet -}$) or *o*-semiquinone radicals (Flowers-Geary et al., 1996). Whether the observed toxicity is the direct result of the reaction between the quinone and cellular targets, or indirectly due to the production of reactive oxygen species (ROS) appears to depend on the structure of the PAH and position of the oxygen atoms (Flowers et al., 1997, Shimada et al., 2004b, Zielinska-Park et al., 2004). Various studies have illustrated the differences in reactivity between *ortho*, *meta*, and *para* positioned oxygen atoms with the greatest being oxygens in the *ortho* position (Flowers et al., 1997, Shimada et al., 2004b).

PAHs have been examined for sub-lethal effects in reproductive tissues of various animals including humans. Early work using mouse embryonic cells demonstrated the ability of a variety of PAHs to induce irreversible morphological changes after exposure

(Kamei, 1980). For instance, benzo[a]pyrene (BaP) has been used to show that both fertility and primordial oocyte number can decline with exposure (Mattison et al., 1983). Other studies have revealed that many different adult and fetal tissue and cell types are sensitive to PAHs including maternal mammary glands (Moore and Gould, 1984, Werts and Gould, 1986), adult spermatogenic cells (Georgellis et al., 1989), fetal liver and lung (Hatch et al., 1990), maternal and fetal white blood cells (Whyatt et al., 2000), and adult lung tissues (Tao et al., 2003). While the literature on BaP exposure is extensive because it is a model carcinogen, few other PAHs including oxyPAHs have been tested in regard to mammalian reproductive sensitivity.

Metals are another class of environmental contaminant through their use in industrial manufacturing and mining that can be found in hazardous waste sites and as soil, air and water pollutants (Jacobson and Turner, 1980). Many sites have been assessed to determine the amounts of metals present in urban, industrial and natural areas. Some metals have been found repeatedly of which Cu, Cd, Hg, Ni, and Zn are of great concern (Karthikeyan et al., 2006, Karar et al., 2006, Madrid et al., 2006). Madrid et al (Madrid et al., 2006) sampled soils from various park sites across Europe and found that concentrations of Ni (11 – 207 mg/kg soil), Zn (46 – 225 mg/kg soil), and Cu (18 – 85 mg/kg soil) varied widely. The authors suggested that increasing park age and industrial activity appeared to correlate with the increasing levels of metals observed. Similarly, Karar et al (Karar et al., 2006) found that Cd (2 – 5 ng/m^3), Zn (483 – 529 ng/m^3), and Ni (7 – 8 ng/m^3) in air samples varied according to collection area with the highest levels found in industrial locales. Inorganic Hg is of special concern due to its volatility. Atmospheric deposition is considered the most important exposure route for soil and

water Hg contamination. Recently, (Lyman et al., 2007) developed a novel method for determining Hg deposition in dry environments. From their research, they found that upwards of 3.95 μg/m^2 was deposited on soil in various sites in Nevada (Lyman et al., 2007). From deposition, the probability of Hg leaching into groundwater after rainfall or adsorbing to soil particles is likely given that Hg is very water soluble. Couple solubility with its widespread deposition and Hg is a cause for concern to both plant and wildlife.

While exposure to metals is one area of concern, the ability of organisms to take up metals has been examined as well. Studies have found detectable levels of metals in most tissues of the human body with the highest concentrations found in the kidney and liver (Cu – 6.52 ppm liver, Zn – 65.31 ppm liver, Cd – 15.62 ppm kidney) (Saltzman et al., 1990). Regarding metal burdens in women specifically, tests have revealed Cu (0.2 μM), Zn (1.7 μM), and Cd (2.7 μM) present in amniotic fluid (Wathen et al., 1995) as well as Cd (0.5 μg/L) and Hg (4.2 μg/L) in urine samples (Brender et al., 2006). Osman et al (Osman et al., 2000) determined that some metals such as Pb and Zn but not Cd could easily cross the placenta into the fetus. Therefore, risk of metal toxicity to both mother and fetus depends on many factors including the innate toxicity of the metal, elimination from the body, and its ability to cross the placental barrier.

Predicting metal toxicity has led to the development of several models that attempt to account for bioavailability, metal speciation, and biological effect (Paquin et al., 2002). One of the first models proposed was the free-ion activity model (FIAM) which suggested that a biological response can be explained through metal speciation and interactions with the organism based on chemical equilibria (Morel, 1983). While the FIAM was more of a conceptual model, the gill surface interaction model (GSIM)

predicted metal bioavailability which provided a quantitative framework to interpret toxicity of individual and mixtures of metals (Pagenkopf, 1983). Both features were incorporated into the more readily accepted biotic ligand model (BLM).

The BLM accounts for metal speciation, competition with other metals, and reactions with other organic or inorganic compounds to predict bioavailability (Di Toro et al., 2001). Furthermore, metal toxicity is predicted by the BLM based on the formation of metal-biological ligand complexes. The BLM assumes that the target of metal interaction is in direct contact with the external environment and that the effect is proportional to the concentration of the metal bound to the target site (Vijver et al., 2004). One of the main assumptions of the BLM is that to exert its toxic effect, the metal must remain bound to the site of action and thus toxicity requires a ratio of metal:ligand as 1:1. This assumption does not account for toxicity observed at levels far below the level required for biotic ligand complexation, therefore alternative mechanisms must be considered. Additionally, the BLM is well-suited to describing acute toxicity of metals; however, the derived ligand-binding constants may not be applicable for predicting long-term exposure to chronic levels of metals.

Another mechanism of metal toxicity includes production of ROS. Many metals are redox active in biological systems, a property employed by organisms in enzymatic reactions. However, uncontrolled ROS generation leads to cellular damage in the form of lipid peroxidation of membranes, DNA adduct formation, and protein denaturation (Halliwell and Gutteridge, 1989). Organisms have developed families of defensive mechanisms including superoxide dismutases, peroxidases, and catalases (Fridovich, 1998). Antioxidants such as ascorbate and tocopherol are employed by organisms to

control free radical chain reactions (Fridovich, 1998). Organisms have learned to use ROS for a multitude of beneficial purposes as well. Many studies have demonstrated the use of ROS in normal cell growth, pathogenic defense, programmed cell death and cellular senescence (Dröge, 2002, Fleury et al., 2002, Finkel, 2003, Apel and Hirt, 2004, Fridovich, 2004). This dichotomy illustrates the delicate redox balance of the cell. The presence of redox active metals in organisms can affect the redox balance.

Some divalent metals, such as Cu^{2+} and Zn^{2+}, have known biological uses however; many more metals have no known biological function including Cd^{2+}, Ni^{2+} and Hg^{2+}. Even metals that are essential trace elements for organisms can be toxic which explains the myriad of protective mechanisms developed to tightly regulate metal concentration and distribution. Organisms have developed different strategies for regulating essential and non-essential metal concentrations. Essential metal concentrations can be regulated by either limiting uptake or increasing excretion/storage (Rainbow, 2007). Non-essential metal concentrations are controlled through increased excretion and internal storage. Unfortunately, whole body concentrations of non-essential metals generally increase with increasing external concentration. At the cellular level, mechanisms have evolved to minimize accumulation of non-essential metals and optimize use of essential metals. Some of these strategies include binding to thiol-containing proteins (i.e. metallothioneins), sequestration in vesicles (i.e. lysosomes), and binding to small organics (i.e. glutathione) (Vijver et al., 2004).

Cu is an important metabolic metal essential for living systems as a cofactor for key enzymes including cytochrome-c-oxidase (ATP synthesis), catalase, superoxide dismutase, and alkaline phosphatase. It is stored in the liver bound to metallothionein

and excreted through the bile bound to the transport protein ceruloplasmin. Cu at high concentrations has been implicated in altering protein structure by interacting with thiol and carbonyl groups (Kumar and Prasad, 2000, Kaur et al., 2006). Excess levels of Cu have also been linked to plasma membrane lipid peroxidation, nucleic acid damage, and apoptosis (Yruela et al., 1996, Karan et al., 1998, Halliwell and Gutteridge, 1989, Pourahmad and O'Brien, 2000, Gyulkhandanyan et al., 2003).

Zn^{2+} is very important for normal cell function in general and necessary for growth and development of the fetus. Zn^{2+} is required for Zn-containing proteins that are used in regulation of gene expression, protein synthesis, intracellular protein trafficking and immune responses (Bao et al., 2003). Deficiency in maternal Zn^{2+} levels can cause functional abnormalities in the fetus (Beach et al., 1983). Organisms have developed several mechanisms to strictly regulate intracellular Zn^{2+} levels through Zn^{2+} transporters and Zn-binding proteins (Luizzi et al, 2001). Sudden cardiac death and osteopenia have been reported in studies with mice lacking in Zn^{2+} transporters (Inoue et al., 2002). Of the 7 Zn^{2+} transporters identified, rat placental tissues contain all 7 isoforms and the expression profiles change during pregnancy (Asano et al., 2004).

Zn^{2+} appears to be toxic at a wide range of concentrations and toxicity is dependent on cell type and assay used. Steinebach and Walterbeek (Steinebach and Wolterbeek, 1993) found that Zn^{2+} increased glutathione and metallothionein levels as well as increased superoxide dismutase (SOD) activity suggesting higher ROS production. Recently, Zn^{2+} has been implicated in cellular signalling of apoptosis and necrosis, although induction of apoptosis appears to be caspase-3 independent (Hamatake et al., 2000, Bossy-Wetzel et al., 2004). Land and Aizenman (Land and Aizenman, 2005)

found that accumulation of intracellular Zn^{2+} is a necessary component of the cell-death cascade in neurons.

Both Cu^{2+} and Zn^{2+} are redox active and this strategy is exploited by many enzymes such as Cu/Zn-superoxide dismutase (Cu/Zn-SOD) for electron transfer. It is this ability to redox cycle that can produce hydroxyl ($OH^{\bullet}$) and $O_2^{\bullet -}$ radicals in a Fenton-like reaction which can then in turn react with proteins, lipids, and DNA (Halliwell and Gutteridge, 1989, Harrison et al., 1999). Both Cu^{2+} and Zn^{2+} can be directly toxic through replacement of natural ligands in metal-containing proteins (Piao et al., 2003). Also, Cu^{2+} and Zn^{2+} can alter plasma membrane fluidity by linking to phosphate moieties of phospholipids or interacting with thiol or carbonyl groups of membrane proteins (Kumar and Prasad, 2000, Kaur et al., 2006). Additional damage has been observed in mitochondrial metabolism, cytokine secretion, and steroid synthesis (Riley et al., 2003, Bao et al., 2003, Piao et al., 2003, Galhardi et al., 2004).

Cd^{2+} has been studied rigorously in toxicology due to its wide distribution in the environment as well as it having no known biological function. In terms of reproductive toxicology, studies have tested Cd^{2+} in steroidogenic cells of various animals including rat (Paksy et al., 1990, Paksy et al., 1992), mouse (Mgbonyebi et al., 1993, Mgbonyebi et al., 1994a, Mgbonyebi et al., 1994b, Mgbonyebi et al., 1998a, Mgbonyebi et al., 1998b), pig (Henson and Chedrese, 2004), fish (Leblond and Hontela, 1999) and human (Kawai et al., 2002). Early studies on rats *in vivo* showed that not only could Cd^{2+} accumulate rapidly in ovarian tissue, but also depress serum progesterone levels in a time and dose dependent manner (Paksy et al., 1990). *In vitro* studies have demonstrated the ability of Cd^{2+} to interfere with basal steroidogenesis (Mgbonyebi et

al., 1994a), hormone stimulated steroidogenesis (Paksy et al., 1992, Mgbonyebi et al., 1994b) or steroidogenic enzyme function (Kawai et al., 2002).

Ni^{2+} is another metal that has been shown to have deleterious effects in plants (Li and Xiong, 2004), invertebrates (Pane et al., 2004), and mammals. Reproductive effects have been demonstrated as perinatal toxicity in rat pups (Smith et al., 1993), blastocyst development (Paksy et al., 1999), oxidative DNA lesions in neonatal rats (Zhou et al., 2001), and mammalian adult sperm production and quality (Danadevi et al., 2003). Of further concern are studies that have detailed the presence and possible accumulation of Ni^{2+} in placental tissue (Reichrtova et al., 1998) and a component of human breast milk, colostrum (Turan et al., 2001)

A third non-metabolic metal of interest, Hg^{2+}, is able to cross the placenta from mother to fetus (Bjornberg et al., 2005) and has been shown to accumulate in the placenta (Shimada et al., 2004a). Interestingly, the role of Hg^{2+} as an immunotoxicant appears to be both tissue and age dependent. Silva et al (Silva et al., 2005a) determined that in rats, cytokine production was decreased in splenocytes from 10 but not 7 day old mice pups. Further investigation revealed that prenatal exposure to Hg^{2+} caused a persistent, inhibitory effect on cytokine production in thymocytes, lymph cells, and splenocytes in female mice (Silva et al., 2005b). Finding the mechanism behind metal toxicity is of great importance. For instance, Marchi et al (Marchi et al., 2004) determined that Cu caused lysosomal membrane destabilization in mussels probably through inhibition of cytosolic phospholipase A2, an enzyme required for lysosome fusion. However, another study using the same concentrations of Cu as Marchi et al (Marchi et al., 2004) determined that the mechanism behind Cu-mediated toxicity to rat

lysosomes was through ROS generation (Pourahmad et al., 2001). These apparently opposing studies illustrate the need to elucidate the mechanism of toxicity for each metal in each situation and that generalizations cannot be made.

The rat corpus luteum (CL) has been used for many years to study various aspects of ovarian structure and function including viability, steroidogenic pathways, and apoptosis; also known as regression (Wu et al., 1993, Sawada and Carlson, 1994, Sawada and Carlson, 1996). One of the main techniques used in CL experimentation is the primary cell suspension culture. This technique was used recently to demonstrate the ability of PHEQ to inhibit LH-stimulated progesterone secretion and increase ROS production (Nykamp et al., 2001). In addition to the use of CL cells as a model cell system, two fluorescent dyes will be used to assess cytotoxicity. alamarBlue™ (resazurin) has been used to determine the metabolic activity of cells (Ahmed et al., 1994, Nociari et al., 1998, Dayeh et al., 2003). In general, the ratios of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD increase in actively proliferating cells compared to non-proliferating cells. Viable cells actively take up alamarBlue™/resazurin whereupon it can be reduced by NADPH, FADH, FMNH, NADH, and cytochromes to resorufin with a change in colour from blue (non-fluorescent) to pink (fluorescent at 590 nm) that can be monitored both spectrophotometrically and fluorescently (Ahmed et al., 1994, O'Brien et al., 2000, Dayeh et al., 2003). The benefits of using this dye compared to other methods include not destructive to cells, non-radioactive, stable in its reduced form, and greater sensitivity when monitored fluorescently (O'Brien et al., 2000, Dayeh et al., 2003, Parrot et al., 2003, Martina Holst and Oredsson, 2005).

The second dye used in this study is 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM). This dye in its non-polar, non-fluorescent form passively diffuses into cells whereupon cellular esterases can cleave the acetate moiety producing a polar compound that fluoresces at 530 nm (Dayeh et al., 2005). A decline in fluorescence is interpreted as a loss of membrane integrity (Dayeh et al., 2005). Another benefit to using these dyes compared to other methods is that CFDA-AM and alamarBlue can be used concurrently since their excitation and emission wavelengths differ saving time and resources.

The objective of this study is to determine the effect of PAH and metal mixtures in CL cells. The effects of 2 PAHs (PHEQ and 1,2-dhATQ) alone and in mixtures with metals (Cu, Cd, Hg, Zn, or Ni) on CL cells will be analyzed. The parameters measured include metabolic activity (MA) and membrane integrity (MI).

3.2 Materials and Methods

3.2.1 Animals

A breeding colony of Wistar rats was maintained by the Department of Biology, University of Waterloo. The care and use of the animals was approved by the Canadian Council on Animal Care. For this study, 25 to 30 day old immature female Wistar rats were superovulated with a subcutaneous (sc) injection of 50 IU of pregnant mare serum gonadotropin (PMSG – Intervet, Whitby, ON) followed 65 hours later with a sc injection of 50 IU of human chorionic gonadotropin (hCG).

3.2.2 Reagents

Most reagents were purchased from Sigma (St. Louis, MO) including Minimal Essential Medium Eagle Joklik modification (MEM), PHE, PHEQ, ATH, 1, 2-dhATQ, $CuSO_4$, $CdCl_2$, $NiCl_2$, $ZnCl_2$, and $HgCl_2$. Earle's Balance Salt Solution (EBSS) was obtained from Life Technologies (Burlington, ON) and DNaseI from Roche (Laval, QC). Reagents used for the viability testing included alamarBlue™ purchased from BioSource, International (Carmarillo, CA) and 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM) obtained from Molecular Probes (Eugene, Oregon). Stock solutions of PHE, PHEQ, ATH, and 1, 2-dhATQ were prepared in dimethyl sulfoxide (DMSO) as described previously (Nykamp et al., 2001). Stock solutions of all metals were prepared in sterile distilled, deionized H_2O.

3.2.3 Preparation of corpora lutea cells

Ovaries were removed from superovulated rats 4 days after hCG injection. Cells were dispersed in a manner similar to the procedure described previously (Carlson et al., 1995). Ovaries were dissected from surrounding tissue, minced and suspended in EBSS. Corpora lutea were dispersed using 0.2 % collagenase and 0.02 % DNase I at 37°C for 30 minutes in a shaking water bath. The atmosphere was enriched with 95 % air and 5 % CO_2. After dispersion, the cells were filtered through Nitex (75 µm) and then centrifuged at 200 x g for 6 minutes. Sedimented cells were suspended and washed in MEM before recentrifugation as above. The buffer was decanted and fresh MEM was added to resuspend cells. The cells were pre-incubated for 60 minutes in MEM with added L-glutamine at 37°C in the 95 % air and 5 % CO_2 enriched atmosphere. The cells were then centrifuged as before and resuspended in phosphate buffered saline + 5 g/L glucose (PBSG) before an aliquot was removed and examined for viability using Trypan Blue and counted on a hemocytometer. Average viability remained approximately 75 % and sample size was based on the number of viable cells. The cells were then monitored for viability after various treatments.

3.2.4 Viability Assays

alamarBlue™ and CFDA-AM were used to assess the effect of toxicants on cell viability. After assessing CL cell viability with Trypan Blue, the cell concentration was adjusted to 5 x 10^5 cells/ml in PBSG and 1 ml aliquots were incubated with the treatments in 1.5 ml microcentrifuge tubes for 4 hours at 37°C in 95:5 % air:CO_2 atmosphere. At the end of the incubation period, the cells were pelleted at 100 x g, the

media removed, and fresh PBSG containing 5 % v/v alamarBlue™ and 4 nM CFDA-AM was added. 50 μl of CL cell suspension was aliquoted into each well of a 96 microwell plate. The plates were incubated for 30 minutes at 37°C in a 95:5 % air:CO_2 enriched atmosphere. Care was taken to minimize exposure to light since the dyes are light sensitive. The plates were then read on a fluorometric mutliwell plate reader (Cytofluor, Series 4000, PerSeptive Biosystems) at excitation and emission wavelengths of 485 nm and 530 nm for CFDA-AM and 530 nm and 590 nm for alamarBlue™, respectively.

3.2.5 Statistics

Data was analysed using two-way ANOVA followed by Fisher's Least Squares Determination (LSD) to assess significant differences where $p < 0.05$ using SYSTAT 10. For data sets with less than n=3, Student's t-test was used to determine significance ($p < 0.05$).

3.3 Results

All of the viability assays were measured as relative fluorescence units as given by the fluorometric plate reader. This data was then converted to a percent of control to account for variability due to the use of sensitive fluorescent dyes as well as cell suspensions. To minimize the amount of data transformed, only the untreated control was assumed to be 100% while all of the treatments were adjusted accordingly. Figure 3.1 shows a comparison of the raw versus transformed data. While originally done to limit observed standard error, the transformed data provided a convenient method to pictorially compare results from different treatments when scale sometimes prevented such comparisons from being made easily. Data in Figure 3.1 shows that the shape of the curve for the transformed data is unchanged from the raw data.

3.3.1 Comparison of PHEQ and 1,2-dhATQ cytotoxicity on CL cells

Two assays were used to assess cell viability; alamarBlue™ for metabolic activity (MA) and CFDA-AM for membrane integrity (MI). Prior to the mixture studies, the effects of PHEQ or 1,2-dhATQ on CL cell MA and MI were assessed. PHEQ caused a significant decrease in MA at all concentrations tested with up to 70% inhibition observed at the highest concentration used, 10 μM PHEQ (Figure 3.2A). MI was not affected to the same magnitude as MA with only an approximate 55% drop, but the decreases were significant at the two highest concentrations, 1.0 and 10 μM PHEQ (Figure 3.2B). The EC50s calculated for PHEQ were 1.53 μM +/- 0.03 and 9.08 μM +/- 0.23 for MA and MI, respectively (Table 3.1).

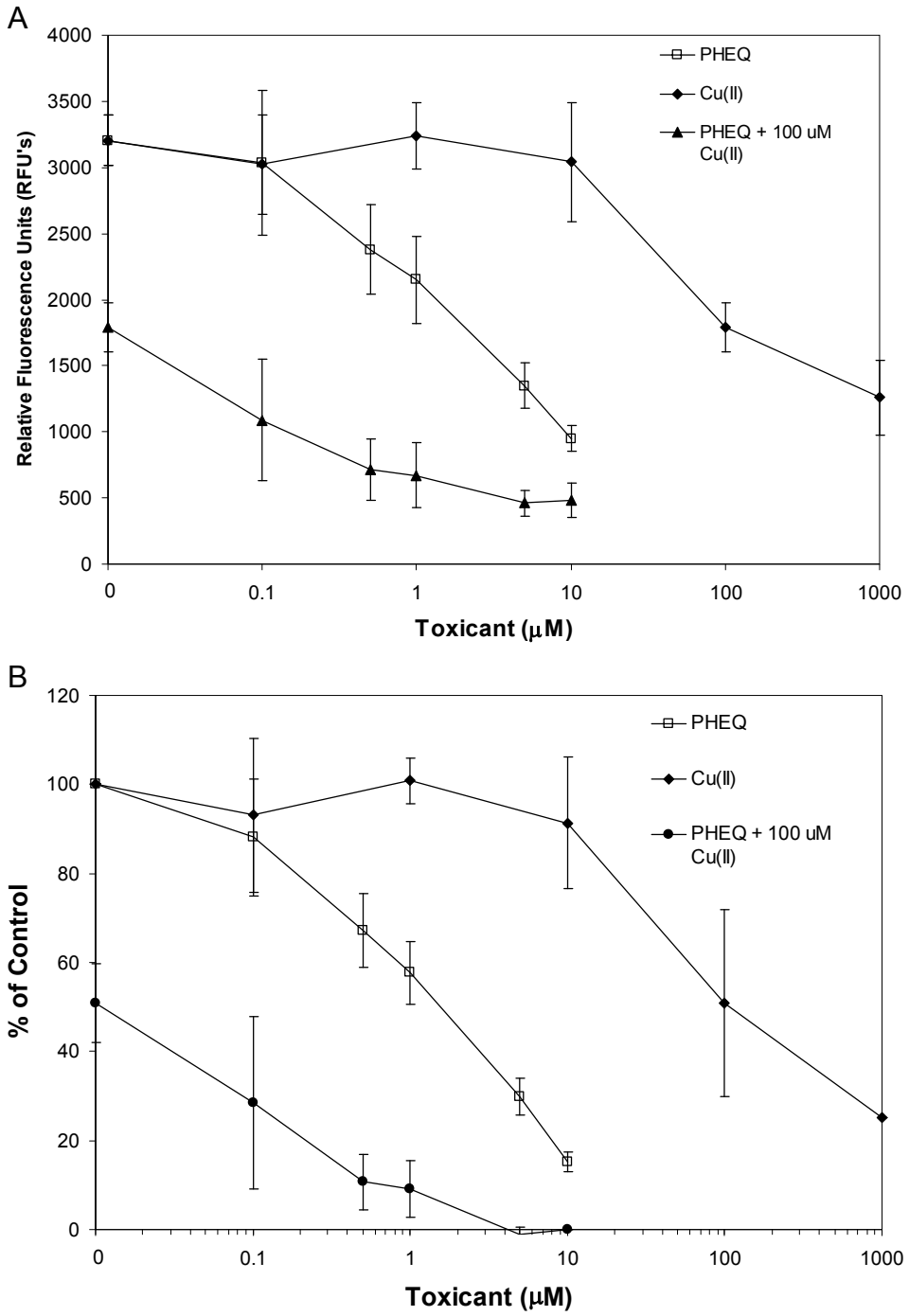
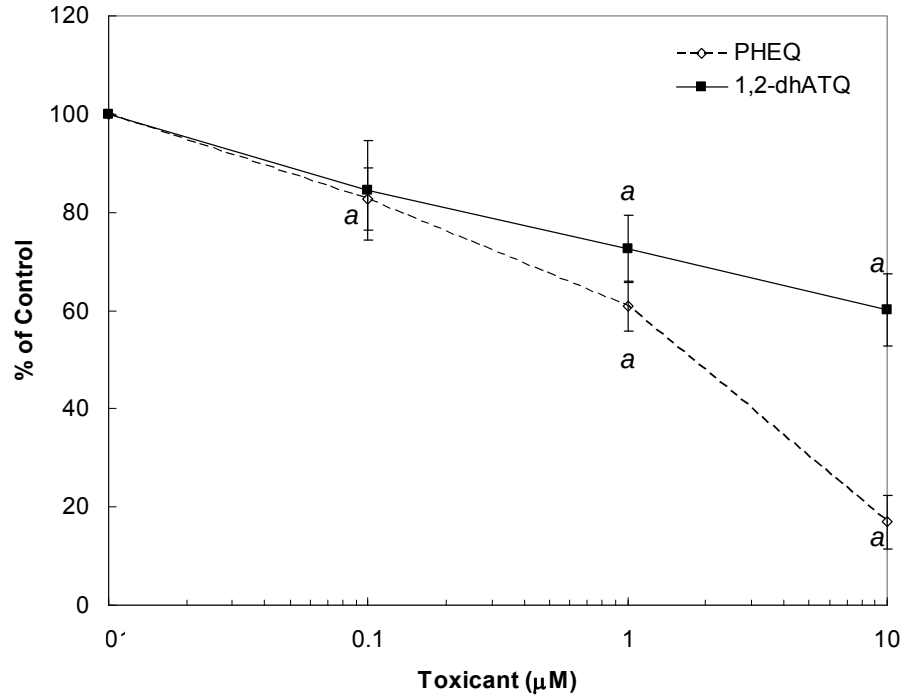


Figure 3.1 Comparison of raw vs. transformed metabolic activity (MA) results. Graphs depict (A) relative fluorescence units versus (B) expression as % of control whereby the untreated control sample is assumed to be 100%. Data shown is PHEQ, Cu^{2+} or PHEQ + 100 μM Cu^{2+} treated CL cells.

A



B

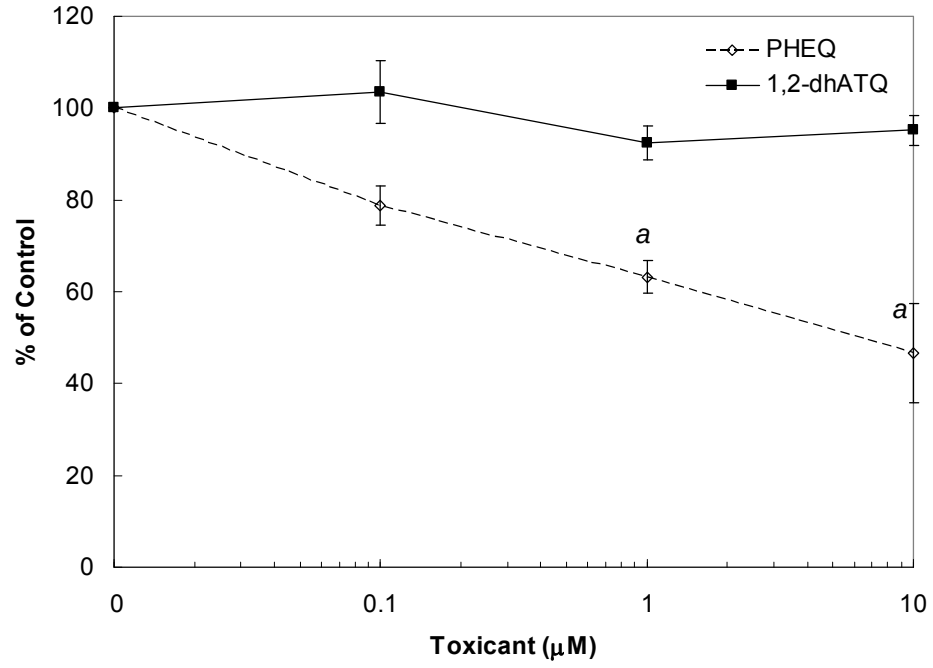


Figure 3.2 Comparison of PHEQ and 1,2-dhATQ treatment on CL cell viability (A) and MI (B). Both compounds inhibit MA, but only PHEQ decreases MI. [a] Significantly different from 0. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents n=6 or more +/- S.E.

Inhibition of MA in CL cells with addition of 1,2-dhATQ was approximately 30% at the highest concentration tested, 10 μM. This inhibition was significant at 1,2-dhATQ concentrations of 1.0 and 10 μM (Figure 3.2A). The estimated EC50 of 42.36 μM 1,2-dhATQ +/- 0.12 for MA was extrapolated from the graph. No significant decrease in MI was observed with exposure to 1,2-dhATQ (Figure 3.2B). No EC50s could be calculated from the data given, and in this instance, 1,2-dhATQ was considered non-toxic based on MI (Table 3.1). The parent compound of PHEQ, PHE, also was tested to determine cytotoxicity to CL cells. Exposure of CL cells to concentrations of PHE up to 10 μM had no significant effect on either MA or MI (Figure 3.4).

3.3.2 Effect of metals on CL cell viability

Five divalent metal cations were tested for their acute toxicity to CL cell MA and MI. The metals chosen for testing were Cu^{2+}, Cd^{2+}, Hg^{2+}, Ni^{2+}, and Zn^{2+}. The concentration-response relationships were plotted using MA and MI as endpoints (Figure 3.3). After analyzing the data, EC50s were calculated and the order of acute toxicity to the MA of CL cells was $Hg^{2+} > Cd^{2+} > Zn^{2+} \approx Ni^{2+} > Cu^{2+}$ with EC50s of 2.16, 6.61, 24.9, 21.3, and 155.3 μM, respectively. MI also was affected by exposure to metals with the order of toxicity being $Hg^{2+} \approx Zn^{2+} > Cd^{2+} > Cu^{2+} > Ni^{2+}$. EC50s for MI that could be determined were calculated at 2.63, 2.25, 4.23, and 971.5 μM, respectively. Since Ni^{2+} was essentially non-toxic to CL cell MI, no EC50 could be calculated.

In general, 2 patterns emerged when analyzing the metal-treated CL cell MA data graphically; (1) a general decrease or (2) a sudden decline in MA with increasing metal concentration (Figure 3.3A). Of the 5 metals tested, Cd^{2+}, Ni^{2+}, and Cu^{2+} slowly inhibited

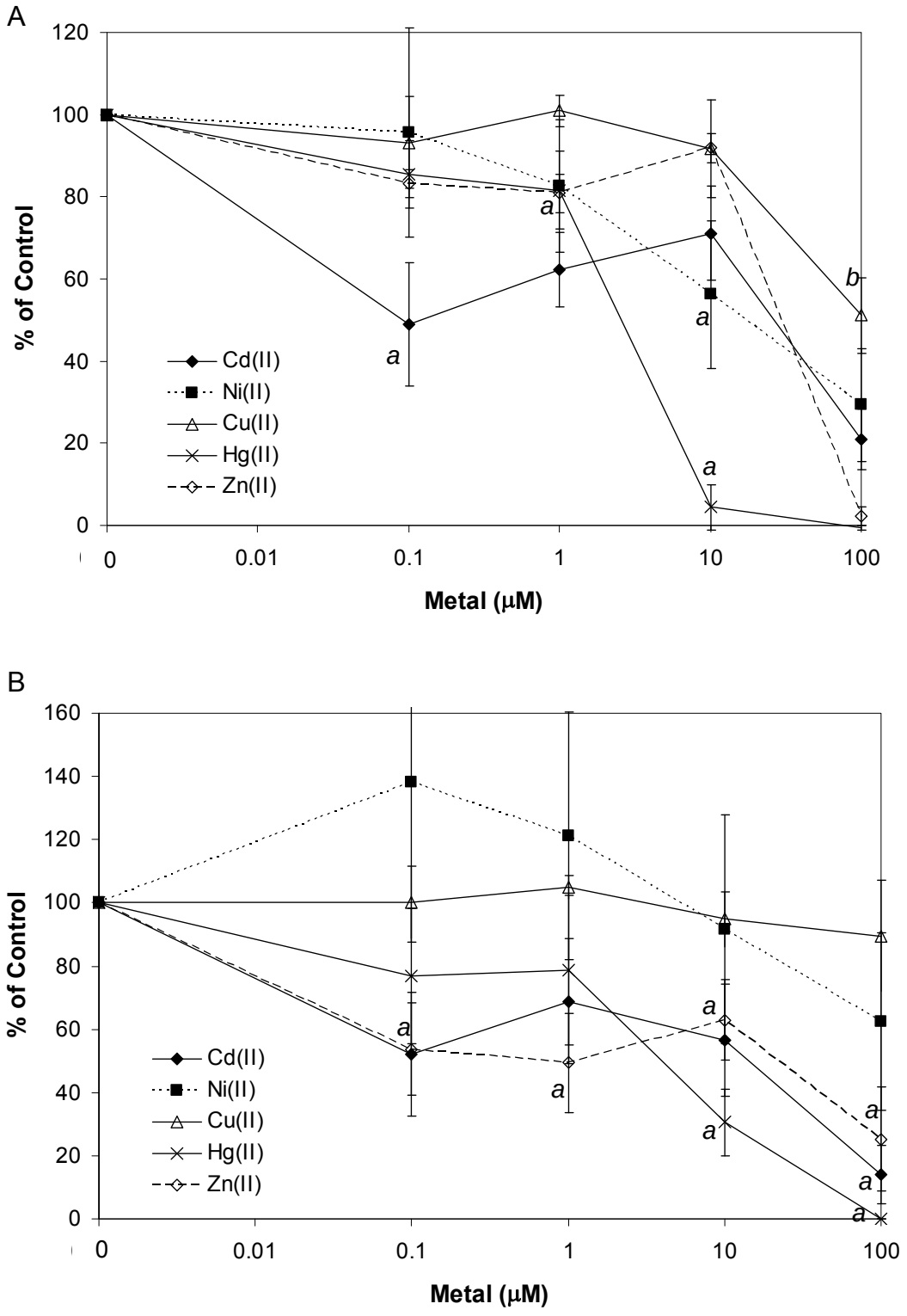


Figure 3.3 Comparison of metal toxicity to MA (A) and MI (B) in CL cells. [a] Significantly different from 0 as determined using two-way ANOVA followed by Fisher's LSD post-hoc test ($p < 0.05$). [b] All data at this point significantly different from 0. Data represents n=4 +/- S.E.

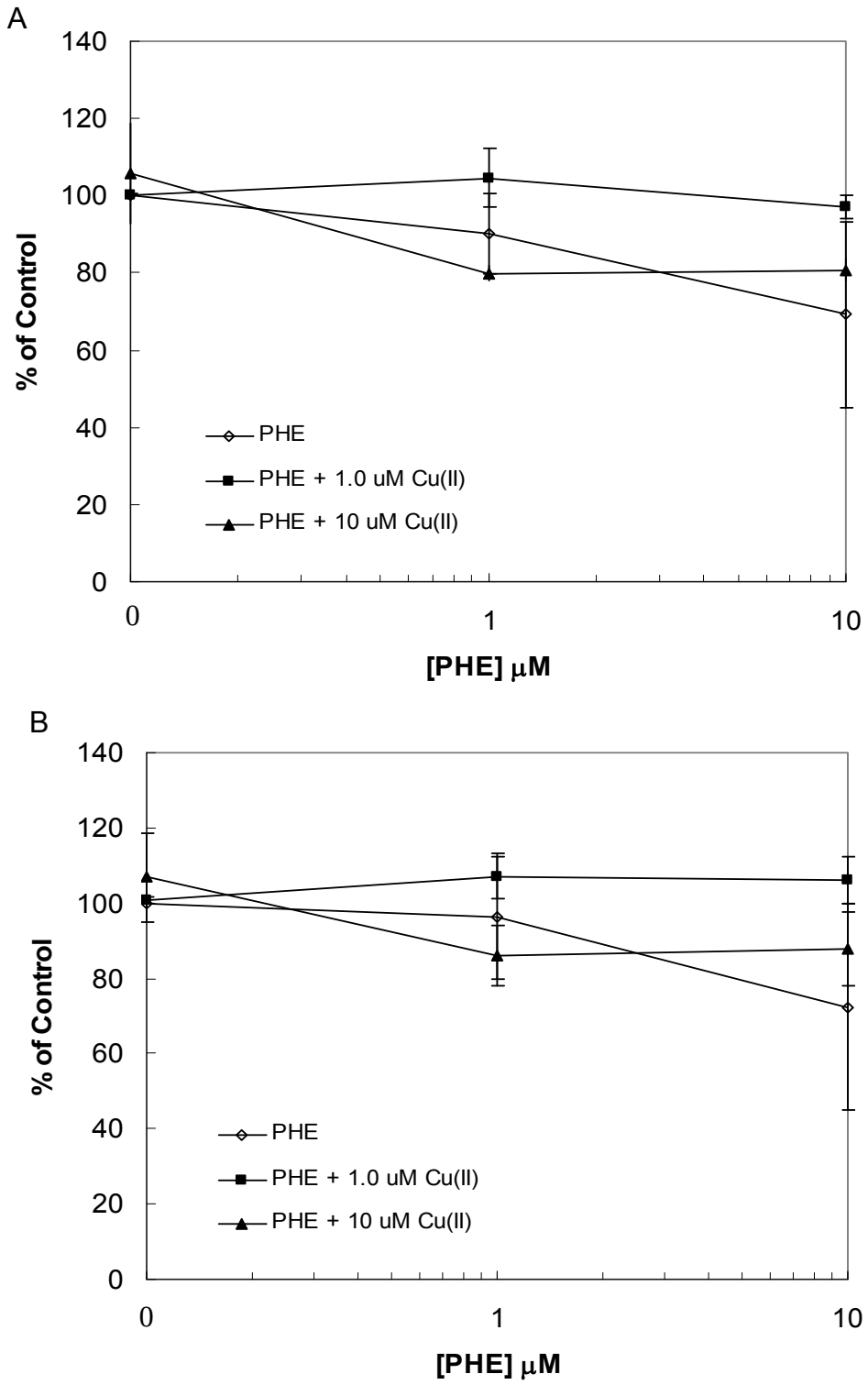


Figure 3.4 Comparison of PHE cytotoxicity with PHE alone or added Cu^{2+}. No significant difference in either MA (A) or MI (B) was observed between data points when tested by two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents n=2 +/- S.E.

MA in CL cells as metal concentration increased. Conversely, Hg^{2+} and Zn^{2+} appeared to have little effect on MA until a sharp decline was observed in MA at 10 μM for Hg^{2+}-treated cells and 100 μM for Zn^{2+}-treated cells (Figure 3.3A). No such difference was observed for MI in metal-treated CL cells.

3.3.3 Effect of PHEQ or PHE/metal mixtures on CL cell viability

The five metals tested previously were used to study the effects of PHEQ/metal mixtures on CL cell MA and MI. The parent compound of PHEQ, PHE was tested only in combination with Cu^{2+}. When PHE was co-incubated with 1.0 or 10 μM Cu^{2+}, no significant decrease in either MA or MI was observed compared to no treatment added (Figure 3.4). Additionally, no change in toxicity to either MA or MI was observed when combining Cu^{2+} with PHE compared to PHE added alone. Since no EC50 could be calculated from the data, PHE + Cu^{2+} was considered non-toxic to CL cells under the conditions used (Table 3.1).

Mixtures of PHEQ and Cu^{2+} were much more toxic than either compound tested alone (Figures 3.5 and 3.6). Three concentrations of Cu^{2+} (1.0, 10, and 100 μM) were combined with a range of PHEQ concentrations. The toxicity of PHEQ to MA was not dramatically altered with the addition of 1.0 μM Cu^{2+}. However, increasing Cu^{2+} to 10 or 100 μM significantly increased PHEQ-mediated toxicity to MA. EC50s reflected this change in toxicity from PHEQ alone to PHEQ + 10 or 100 μM Cu^{2+} (1.53 μM vs. 0.29 or 0.01 μM PHEQ – Table 3.1). Overall, a 150% decrease in MA EC50 was observed between PHEQ exposure alone compared to PHEQ + 100 μM Cu^{2+}.

MI was similarly affected when CL cells were exposed to both PHEQ and Cu^{2+}. Again, combining a low concentration of Cu^{2+} (1.0 μM) with PHEQ did not change CL cell MI, however, increasing Cu^{2+} to 10 and 100 μM significantly lowered PHEQ-mediated toxicity to MI (Figure 3.5). When examining EC50s, the addition of 10 μM Cu^{2+} to PHEQ-treated cells decreased MI by 50% (5.35 μM PHEQ) while 100 μM Cu^{2+} combined with PHEQ lowered MI by 100% (0.09 μM PHEQ) compared with PHEQ treatment alone (9.08 μM PHEQ).

Mixtures of PHEQ and Cd^{2+} were tested as well for their combined effect on CL cell MA and MI. Two concentrations of PHEQ (0.1 or 1.0 μM) were mixed with various concentrations of Cd^{2+}. Compared to Cd^{2+} treatment alone, no significant change was observed in CL cell MA or MI with the co-exposure to Cd^{2+} and PHEQ at any concentration (Figure 3.7). From the graphs, no significant difference at any concentration of Cd^{2+} was observed between Cd^{2+} treatment alone and Cd^{2+} + PHEQ. However, there was an apparent 50% decrease between Cd^{2+} alone (6.61 μM Cd^{2+}) and Cd^{2+} + 0.1 μM PHEQ (2.91 μM Cd^{2+}) in EC50s for MA calculated from the data. No EC50 could be determined for Cd^{2+} + 1.0 μM PHEQ since MA curve began at less than 50% (Figure 3.7A). MI of CL cells gradually decreased with increasing Cd concentration (Figure 3.7B). Addition of 0.1 or 1.0 μM PHEQ to Cd^{2+}-treated cells did not significantly alter MI compared to Cd^{2+} treatment alone (Figure 3.7B). While the EC50 of Cd^{2+} decreased from 4.23 μM to 1.08 μM with the addition of 1.0 μM PHEQ, EC50 of Cd^{2+} increased to 14.62 μM in the presence of 0.1 μM PHEQ (Table 3.1).

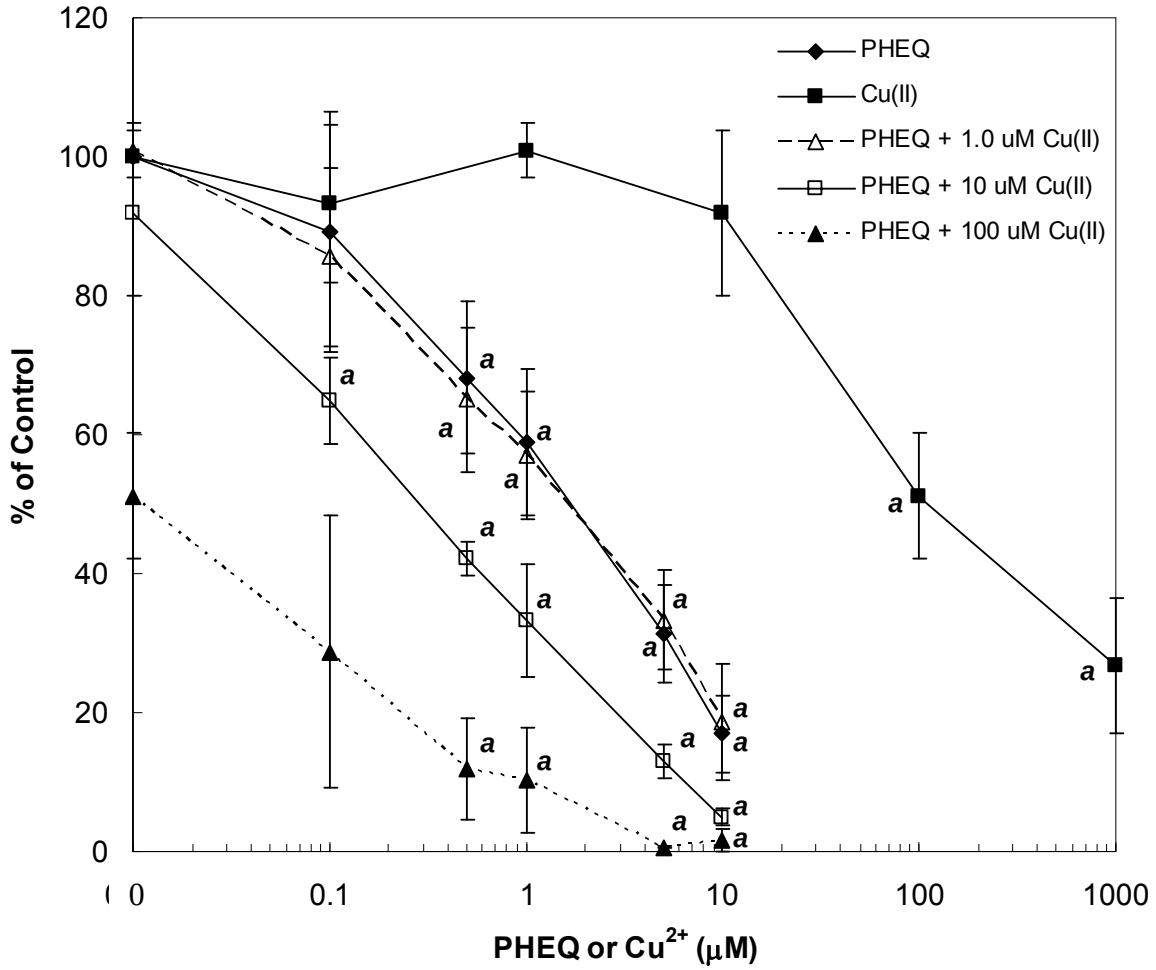


Figure 3.5 Effect of PHEQ and Cu^{2+} on CL cell MA. PHEQ alone inhibits MA in a concentration-response relationship. Increasing PHEQ toxicity is observed with increasing [Cu^{2+}] as measured by decreasing MA. [a] Significantly different from 0 PHEQ. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

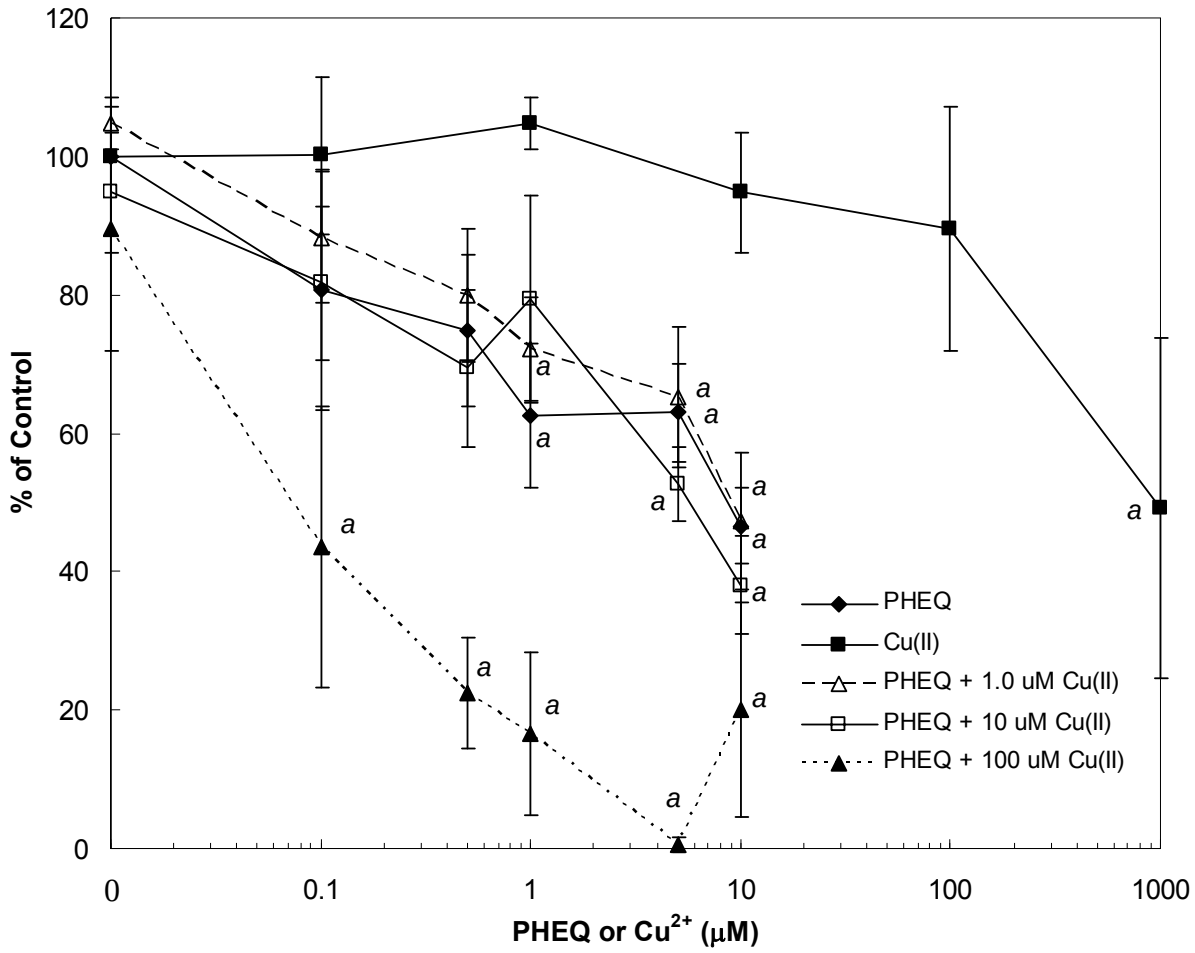


Figure 3.6 Effect of PHEQ and Cu^{2+} on CL cell MI. PHEQ alone diminishes MI in a dose-response relationship. Increasing PHEQ toxicity is observed with increasing [Cu^{2+}] as measured by decreasing MI. [a] Significantly different from 0 PHEQ. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

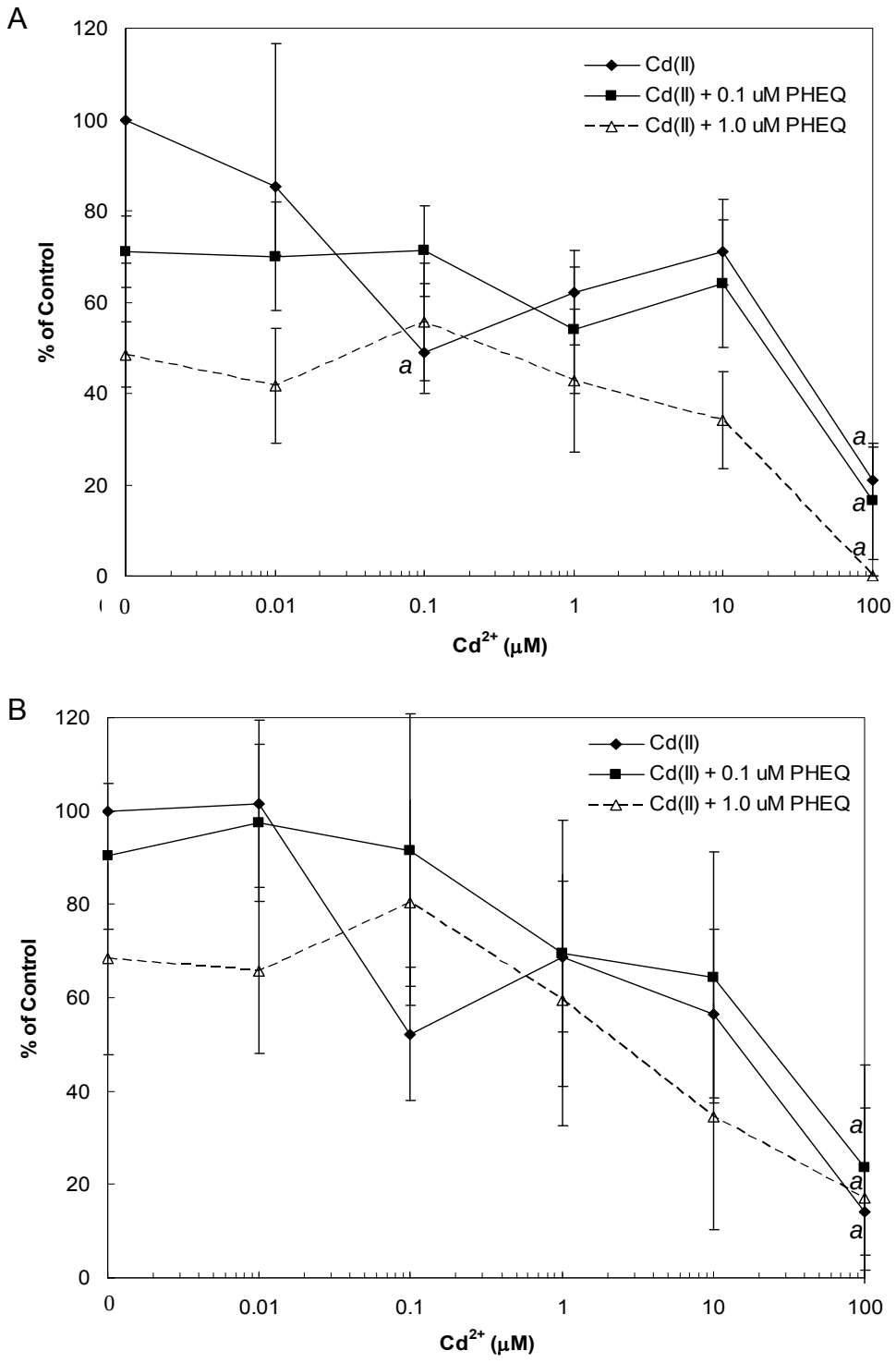


Figure 3.7 Effect of PHEQ and Cd^{2+} on CL cell cytotoxicity. Alone Cd^{2+} inhibits both MA (A) and MI (B). Addition of PHEQ causes a further decrease. [a] Significantly different from 0 µM PHEQ. Data represents mean of n=5 ± S.E. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

A third metal tested with PHEQ was Ni^{2+}. Various concentrations of Ni^{2+} were combined with either 0.1 or 1.0 μM PHEQ and assessed for their effect on CL cell MA and MI. While exposure to Ni^{2+} caused a gradual decrease in MA, addition of either 0.1 or 1.0 μM PHEQ did not appear to enhance toxicity of Ni^{2+} significantly (Figure 3.8A). MI was not affected significantly by exposure to Ni^{2+} alone or in combination with either 0.1 or 1.0 μM PHEQ (Figure 3.8B). Neither Ni^{2+} alone nor Ni^{2+} with 0.1 μM PHEQ were toxic to CL cell MI but Ni^{2+} with 1.0 μM PHEQ was toxic (EC50 = 0.18 μM Ni^{2+}). Hg^{2+} was another metal tested in combination with 0.1 or 1.0 μM PHEQ and their effect on CL cell MA and MI determined. The concentration-response curve displayed a sharp drop for both MA and MI when CL cells were exposed to Hg^{2+} alone (Figure 3.9). Addition of 0.1 μM PHEQ with various concentrations of Hg^{2+} did not significantly enhance Hg^{2+}-mediated toxicity (Figure 3.9A). However, combining 1.0 μM PHEQ with 1.0 μM Hg^{2+} did appear to cause a greater than additive decrease in MA compared to 1.0 μM Hg^{2+} treatment alone (Figure 3.9A). No significant difference in Hg^{2+}-treated CL cell MI was observed with the addition of either 0.1 or 1.0 μM PHEQ.

The last metal tested with PHEQ was Zn^{2+}. MA gradually decreased with increasing Zn^{2+} until a sharp drop was observed with the highest concentration, 100 μM (Figure 3.10A). Combining 0.1 or 1.0 μM PHEQ with Zn^{2+} did not enhance Zn^{2+}-mediated toxicity beyond additivity. MI of CL cells gradually decreased to approximately 30% of control when exposed to Zn^{2+} (Figure 3.10B). While addition of 0.1 or 1.0 μM PHEQ decreased MI in Zn^{2+}-treated CL cells to 20 and 10%, respectively, this enhanced toxicity was not greater than additive.

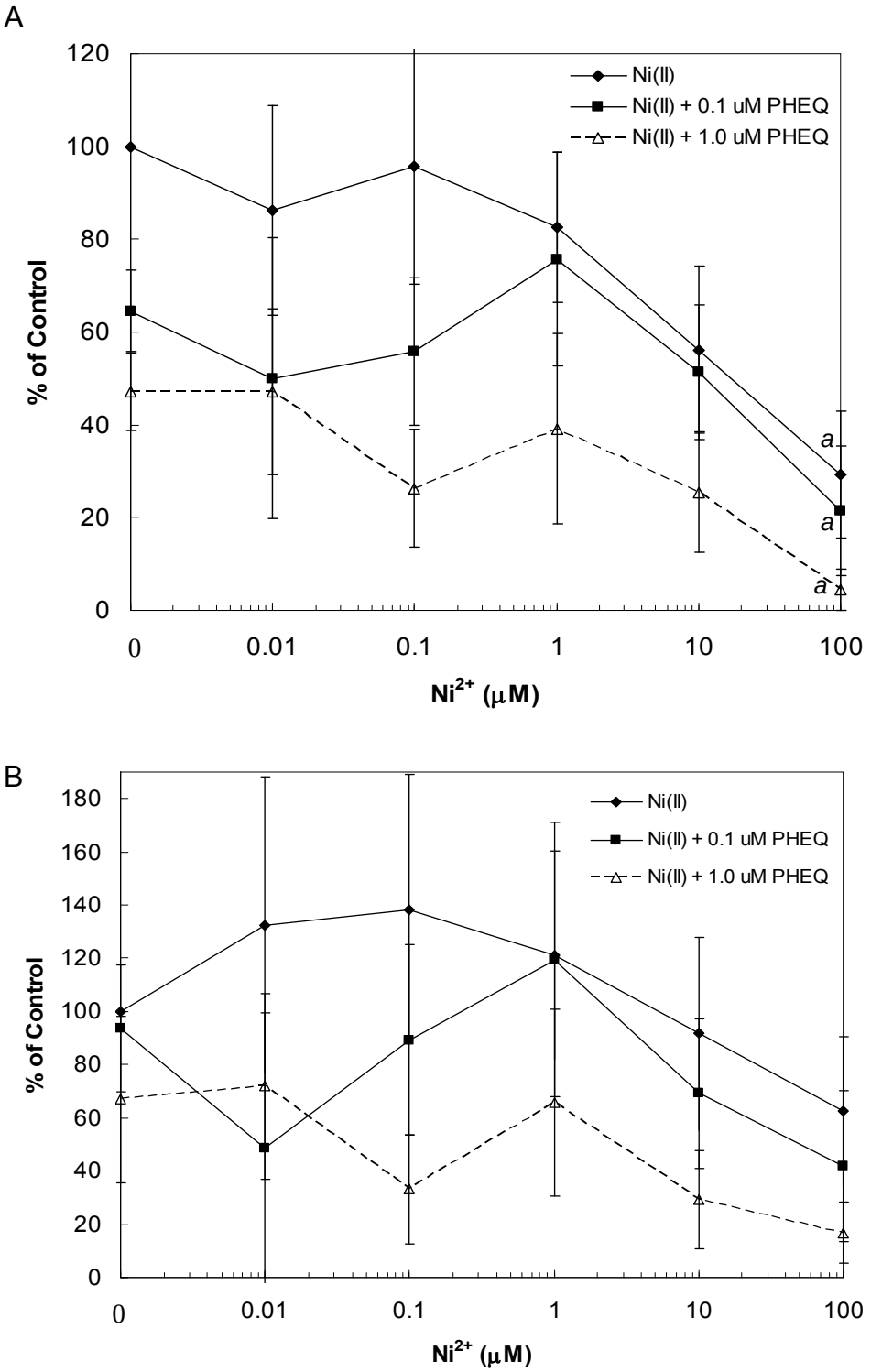


Figure 3.8 Effect of PHEQ and Ni^{2+} on CL cell cytotoxicity. Ni^{2+} alone inhibits MA (A) but not MI (B). Addition of PHEQ causes a further inhibition (A). *a* Significantly different from 0 μM PHEQ. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

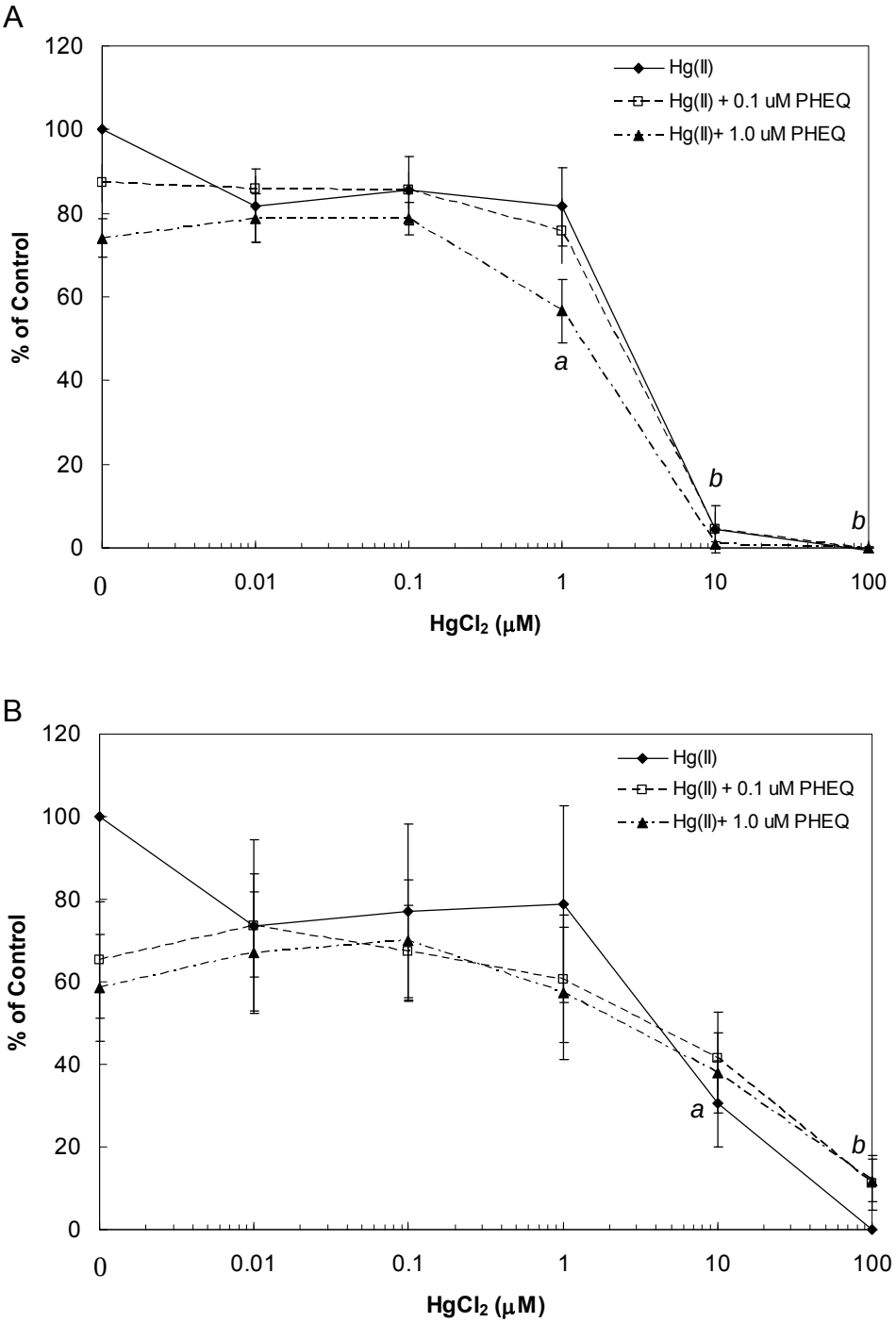


Figure 3.9 Effect of PHEQ and Hg^{2+} on CL cell cytotoxicity. Hg^{2+} inhibits MA (A) and MI (B). Only addition of 1.0 μM PHEQ causes a further inhibition. ***a*** Significantly different from 0. ***b*** All data at this point significantly different from 0. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

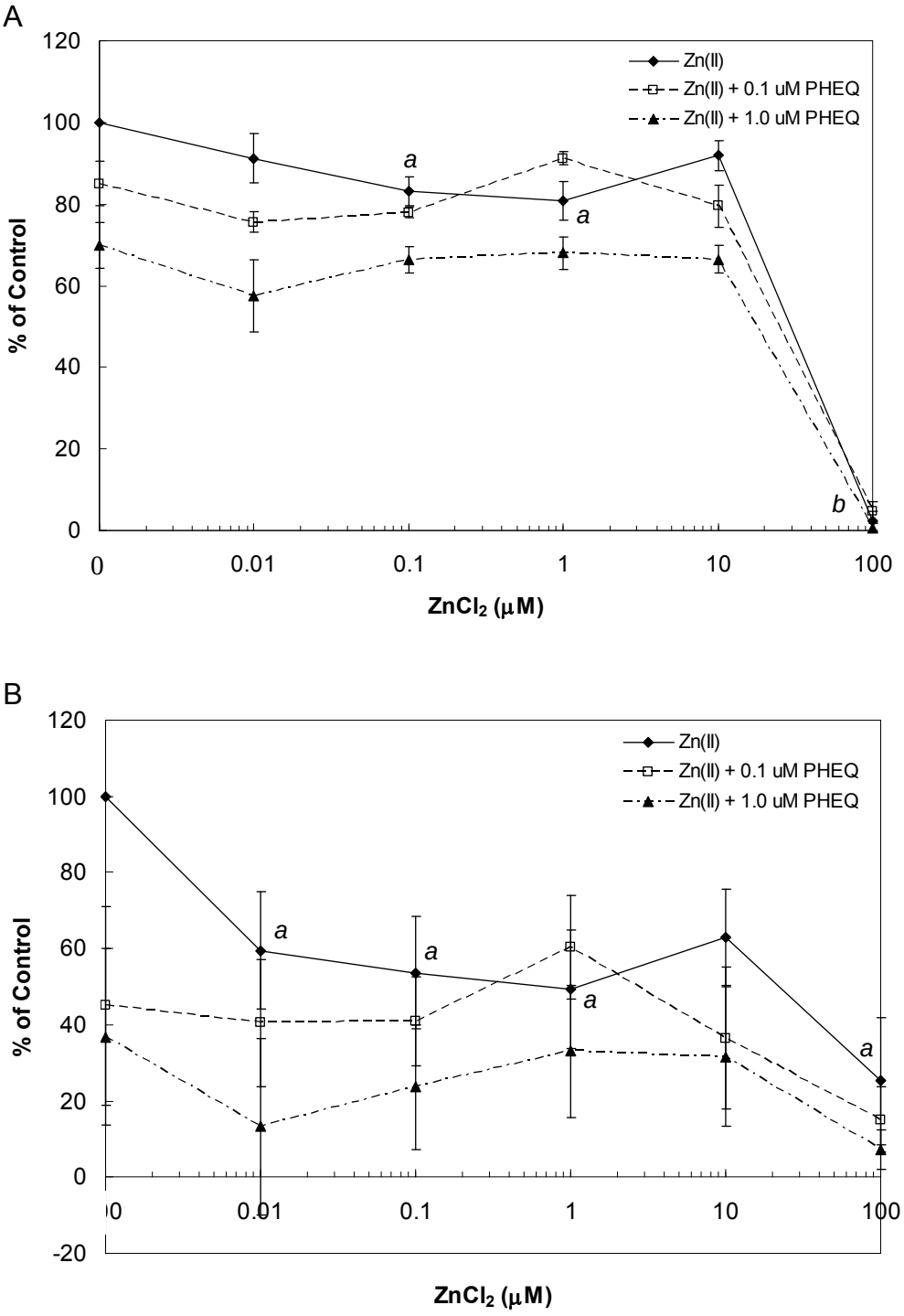


Figure 3.10 Effect of PHEQ and Zn^{2+} on CL cell cytotoxicity. Zn^{2+} inhibits MA (A) and MI (B). Only addition of 1.0 μM PHEQ causes a further inhibition (A). [a] Significantly different from 0 μM PHEQ. [b] All points significantly different from 0 μM PHEQ. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

3.3.3 Effect of 1,2-dhATQ/metal mixtures on CL cell viability

Four metals were combined singly with 1,2-dhATQ to determine the effect of these metal/1,2-dhATQ mixtures on CL cell MA and MI. Of the five metals tested above, only Cu^{2+} was not used. In experiments employing JEG-3 cells, it was shown that neither 1,2-dhATQ nor anthracene (ANT), the parent compound of 1,2-dhATQ, with or without added Cu^{2+} were toxic to JEG-3 cells (Nykamp, 2007 – Chapter 4). Therefore, these experiments were not duplicated in CL cells (Nykamp, 2007 – Chapter 4).

Cd^{2+} combined with various concentrations of 1,2-dhATQ (0.1, 1.0, and 10 μM) was assessed for toxicity to CL cell MA and MI. The addition of 1,2-dhATQ at any concentration to Cd^{2+}-treated cells did not enhance toxicity compared to Cd^{2+} alone (Figure 3.11). While none of the mixtures exhibited greater than additive toxicity for either MA or MI, combining 0.1 μM 1,2-dhATQ with Cd^{2+} appeared to lessen Cd^{2+}-induced toxicity to MI in CL cells (Figure 3.11B). Also, compared to control samples with no toxicant added neither MA nor MI were significantly affected by Cd^{2+}/1,2-dhATQ (1.0 or 10 μM) mixtures until the highest concentration of Cd^{2+} used (100 μM – Figure 3.11).

The effect of 1,2-dhATQ/Ni^{2+} combinations to CL cell MA and MI was tested next. The addition of various concentrations of 1,2-dhATQ to Ni^{2+}-treated cells did not cause a greater than additive toxicity for CL cell MA (Figure 3.12A). Interestingly, co-exposure of Ni^{2+} with 0.1 μM 1,2-dhATQ appeared to cause synergistic toxicity to CL cell MI at 1.0, 10, and 100 μM Ni^{2+} (Figure 3.12B). Also, the general trend in MI observed with Ni^{2+} treatment was an increase in MI with increasing Ni^{2+} concentration (Figure 3.12B). Neither 0.1 nor 1.0 μM 1,2-dhATQ changed this trend, but addition of 10 μM 1,2-dhATQ and Ni^{2+} to CL cells did not result in an increase in MI (Figure 3.12B).

Only 2 concentrations of 1,2-dhATQ (1.0 and 10 μM) were combined with Hg^{2+} to test the effect of CL cell MA and MI. The addition of 1.0 μM 1,2-dhATQ to various concentrations of Hg^{2+} did not appear to enhance Hg^{2+}-mediated toxicity to MA (Figure 3.13A). However, synergistic toxicity may have occurred when the concentration of 1,2-dhATQ was increased to 10 μM. Unfortunately, too much variability was present to make a definitive determination. Mixtures of Hg^{2+} and 1.0 or 10 μM 1,2-dhATQ did not exhibit greater than additive toxicity to CL cell MI (Figure 3.13B).

As with PHEQ, Zn^{2+} was also tested in combination with 1,2-dhATQ. Compared to Zn^{2+} treatment alone, the addition of 1,2-dhATQ (1.0 or 10 μM) did not enhance Zn^{2+}-mediated toxicity to either CL cell MA or MI (Figure 3.14). Generally, additive or less than additive toxicity was observed in both MA and MI for all concentrations of Zn^{2+}/1,2-dhATQ mixtures except for 100 μM Zn^{2+} with 1.0 μM 1,2-dhATQ where a slight increase in both MA and MI was observed, though not significant (Figure 3.14).

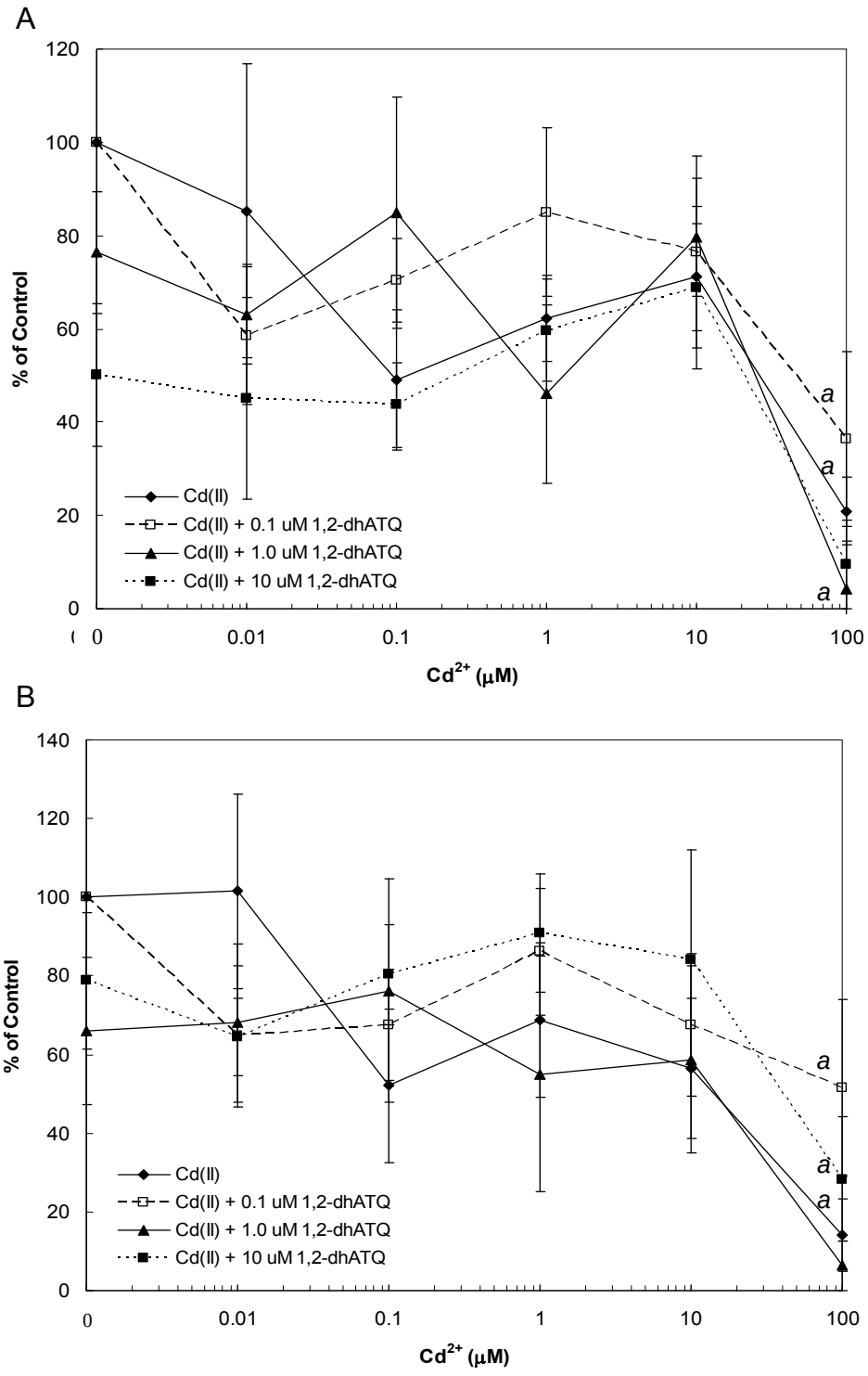


Figure 3.11 Effect of 1,2-dhATQ and Cd^{2+} on CL cell cytotoxicity. Cd^{2+} inhibits MA (A) and MI (B). Addition of 1,2-dhATQ causes a further inhibition. [a] Significantly different from 0 μM 1,2-dhATQ. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

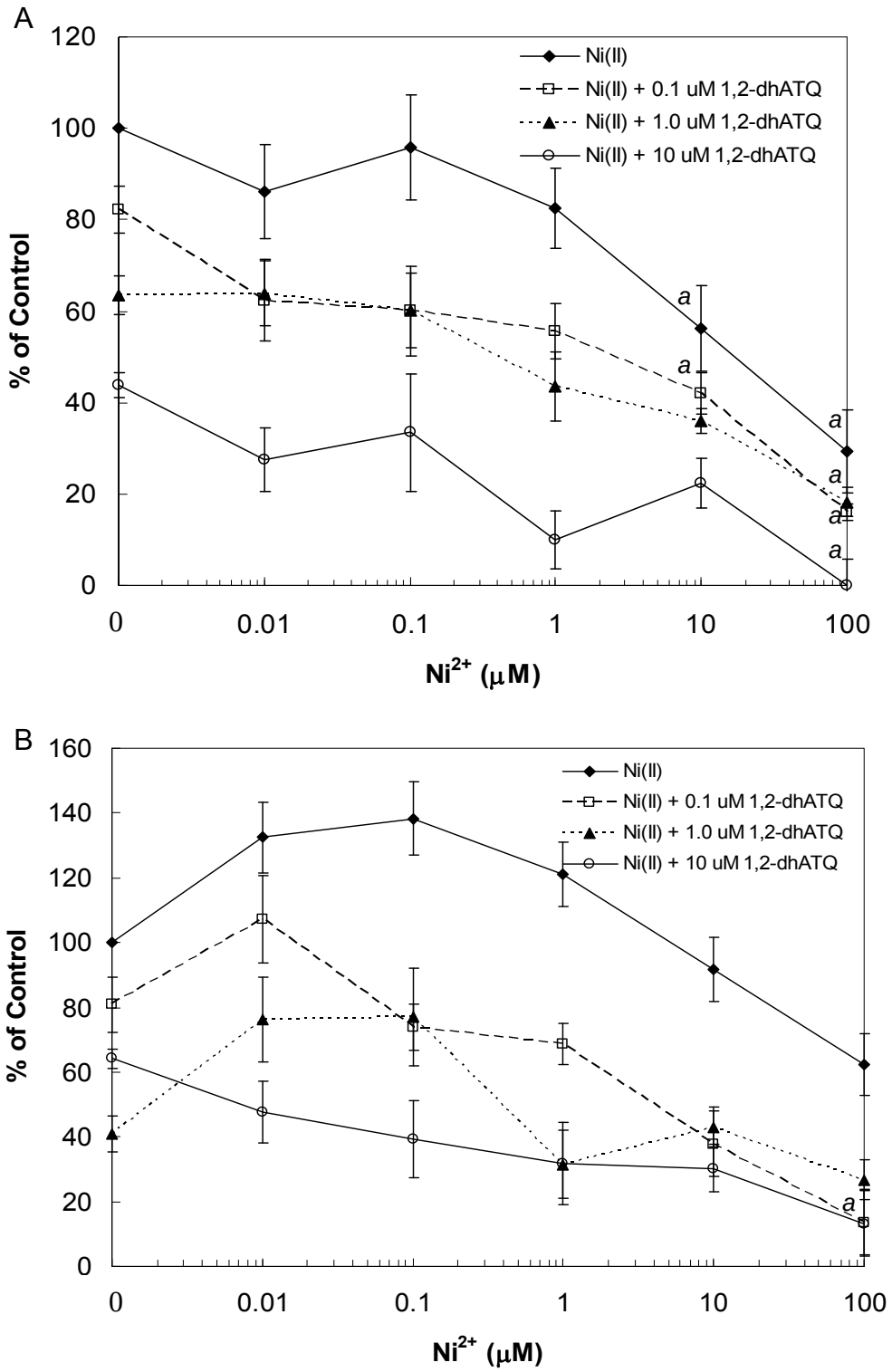


Figure 3.12 Effect of 1,2-dhATQ and Ni^{2+} on CL cell cytotoxicity. Ni^{2+} alone inhibits MA (A) but not MI (B). Addition of 1,2-dhATQ causes a further inhibition (A). ^{a} Significantly different from 0. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

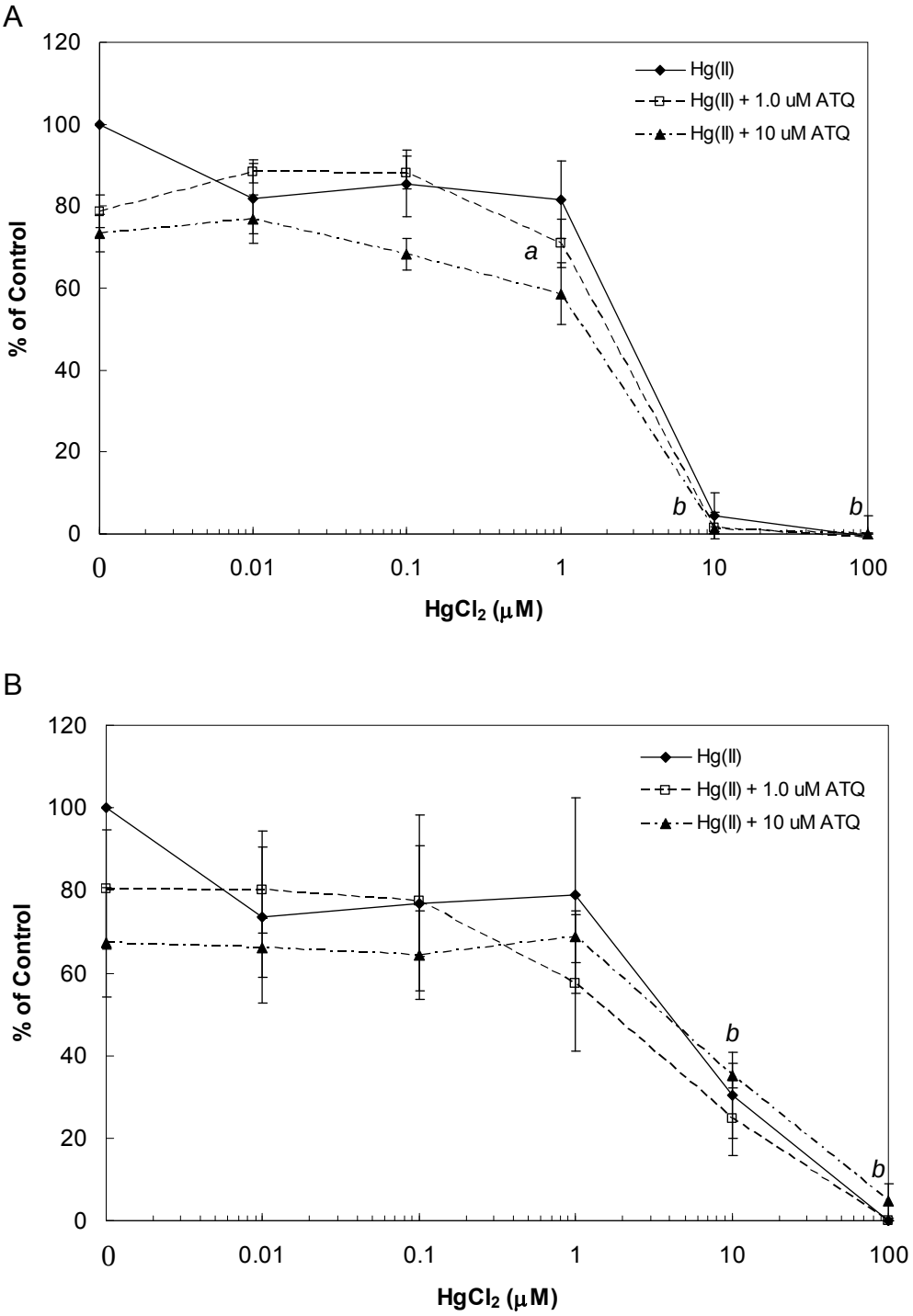


Figure 3.13 Effect of 1,2-dhATQ and Hg^{2+} on CL cell cytotoxicity. Hg^{2+} inhibits MA (A) and MI (B). Only addition of 10 μM 1,2-dhATQ causes a further inhibition of MA (A). [a] Significantly different from 0. [b] All data at this point significantly different from 0. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

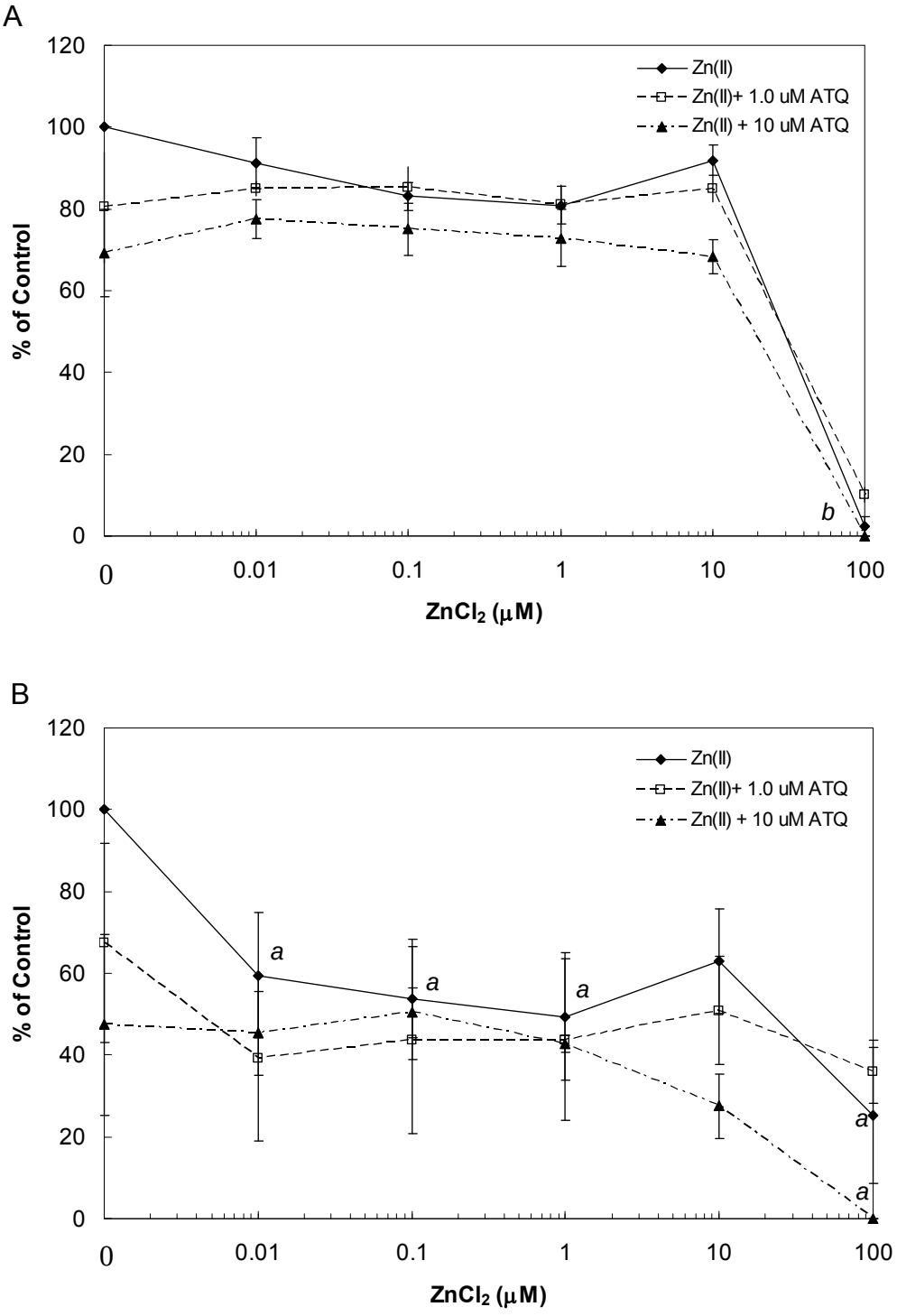


Figure 3.14 Effect of 1,2-dhATQ and Zn^{2+} on CL cell cytotoxicity. Zn^{2+} inhibits MA (A) and MI (B). Only addition of 10 μM 1,2-dhATQ causes a further inhibition (A). *a* Significantly different from 0. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$). Data represents mean of n=4 ± S.E.

Table 3.1 Summary of EC50s for CL cell treatments.

Treatment	Metabolic Activity (μM)	Membrane Integrity (μM)
Cu^{2+}	155.3 +/- 0.12	971.5 +/- 0.06
PHE	NT	NT
PHE + 1.0 μM Cu^{2+}	NT	NT
PHE + 10 μM Cu^{2+}	NT	NT
PHEQ	1.53 +/- 0.03	9.08 +/- 0.23
PHEQ + 1.0 μM Cu^{2+}	1.45 +/- 0.05	10.41 +/- 0.17
PHEQ + 10 μM Cu^{2+}	0.29 +/- 0.04	5.35 +/- 0.16
PHEQ + 100 μM Cu^{2+}	0.01 +/- 0.08	0.09 +/- 0.17
1,2-dhATQ	42.36 +/- 0.12	NT
Cd^{2+}	6.61 +/- 0.78	4.23 +/- 0.52
Cd^{2+} + 0.1 μM PHEQ	2.91 +/- 0.73	14.62 +/- 0.22
Cd^{2+} + 1.0 μM PHEQ	ND	1.08 +/- 0.54
Cd^{2+} + 0.1 μM 1,2-dhATQ	NT	766.3 +/- 1.97
Cd^{2+} + 1.0 μM 1,2-dhATQ	3.46 +/- 1.13	1.46 +/- 0.78
Cd^{2+} + 10 μM 1,2-dhATQ	0.02 +/- 3.36	NT
Ni^{2+}	21.3 +/- 4.5	NT
Ni^{2+} + 0.1 μM PHEQ	1.36 +/- 1.34	NT
Ni^{2+} + 1.0 μM PHEQ	ND	0.18 +/- 0.74
Ni^{2+} + 0.1 μM 1,2-dhATQ	0.68 +/- 0.32	3.28 +/- 0.3
Ni^{2+} + 1.0 μM 1,2-dhATQ	0.19 +/- 0.3	0.28 +/- 1.37
Ni^{2+} + 10 μM 1,2-dhATQ	2.17×10^{-4} +/- 0.92	0.013
Hg^{2+}	2.16 +/- 0.21	2.63 +/- 0.34
Hg^{2+} + 0.1 μM PHEQ	1.93 +/- 0.19	0.89 +/- 0.51
Hg^{2+} + 1.0 μM PHEQ	0.63 +/- 0.37	0.47 +/- 0.63
Hg^{2+} + 1.0 μM 1,2-dhATQ	1.6 +/- 0.23	0.94 +/- 0.28
Hg^{2+} + 10 μM 1,2-dhATQ	0.36 +/- 0.43	0.58 +/- 0.61
Zn^{2+}	24.9 +/- 0.41	2.25 +/- 0.78
Zn^{2+} + 0.1 μM PHEQ	20.73 +/- 0.33	ND
Zn^{2+} + 1.0 μM PHEQ	1.54 +/- 1.12	ND
Zn^{2+} + 1.0 μM 1,2-dhATQ	13.35 +/- 0.37	ND
Zn^{2+} + 10 μM 1,2-dhATQ	4.62 +/- 0.82	ND

NT = not toxic.
ND = not able to determine

3.4 Discussion

PAHs and metals are often co-contaminants in the environment. Effective risk assessment must take into account potential interactions of these contaminants. The objective of this study was to determine the cytotoxicity of oxyPAHs, metals, and oxyPAH/metal mixtures on CL cells as measured by metabolic activity (MA) and membrane integrity (MI). Of the 2 oxyPAHs tested, PHEQ was more toxic than 1,2-dhATQ while the order of toxicity for the metals was $Hg^{2+} > Cd^{2+} > Ni^{2+} \approx Zn^{2+} > Cu^{2+}$. The mixture of metals with oxyPAHs was generally more toxic than either chemical alone. Most mixtures exhibited additive toxicity; however, some showed evidence of synergism. Generally, MA was a more sensitive indicator of cellular impacts than MI.

3.4.1 CL cell viability with exposure to PAHs and oxyPAHs

CL cells were exposed to PHE and PHEQ. Both MA and MI decreased with increasing concentrations of PHEQ but not PHE. In general, this finding is consistent with previous studies using *V. fisherii*, *L. gibba*, and *D. magna* (McConkey et al., 1997, Xie et al., 2006, Lampi et al., 2006). The route of activation for PHE includes both biotic and abiotic mechanisms. Abiotic means such as UV light can transform PHE into an oxyPAH with PHEQ being its major photoproduct (McConkey et al., 1997). With the high probability of PHE being exposed to UV light; dermal absorption, inhalation or ingestion of PHEQ becomes a likely exposure route for organisms. The prevalence of PHE in the environment also makes PHE uptake by an organism likely. While the main biotic route of PAH xenobiotic metabolism usually involves phase I reactions including

cytochrome P450 enzymes, the activation of PHE may also occur via dihydrodiol dehydrogenases (Flowers-Geary et al., 1996). Once in the body, PHE can be readily transformed into PHEQ and is in fact the major metabolite found (Kadry et al., 1995).

There are many possible reasons why PHEQ is more toxic than PHE. The highly hydrophobic nature of PHE is lessened with the addition of oxygen atoms when PHEQ is formed. The greater solubility of PHEQ in water increases the ability of an organism/cell to take up PHEQ resulting in increased exposure. Yet it retains inherent lipophilicity allowing PHEQ to permeate lipid membranes and partition to the cytosol and other organelles. Taken together, these factors result in the increased availability of PHEQ compared to PHE generating a greater risk of toxicity to the organism/cell. PHEQ, as a PAH *o*-quinone, is more electrophilic than its parent PAH. By abstracting electrons from other cellular nucleophiles, PHEQ can react with cellular constituents such as RNA and DNA leading to genotoxicity. A third mechanism involves the redox-cycling ability of PHEQ. PAH *o*-quinones like PHEQ can undergo one-electron redox-cycling catalyzed by cellular reductases such as mitochondrial NADH:ubiquinone oxidoreductase (Flowers-Geary et al., 1996). The *o*-semiquinone anion radical formed can react with molecular oxygen to form ROS thus regenerating to the *o*-quinone. This futile redox-cycling can lead to a change in the redox state of the cell, oxidative damage and eventually, cell death.

The exposure of CL cells to 1,2-dhATQ caused a decrease in MA but not MI. The differing slopes for the concentration-response curves between PHEQ and 1,2-dhATQ is indicative of different mechanisms of toxicity. Previous studies on hydroxyanthra-quinones have shown that toxicity is highly dependent on structure and position of

hydroxyl groups. Lampi et al (Lampi et al., 2006) postulated that tautomerization and hydrogen bonding of some hydroxylated anthraquinones could favour Michael adduct formation with other biological molecules. Recently, Wang et al (Wang et al., 2006) discovered that quinone toxicity was inversely proportional to the degree of substituents present on the aromatic ring. This group also determined that the endoplasmic reticulum was sensitive to adduct formation in the presence of quinone compounds resulting in cell death. This would not be a favoured mechanism for 1,2-dhATQ due to the close proximity of the hydroxyl groups which would sterically hinder Michael adduct formation (Briggs et al., 2003, Wang et al., 2006). However, Babu et al. (Babu et al., 2001) found that treating the higher plant, *L. gibba*, with 1,2-dhATQ resulted in inhibition of photosynthetic electron transport in the chloroplast. The role of 1,2-dhATQ as an electron sink in the CL cell mitochondria would explain the toxicity detected. The difference in toxicity between MA and MI would support the role of 1,2-dhATQ as a localized mitochondrial toxicant.

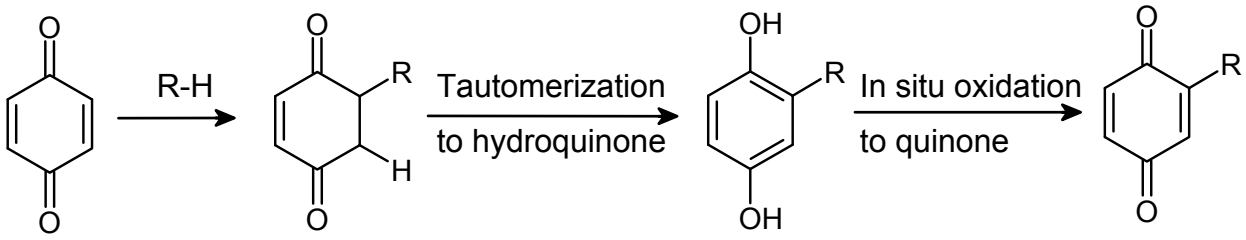


Figure 3.15 Schematic representation of 1,4-benzoquinone Michael adduct formation. Adapted from Briggs et al., 2003 and Wang et al., 2006 .

While both PHEQ and 1,2-dhATQ exhibited greater toxicity toward MA than MI, only PHEQ lowered MI. The futile redox cycling of PHEQ resulted in continuous production of ROS representing a mechanism that involves random toxic events whereby any

cellular component could be a target. The mechanism of action for 1,2-dhATQ represents specific toxicity to the mitochondria with 1,2-dhATQ circumventing the normal electron flow but remaining non-toxic to the cell membrane.

3.4.2 Toxicity of various metals to CL cells

Metals can bind to the outside membrane surface of some cell types or can be actively or passively transported inside the cell (Zalups and Barfuss, 2002). Once inside, metals can be sequestered in intracellular vesicles or through binding to storage proteins like metallothionein (Jacobson and Turner, 1980, Pourahmad and O'Brien, 2000, Zhang et al., 2003, Vijver et al., 2004). If the metals remain free inside the cell, damage occurs through displacement of the natural ligands in proteins or increases in production of reactive oxygen species (Jacobson and Turner, 1980, Chen et al., 1996, Manzl et al., 2004, Xie and Shaikh, 2006a). In general, high concentrations of metals exert toxicity according to the biotic ligand model (BLM) while low levels are indirectly toxic through ROS-mediated damage.

All of the metals tested in this study can generate ROS but not all by redox cycling (Prasad et al., 2006, Shenker et al., 2000, Risso-de Faverney et al., 2004, Pathak and Khandelwal, 2006, Steinebach and Wolterbeek, 1993, Galhardi et al., 2004, Austin et al., 1998, Manzl et al., 2004). One method of ROS production involves cycling of the metal between two redox states whereby the metal catalyzes electron transfer from a donor biomolecule to O_2 generating $O_2^{\cdot-}$ in the process. $O_2^{\cdot-}$ can be reduced further to H_2O_2 and even hydroxyl radicals (•OH) via Fenton-type reactions by metals. Any of these ROS can be extremely damaging to biological constituents. Metals that can easily

redox cycle *in vivo* include Cu^{2+} and Zn^{2+} (Stohs and Bagchi, 1995, Gyulkhandanyan et al., 2003, Bossy-Wetzel et al., 2004).

Another mechanism of metal induced ROS production includes the inactivation of cellular antioxidants. Metals with these properties include Cd^{2+}, Hg^{2+}, and Ni^{2+} (Lund et al., 1993, Garcia-Fernández et al., 2002, Adamis et al., 2004). Adamis et al (Adamis et al., 2004) determined that Cd^{2+} was able to bind glutathione (GSH) and that GSH-Cd conjugates appeared detrimental to *Saccharomyces cerevisiae*. Further evidence of the effects of metals on antioxidant levels may be indicated by studies such as Mahboob et al (Mahboob et al., 2001) and Goering et al (Goering et al., 2002) whereby induction of GSH expression was increased or levels of GSH were lowered in a dose-dependent manner. This indirect means of increasing ROS levels would be dependent on the inherent antioxidant concentrations present in the cell. One antioxidant protein in the cell is thioredoxin which occurs in two isoforms, thioredoxin-1 is cytosolic, and thioredoxin-2 is mitochondrial. Hansen et al (Hansen et al., 2006) determined that greater thioredoxin-2 oxidation compared to thioredoxin-1 occurred with exposure to Cd^{2+} and Hg^{2+}. These observations illustrate that Cd^{2+} and Hg^{2+} have greater oxidative effects in the mitochondria than the cytosol. For metals such as Cd^{2+}, Hg^{2+}, and Ni^{2+}, this mechanism may explain the greater toxicity observed for MA compared to MI.

A third mechanism of metal-mediated ROS generation may involve the displacement of naturally occurring metals in key proteins in the mitochondrial electron transport chain. One example would be the replacement of Fe^{3+} by Cd^{2+} in cytochrome P450 (Jasińska et al., 1996, Bozcaarmutlu and Arinc, 2007). With Cd^{2+} being more of an electron sink than Fe^{3+}, electron flow would be diverted from cytochrome P450 and the

electrons could then react with other available electron acceptors such as O_2 resulting in ROS (Babu et al., 2001, Babu et al., 2005). For non-redox cycling metals, the greater toxicity observed for MA compared to MI would be explained by this mechanism.

An exception to the observed trend in metal-induced toxicity was Zn^{2+}. In the current study, EC50s for Zn^{2+} exposure were higher for MA (25 μM) than for MI (2.2 μM) suggesting that the plasma membrane was a more sensitive target than the mitochondria. Although Zn is essential, Zn homeostasis is tightly regulated through complex uptake and efflux transporter proteins as well as storage proteins under metal-dependent transcriptional control (Laity and Andrews, 2007). Control of gene regulation may be another target of Zn toxicity with the use of Zn in many transcription factors.

Literature values for Zn^{2+} EC50s are highly variable. Riley et al (Riley et al., 2003) and Dayeh et al., 2005 reported similar EC50s ranging from 200 μM for rat lung epithelial cells to 127 μM for *Tetrahymena thermophila*. However, Leblond and Hontela (Leblond and Hontela, 1999) found that interrenal cell suspensions from juvenile rainbow trout were less sensitive with an EC50 of 22.8 mM. Conversely, Dayeh et al (Dayeh et al., 2005) reported an EC50 of 18 μM for rainbow trout gill cell line. In the current study, Zn^{2+} exhibited greater toxicity to membrane permeability than to metabolic activity. Similar results showed that Zn^{2+}-induced damage to rat lung epithelial cells was a result of leaky cell membranes (Riley et al., 2003). This may explain the sensitivity of plasma membranes to Zn^{2+} observed in the current study.

Confounding the search for the mechanism of action for each metal are the high levels of antioxidants found in CL cells. The high β-carotene content of the CL gives it a characteristic yellow colour hence its name corpus luteum which is Latin for yellow body.

Other antioxidants found in CL cells include SOD, glutathione (GSH), ascorbic acid, and catalase (Rapoport et al., 1998, Kodaman and Behrman, 1999, Pepperell et al., 2003). One study found that variations of reduced glutathione (GSH)/oxidized glutathione (GSSG) redox state up to 60 mV occur normally during cell growth and differentiation. However, oxidations greater than 60 mV have been associated with cell death in colon adenocarcinoma HT29 cells (Kirlin et al., 1999). Any fluctuations in the redox state caused by metals would need to overwhelm the antioxidants found in the cytosol of CL cells. Mitochondria also contain antioxidants; however, the smaller volume and presence of critical enzymes makes the mitochondria more sensitive to ROS compared to the cell membrane. This explains why MA is more sensitive than MI. The high levels of antioxidants present in the CL explain the high concentrations necessary to elicit an effect by a metal like Cu^{2+}. Metal-mediated ROS production explains the observed toxicity for Cu^{2+} and is the probable mechanism employed by this metal. The depletion of antioxidants resulting in increased ROS production by metals such as Hg^{2+} and Cd^{2+} that do not redox cycle explains the toxicity observed in both the mitochondria and the cell membrane. Comparing MA and MI EC50s for each metal appear to support the different toxic mechanisms of metals.

3.4.3 Toxicity of metal/PAH mixtures to CL cell viability

Mixtures of various metals with either PHEQ or 1,2-dhATQ showed very different results. Addition of PHEQ to increasing concentrations of Cd^{2+}, Hg^{2+}, Ni^{2+} or Zn^{2+} displayed additive or less than additive toxicity to both MA and MI. This observation was repeated when PHEQ was replaced with 1,2-dhATQ. However, synergistic toxicity was

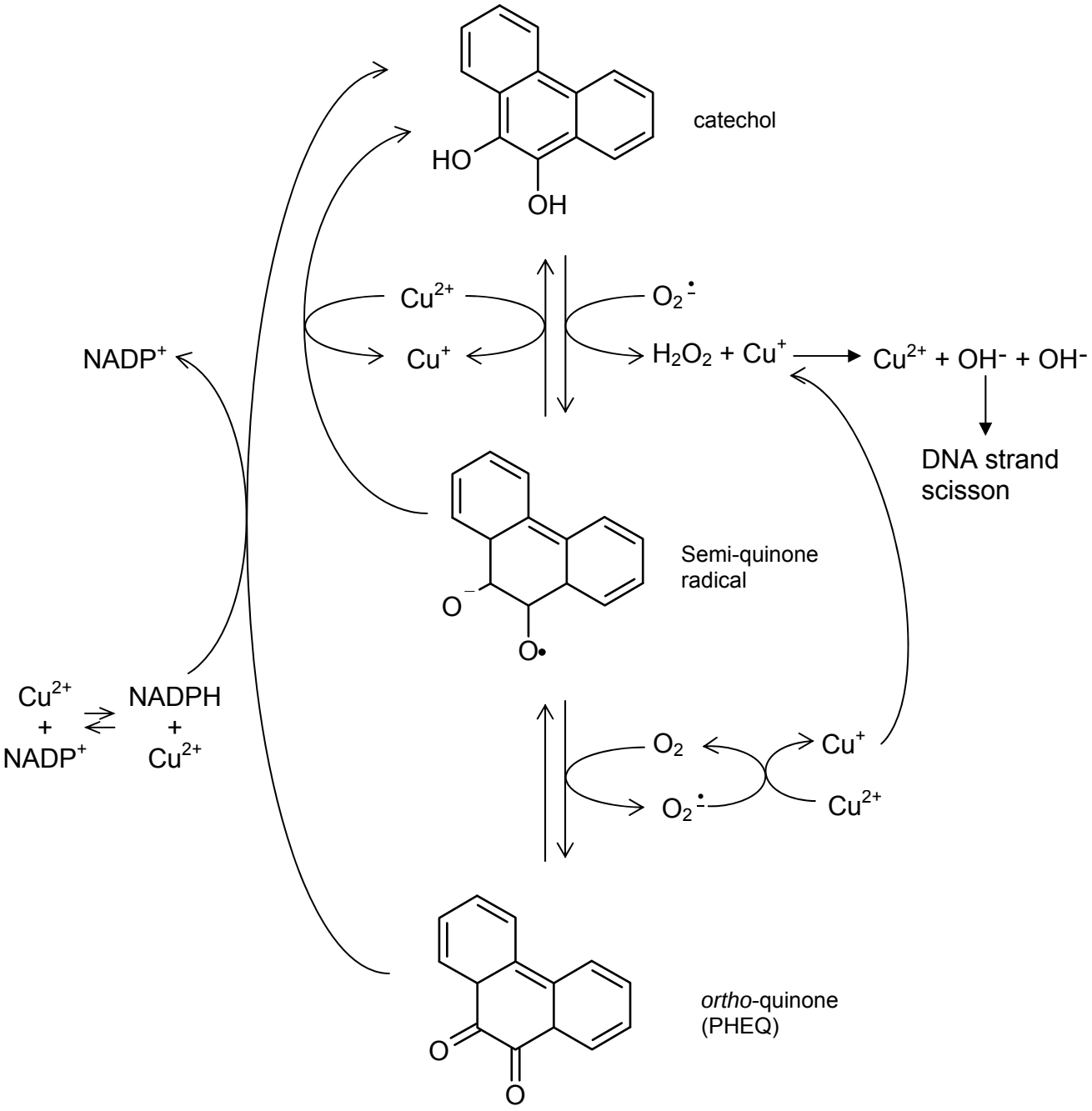


Figure 3.16 Proposed mechanism of Cu^{2+}/PHEQ redox cycling in the presence of NADPH. Redox cycling of PHEQ has a strict requirement for NADPH and Cu^{2+}. NADPH promotes redox cycling by providing reducing equivalents to form the catechol which could then autooxidize to reform the *o*-quinone (Adapted from Flowers et al., 1997).

observed with mixtures of Cu^{2+} and PHEQ. The co-exposure of CL cells to various concentrations of PHEQ with 10 or 100 μM Cu^{2+} resulted in a greater than additive toxicity to MA but not MI except for PHEQ + 100 μM Cu^{2+}. Previously, mixtures of PHEQ and Cu^{2+} have been shown to increase ROS in CL cells by 800% (Nykamp et al., 2001). Such an increase in ROS would overwhelm any antioxidant defence mounted by the cell leading to the observed impairment of MI. The lesser amount of antioxidants present in the mitochondria would be more easily nullified by increased ROS production which supports the observation of MA as more sensitive to toxic insult than MI. The mechanism behind this was postulated by Jarabak et al (Jarabak et al., 1997) and Flowers-Geary et al (Flowers-Geary et al., 1996) in cell-free systems (Figure 3.16). In this scenario, PHEQ can undergo a one electron reduction to produce a semi-quinone radical, which in turn can pass the unpaired electron to electrophilic species such as molecular oxygen to produce the superoxide radical and other related ROS (Flowers et al., 1997, Jarabak et al., 1997, Bolton et al., 2000). Recent work suggests that oxyPAHs and metals may act together to promote toxicity in a catalytic manner involving reactive ROS production targeting DNA and the mitochondria (Babu et al., 2001, Das et al., 1997).

The data presented here show that CL cells are vulnerable to both metal and oxyPAH exposure. The general mechanism of action for both species is most likely ROS production. Parent PAHs such PHE that do not produce ROS may not be directly toxic, but the greater toxicity exhibited by their photoproducts represents a hazard that must be addressed in risk assessment. MA in CL cells was a more sensitive indicator of toxicity than MI for most metals tested except Zn^{2+}. Being a metabolic metal, the cell

may have adapted means of mitigating Zn^{2+}-induced toxicity in the mitochondria but not at the cell membrane. The EC50s calculated in this study support the idea of metal-induced ROS production for toxicity rather than the BLM since the concentrations of metals used showed toxicity at levels far below that required for the BLM.

Most of the mixtures of oxyPAHs and metals tested did not display more than additive toxicity to CL cells. However, PHEQ and Cu^{2+} mixtures exhibited synergistic toxicity to both MA and MI in CL cells due to futile redox cycling and production of damaging ROS. This combination thus may represent a unique threat that must be considered during site-specific risk assessment.

The use of the CL represents a unique opportunity for studying reproductive toxicology in that toxic events at the cellular level can have implications at the physiological level. In the broadest definition, all of the toxicants tested represent potential endocrine disrupters due to their detrimental effect on a key endocrine organ.

Chapter 4 – Toxicity of Polycyclic Aromatic Hydrocarbons, Metals and Mixtures in a Placental Cell Line, JEG-3

4.1 Introduction

The human placenta is responsible for the exchange of nutrients, the elimination of wastes, and the production of hormones used for the maintenance and development of the fetus. The regulation of placental progesterone production is of great interest with regard to preserving pregnancy, onset of labour, timing of delivery and fetal growth (Gellersen and Brosens, 2003, Bulletti and de Ziegler, 2006). The placenta is composed of both maternal and fetal elements (Fazleabas and Strakova, 2002). After an oocyte is fertilized, the cleaving embryo, blastocyst, differentiates into two groups of cells; a peripheral outer cell mass and a central inner cell mass (Fazleabas and Strakova, 2002). The outer cell mass or trophoblast constitutes the main source of the placenta while the inner cell mass develops into the fetus. As the blastocyst implants itself into the endometrial lining of the uterus, the epithelial cells of the endometrium are transformed into decidual cells which accumulate lipid and glycogen (Fazleabas and Strakova, 2002). Higher lipid concentrations in the decidual cells are the source for steroidogenesis (Malassine and Cronier, 2002). The decidual cell layer thickens and becomes highly vascularized to provide adequate circulation of blood to the developing fetus.

JEG-3 cells are a placental choriocarcinoma cell line of trophoblastic origin (Lala et al., 2002). Although transformed, these cells retain many of the characteristics necessary for placental function (Burnside et al., 1985, Graham and Lala, 1992, Huang

and Miller, 2004). Studies using this cell line have established the regulation of various steroidogenic enzymes and co-factors involved in placental steroidogenesis (Ritvos, 1988, Ringler et al., 1989, Ritvos and Voutilainen, 1992a, Ritvos and Voutilainen, 1992b, Tremblay and Beaudoin, 1993, Black et al., 1993).

With the increased interest in endocrine disrupting chemicals (EDCs), JEG-3 cells have provided an attractive alternative to whole animal testing. Further use of JEG-3 cells has demonstrated the effect of contaminants on metallothionein and apoptosis induction, aromatase activity, epidermal growth factor levels, and human chorionic gonadotropin (hCG) secretion (Zhang et al., 1995, Zhang and Shiverick, 1997, Letcher et al., 2000, Vinggaard et al., 2000, McAleer and Tuan, 2001a, McAleer and Tuan, 2001b, Olivares et al., 2002, Nativelle-Serpentini et al., 2003, Allèra et al., 2004). More recently, studies using cell lines have examined the detrimental effects of pesticides, metals, and polycyclic aromatic hydrocarbons (Zhang et al., 1995, Eneman et al., 2000, Vinggaard et al., 2000). One study found that 7 PAHs of varying molecular weight were present in the placentas of all women tested with no relation to differing environmental conditions (Gladen et al., 2000). While no associations were observed between placental PAH concentration and birth weight of the infant, this study shows the prevalence of PAHs in relation to the reproductive system.

Many experiments analysing the effects of contaminants have been limited to single exposure studies, however, compounds such as PAHs and metals are commonly found in combination (Kamalakkannan et al., 2005, Brown and Peake, 2006, Gravato et al., 2006, Dai et al., 2007, Wichmann et al., 2006). Phenanthrene (PHE) is a common PAH found in many populated settings. Sources of PHE include smelting furnaces and traffic

emissions (Xing et al., 2006, Ciganek et al., 2004). When exposed to UV light, PHE is converted to the more reactive derivative 9,10-phenanthrenequinone (PHEQ) (Huang et al., 1995). Studies have demonstrated the ability of PHEQ to redox cycle with reactive oxygen species (ROS) being produced (Flowers-Geary et al., 1996, Nykamp et al., 2001, Shimada et al., 2004b). Interestingly, superoxide dismutase (SOD) can inhibit PHEQ redox cycling, but the addition of Cu^{2+} can restore cycling to pre-SOD rates (Jarabak et al., 1998). Another common PAH, anthracene (ANT) can also undergo oxidation reactions to form many quinone derivatives (Mallakin et al., 1999). One derivative of interest, 1,2-dihydroxyanthraquinone (!,2-dhATQ), has been shown to inhibit growth in the aquatic plant *Lemna gibba* (Mallakin et al., 1999).

In terms of human reproduction, the investigation of many environmental compounds has been overlooked in favour of pharmaceutical or recreational chemicals. Since studies have shown that humans have been exposed to environmental contaminants even prior to conception has made investigating the effect of toxicants such as PAHs and metals a priority. Therefore, the objective of this study was to determine whether two modified PAHs, PHEQ and 1,2-dhATQ, alone or in combination with metals, Cu^{2+} or Hg^{2+}, have a detrimental effect on steroid hormone production and the placenta two key components of the female, mammalian reproductive system. To this end, JEG-3 cells were used as the experimental model.

4.2 Materials and Methods

4.2.1 Reagents

Most reagents were purchased from Sigma (St. Louis, MO) including Minimal Essential Medium Eagle (MEM), sodium pyruvate, gentamycin, PHE, PHEQ, ANT, 1, 2-dhATQ, $CuCl_2$, and $HgCl_2$. Versene and MEM non-essential amino acids were obtained from Life Technologies. Reagents used for the viability testing included alamarBlue™ purchased from BioSource, International (Carmarillo, CA) and 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM) obtained from Molecular Probes (Eugene, Oregon). The chemicals used for the buffer in the radioimmunoassay were obtained from Sigma as well as charcoal (Norit A) and non-radioactive progesterone. [1, 2, 6, 7, 16, 17-^{3}H]Progesterone was purchased from Amersham BioSciences. Stock solutions of PHE, PHEQ, ANT, and 1, 2-dhATQ were prepared in dimethyl sulfoxide (DMSO) as described previously (Nykamp et al., 2001) . Stock solutions of $CuCl_2$ and $HgCl_2$ were prepared in sterile distilled, deionized H_2O.

4.2.2 Cell Culture and Treatment

JEG-3 cells were obtained from the American Tissue Culture Collection (ATCC). Cells were grown in 75 cm^2 tissue culture flasks using MEM supplemented with 10% FBS, 0.2 mM MEM non-essential amino acids, 2 mM L-glutamine, 0.5 mM sodium pyruvate, and 1 µl/ml gentamycin. Cells were incubated in a humidified atmosphere supplemented with 5% CO_2 at 37°C. Sub-culturing of cells included washing with versene twice prior to addition of trypsin. Cells were re-suspended in MEM with 10%

FBS and plated at a cell density of 2 x 10^5 cells/ml in 48 well Falcon plates with a total volume of 0.5 ml. Prior to treatment with the chemicals of interest, cells were assessed for viability and attachment to the plate using a phase contrast microscope. After removing media containing FBS, treatments were added to the wells containing MEM without added FBS. All PAHs were diluted in DMSO to a working concentration from stock before addition of 2.5 µl per well. The final dilution for all PAH concentrations in each well was 1/200. Working solutions of $CuCl_2$ or $HgCl_2$ were prepared in sterile, purified water and 5 µl of each concentration was added per well with a final dilution of 1/100. All experiments included a DMSO control. Cells were incubated with the treatments for 24 hours under the same conditions as before.

4.2.3 Viability Assays

For JEG-3, alamarBlue™ and CFDA-AM were used to assess the effect of toxicants on cell viability. Immediately prior to testing, the media was removed and 200 µl of L-15/ex containing 5 % v/v alamarBlue™ and 4 nM CFDA-AM was added to each well. The plates were incubated for 30 minutes under the same conditions as above. Care was taken to minimize exposure to light since the dyes are light sensitive. The plates were then read on a fluorometric multiwell plate reader (Cytofluor, Series 4000, PerSeptive Biosystems) at excitation and emission wavelengths of 485 nm and 530 nm for CFDA-AM and 530 nm and 590 nm for alamarBlue™.

4.2.4 Progesterone Radioimmunoassay (RIA)

Cells used for progesterone RIA were plated in 48 well microplates and grown to confluency in MEM containing 10 % FBS prior to treatment. To determine the effect of PHEQ, PHE, ATH, 1, 2-dhATQ or $CuCl_2$ (singly or in combination) on progesterone secretion, JEG-3 cells were incubated with various concentrations of toxicant in MEM without added FBS for 24 hours at 37°C / 5% CO_2. For this study, each experiment was replicated 3 or more times on different days consisting of typically 5 replicates per plate. After incubation, the samples were removed and placed in microcentrifuge tubes to be centrifuged (1000 g, 3 min) for separation of cells from medium. The supernatant was decanted and stored at -20°C until quantification of progesterone by RIA using the procedure described previously (Carlson and Gole, 1978). None of the PAHs were found to affect the binding of the anti-progesterone antibody in the assay.

4.2.5 Statistics

Data was analysed using two-way ANOVA followed by Fisher's Least Squares Determination (LSD) to assess significant differences where $p < 0.05$ using SYSTAT 10. For data sets with less than n=3, Student's t-test was used to determine significance ($p < 0.05$).

4.3 Results

Both PHEQ and 1,2-dhATQ decreased JEG-3 metabolic activity (MA) and membrane integrity (MI) after 24 hour exposure. Original experiments tested different incubation periods (24 versus 48 hour exposure) and found little difference to either MA or MI (Figure 4.1). With MA, the EC50 remained at 2.0 μM. However, the EC50 for MI decreased dramatically to 9 μM. Because MA appeared to be the more sensitive of the two parameters studied and there was no change between 24 and 48 hour exposure, the remainder of the experiments were conducted with 24 hour incubation periods. Additional experiments tested the effect of culture age on response to toxicant exposure. As culture age increased, MA and MI sensitivity to PHEQ decreased (Figure 4.2). From this data, a median age of 5 days was selected to perform the remainder of the toxicity testing.

No change was observed in cells treated with PHE, ANT or Cu^{2+} alone or in combination. This was true as well for progesterone secretion. Addition of extracellular copper chelator, bathocuproine (BC), but not intracellular chelator, neocuproine (NC), diminished PHEQ + Cu^{2+} toxicity.

4.3.1 Effect of PHE, PHEQ and Cu^{2+} on JEG-3 cells

The potential for PHE, PHEQ and Cu^{2+} to decrease cell viability was tested in JEG-3 cell line. Initial experiments showed that after 24 hours the addition of PHE or Cu^{2+} alone had little effect on JEG-3 cell MA (Figure 4.3). In another 24 hour test, co-incubation of PHE and Cu^{2+} with JEG-3 cells also showed little effect on MI (Figure 4.3). However, initial experiments with PHEQ demonstrated a significant and dramatic

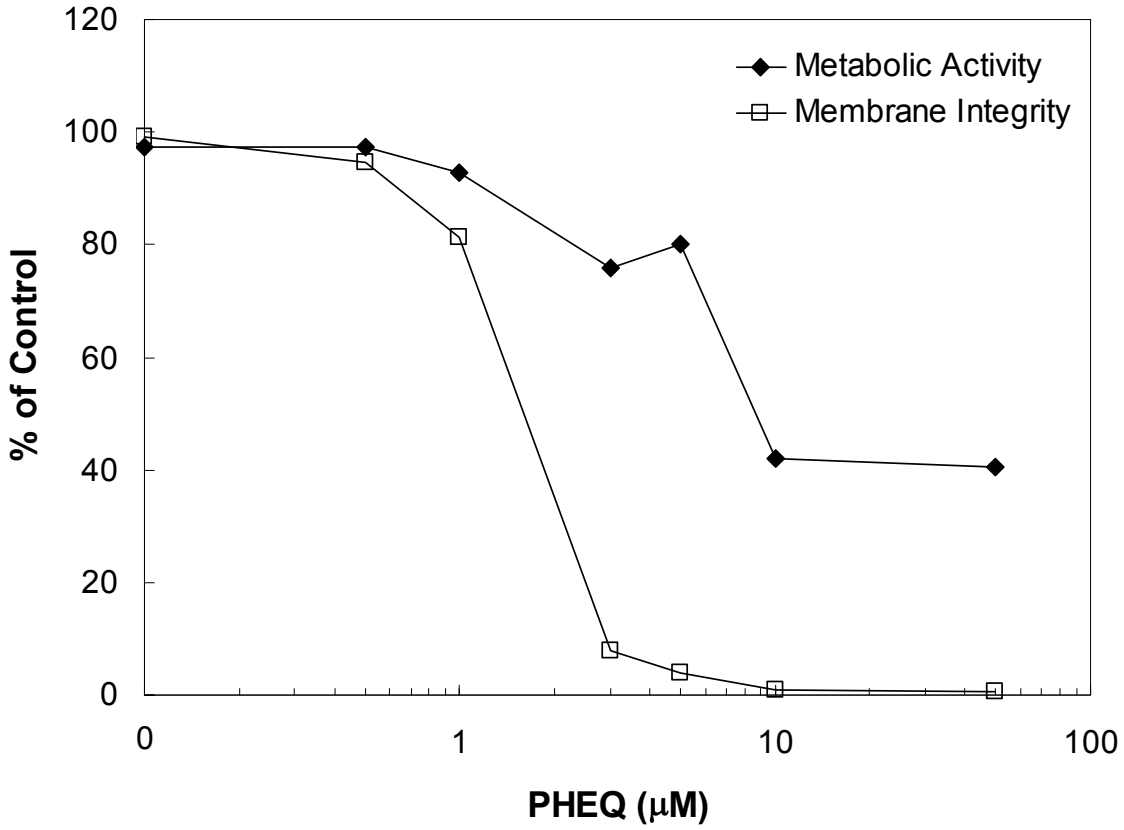


Figure 4.1 Effect of PHEQ on JEG-3 viability after 48 hour exposure. JEG-3 cells grown for 5 days to confluency prior to testing. MA appeared to decrease in a concentration dependent manner. MI decreased as well but higher concentrations of PHEQ were required to elicit an effect compared to MA. Little difference observed in response of JEG-3 cells to PHEQ treatment 48 hour incubations compared to experiments conducted with 24 hour exposure. Data represents n=1.

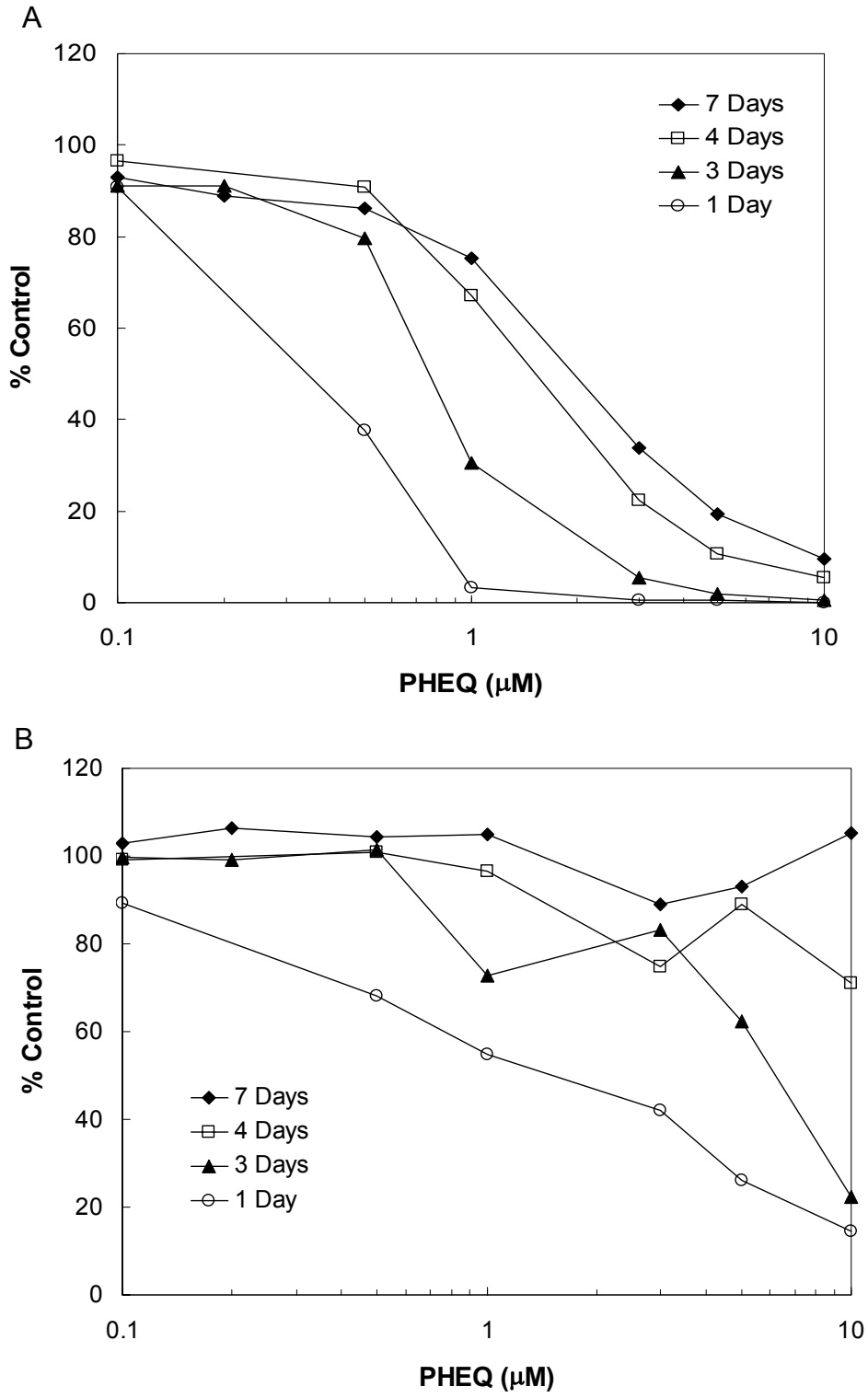


Figure 4.2 Effect of culture age on toxicity of PHEQ in JEG-3 cells. Graphs depict MA (A) and MI (B) for cells that were cultured for various time periods prior to PHEQ exposure. Younger cells (days 1 and 3) appear to be more sensitive to toxic insult. Little difference observed between days 4 and 7 for MA and MI. Data represents n=1.

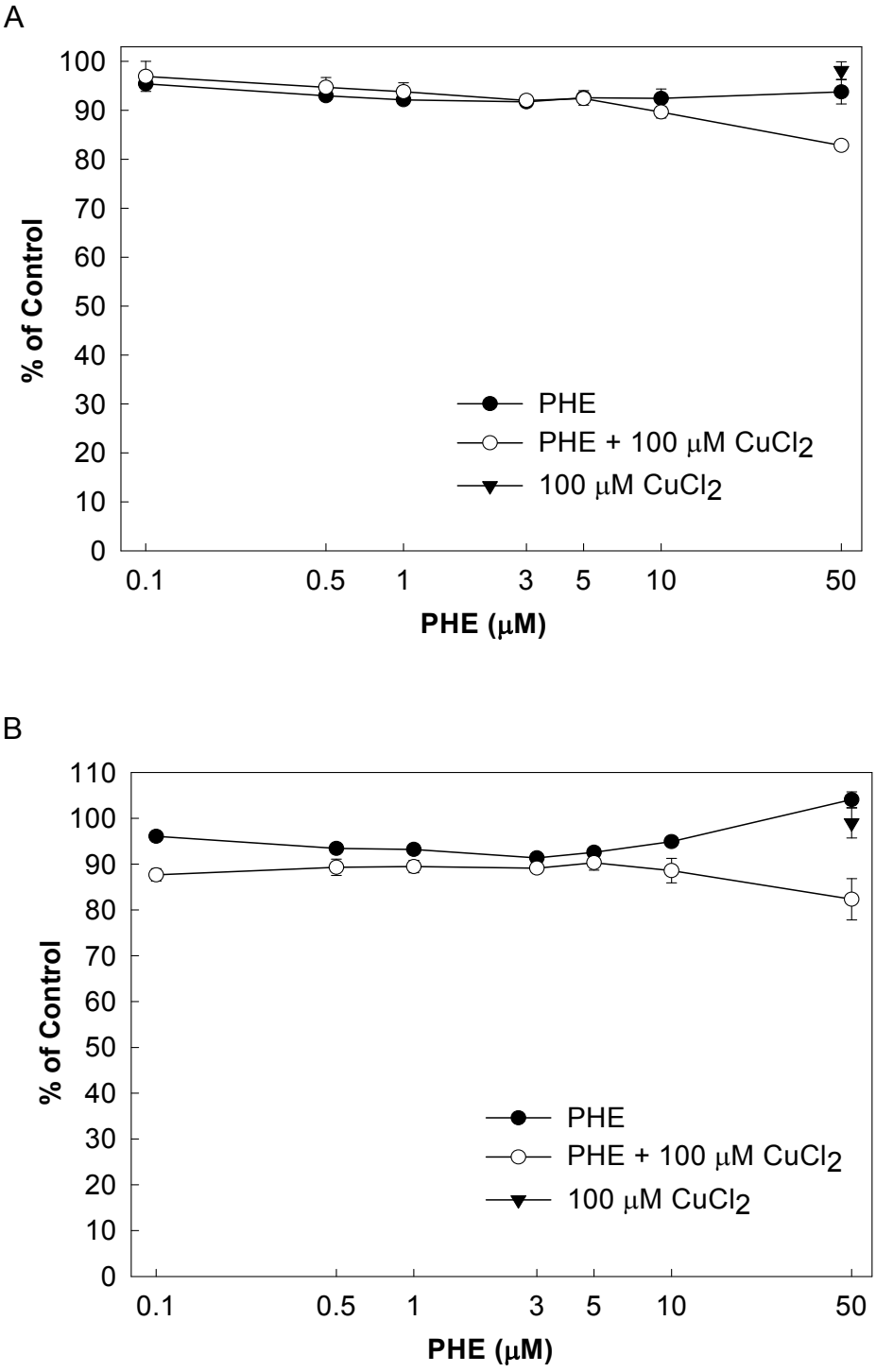


Figure 4.3 Effect of PHE and/or $CuCl_2$ on (A) metabolic activity and (B) membrane integrity in JEG-3 cells. Concentrations up 50 μM PHE with and without 100 μM $CuCl_2$ appear to have no effect on either MA or MI in JEG-3 cells after 24 hour exposure as measured by alamarBlue™ and CFDA-AM. Data represents mean of at least n=2 ± S.E. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

decrease in cell MA after incubation with the chemical for 24 hours (Figure 4.4). The EC50 was calculated at 2.0 μM (Table 4.1). Conversely, after 24 hours, MI was not as affected by PHEQ (Figure 4.4) with an EC50 of 110 μM (Table 4.1). Combining PHEQ with 100 μM Cu^{2+} caused an even greater decrease in both MA and MI, with EC50s for PHEQ of 0.1 μM and 0.5 μM, respectively (Figure 4.4). Due to the rapid decrease in viability observed with the combination of PHEQ and 100 μM Cu^{2+}, JEG-3 cells were treated with varying concentrations of Cu^{2+} and a low concentration of PHEQ (0.5 μM). No significant change in either MA or MI was detected until the highest concentrations of Cu^{2+} were used (Figure 4.5). The Cu^{2+} EC50s for MA and MI were calculated at 64.8 and 74.5 μM, respectively (Table 4.1).

The kinetic effect of PHEQ with and without Cu^{2+} was assessed over a 24 hour incubation period. For PHEQ exposure, MA decreased as incubation time increased with the greatest toxicity observed after 24 hours (Figure 4.6A). Membrane integrity remained unaffected after PHEQ exposure regardless of incubation time except after 8 hour incubation period which showed a significant decrease (Figure 4.6B). When incubated with both PHEQ and 100 μM Cu^{2+}, MA decreased with increasing incubation time. Compared to other incubation periods tested, the greatest inhibition appeared to occur after 24 hours (Figure 4.7A). Membrane integrity also appeared to follow the same trend as MI decreased with increasing incubation time (Figure 4.7B).

Cells were tested to determine their ability to recover after exposure to PHEQ with and without Cu^{2+}. The cells were treated for 24 hours, examined for viability, and then fresh media without toxicants was added. After allowing 24 hours for recovery, viability was re-assessed. For cells treated with PHEQ, little difference was observed between

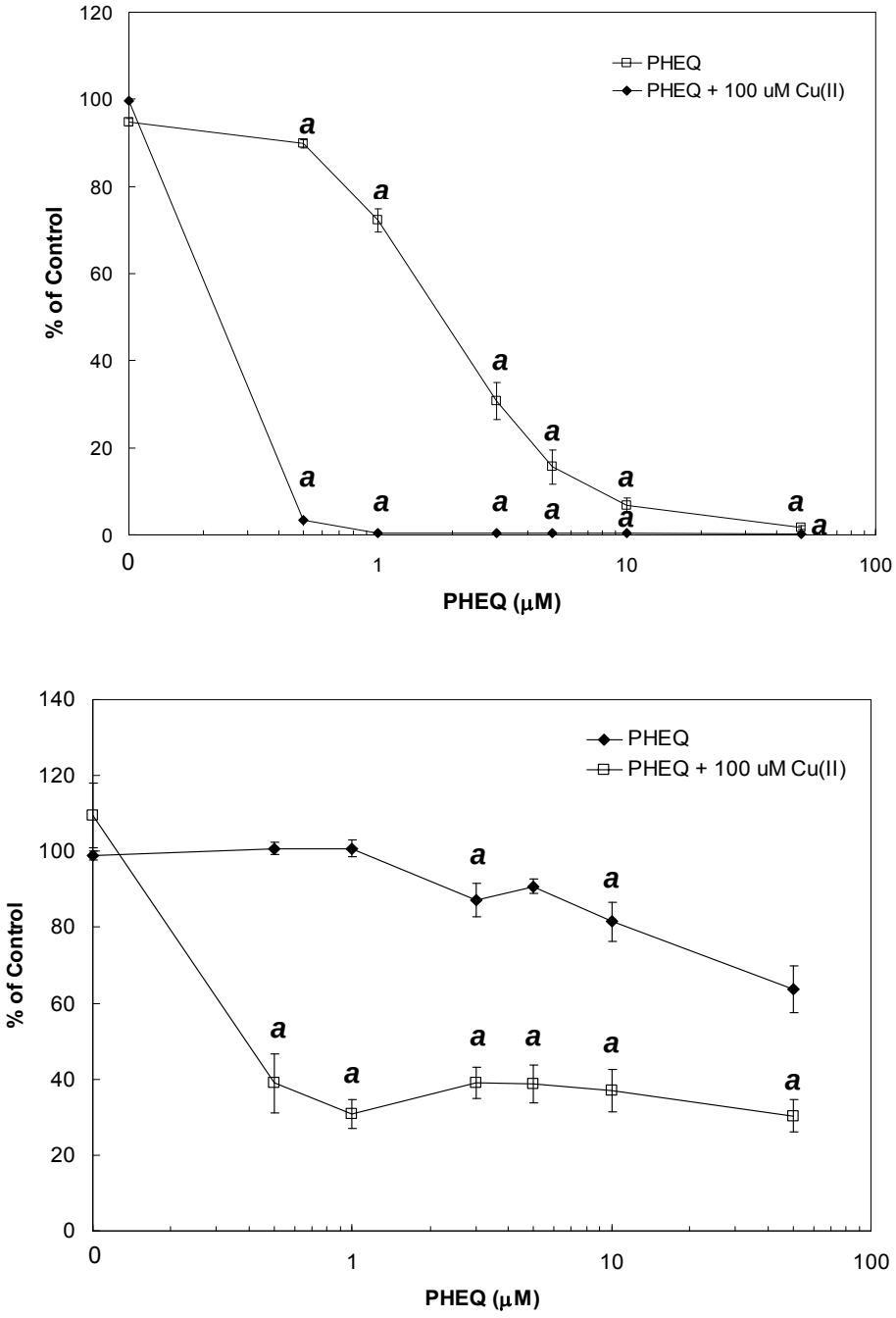


Figure 4.4 Effect of PHEQ and PHEQ + 100 μM Cu^{2+} on JEG-3 MA and MI after 24 hour exposure. JEG-3 cells grown for 5 days to confluency prior to testing. Incubation of JEG-3 cells with concentrations of ≥ 1.0 mM PHEQ showed significant inhibition of (A) MA as measured by alamarBlue™ and (B) MI as measured by CFDA-AM Addition of 100 μM Cu^{2+} significantly increased inhibition of MA and MI at all concentrations of PHEQ. [a] Significantly different from 0 μM PHEQ. Data represents average of n=4 ± SE. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

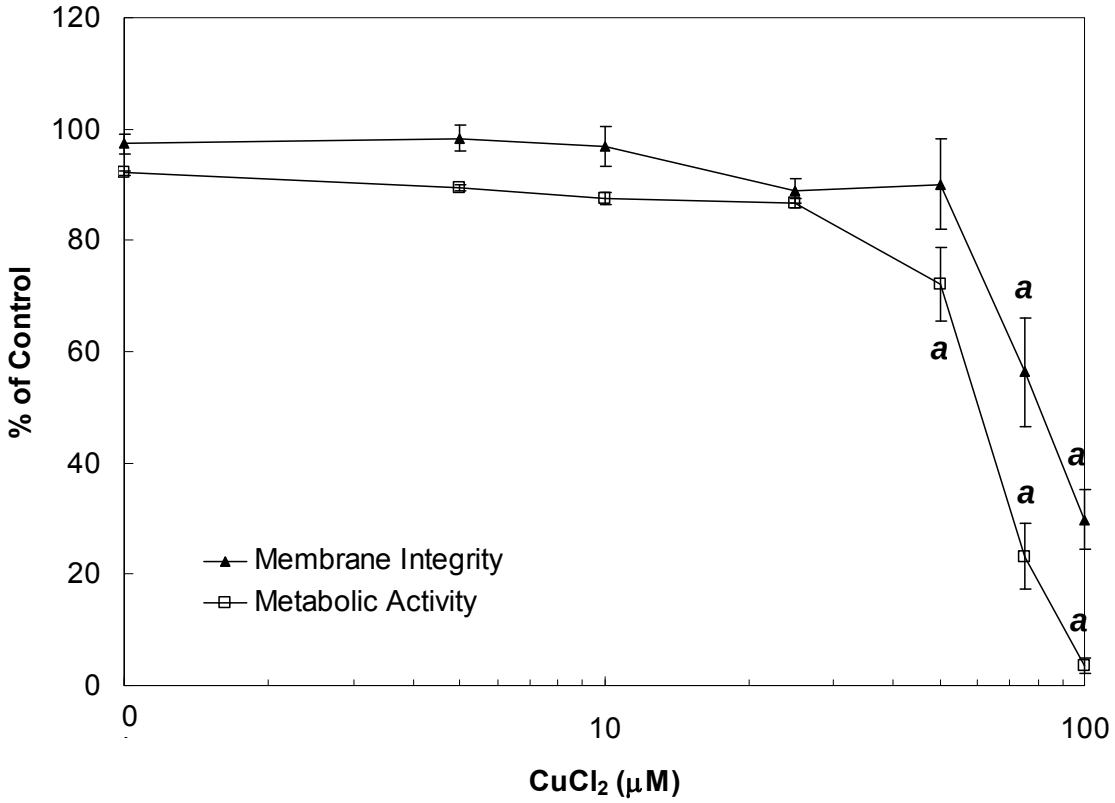


Figure 4.5 Effect of Cu^{2+} and 0.5 μM PHEQ on JEG-3 viability after 24 hour exposure. JEG-3 cells grown for 5 days to confluency prior to testing. No significant change in 0.5 μM PHEQ treated cells for either MA or MI was found as concentration of copper increased until 50 μM $CuCl_2$ for MA and 75 μM $CuCl_2$ for MI. [a] Significantly different from 0.5 μM PHEQ. Data represents average of n=5 ± SE. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

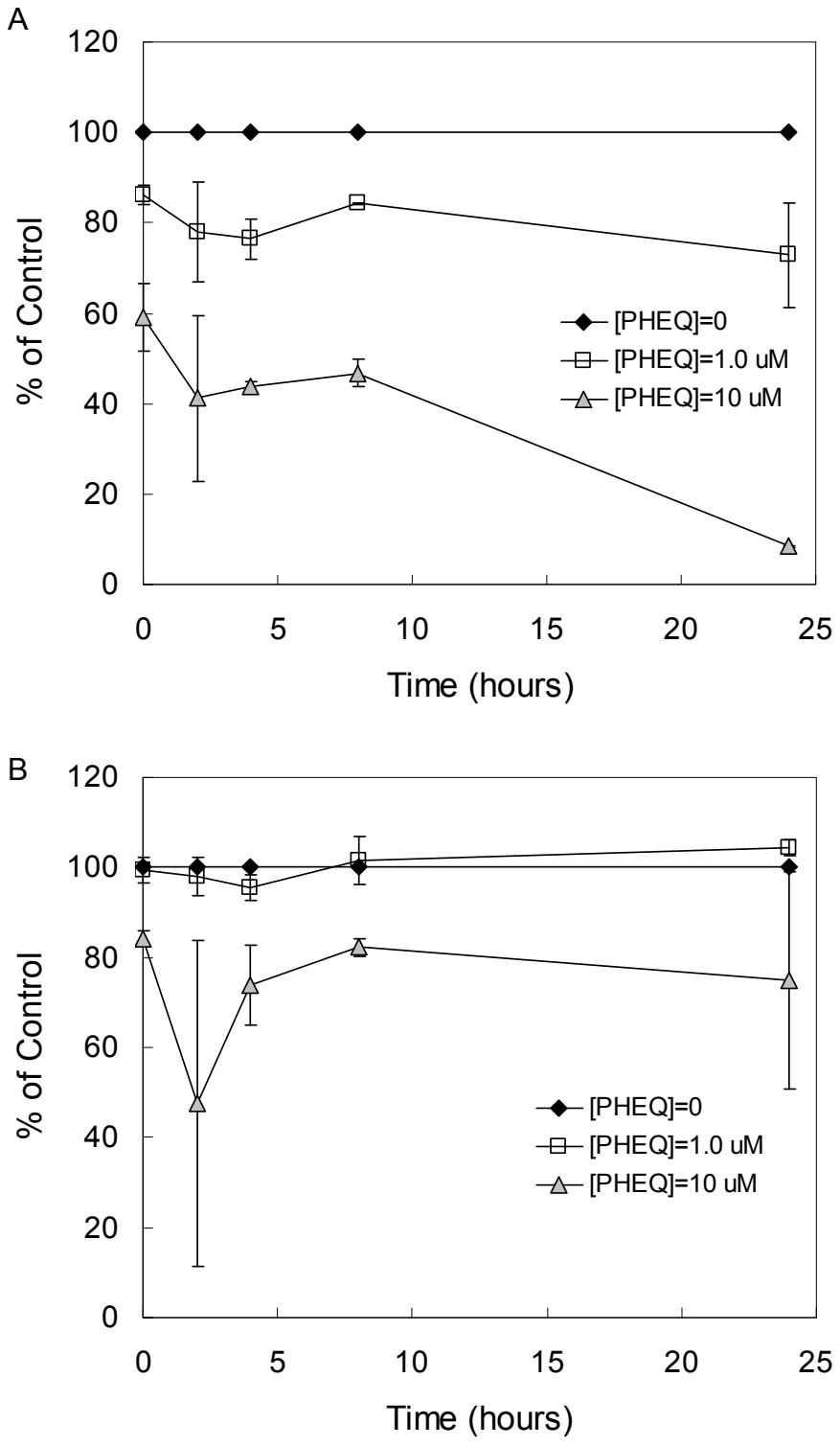


Figure 4.6 Kinetic effect of PHEQ on JEG-3 viability. Graphs depict (A) MA and (B) MI. Initial decrease in both MA and MI appears to occur immediately after exposure to PHEQ. Full inhibition of MA in 10 μM PHEQ treated cells appears to occur between 8 and 24 hours. Data represents mean of n=2 ± S.E.

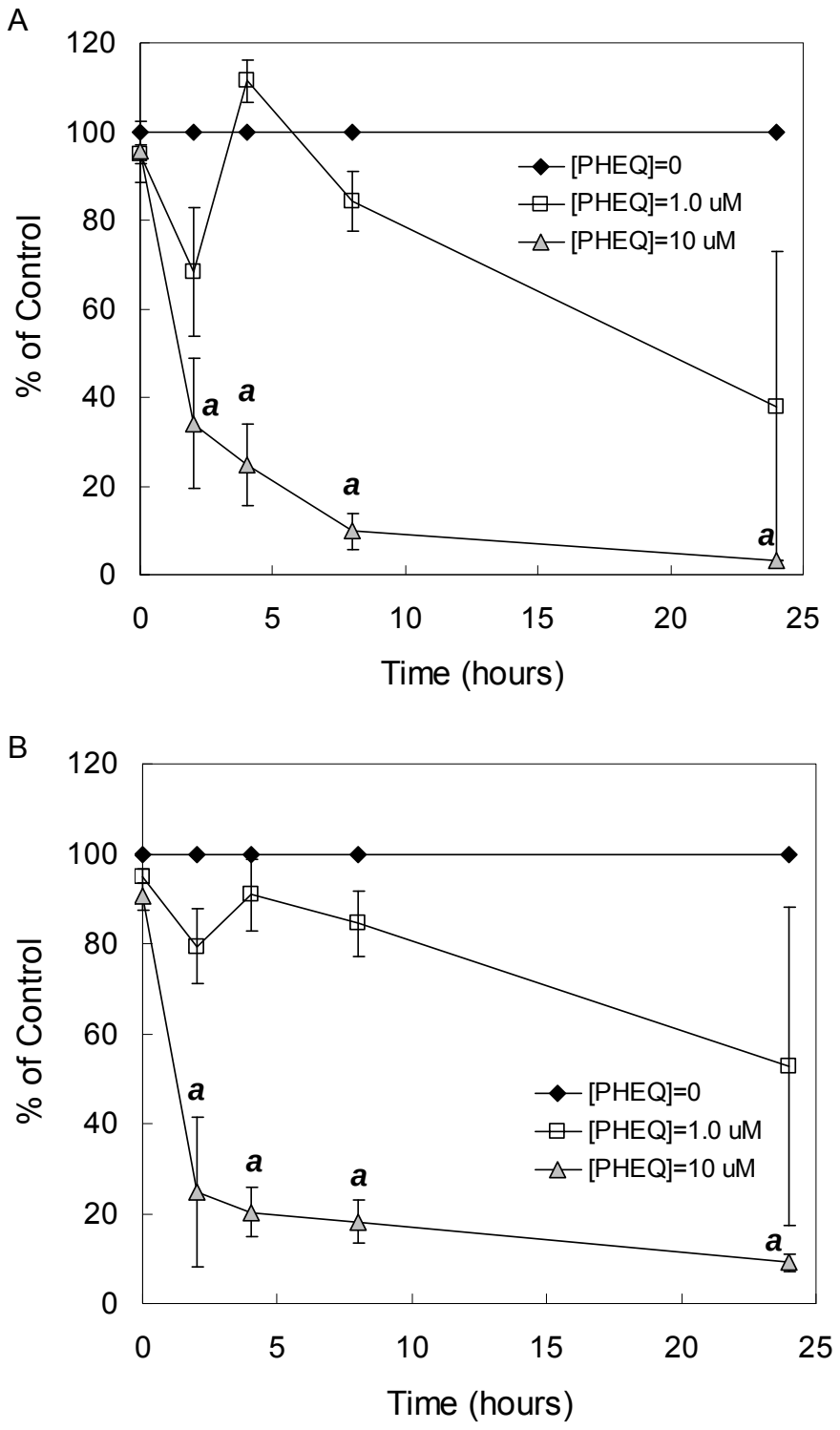


Figure 4.7 Kinetic effect of PHEQ + 100 μM Cu^{2+} on JEG-3 viability. Most inhibition of both MA (A) and MI (B) occurs within the first 2 hours of exposure to 10 μM PHEQ + 100 μM Cu^{2+}. [a] Significant decrease in MA and MI with 10 μM PHEQ + Cu^{2+} at 2 hours of exposure compared to time 0. Data represents mean of n=2 ± S.E. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

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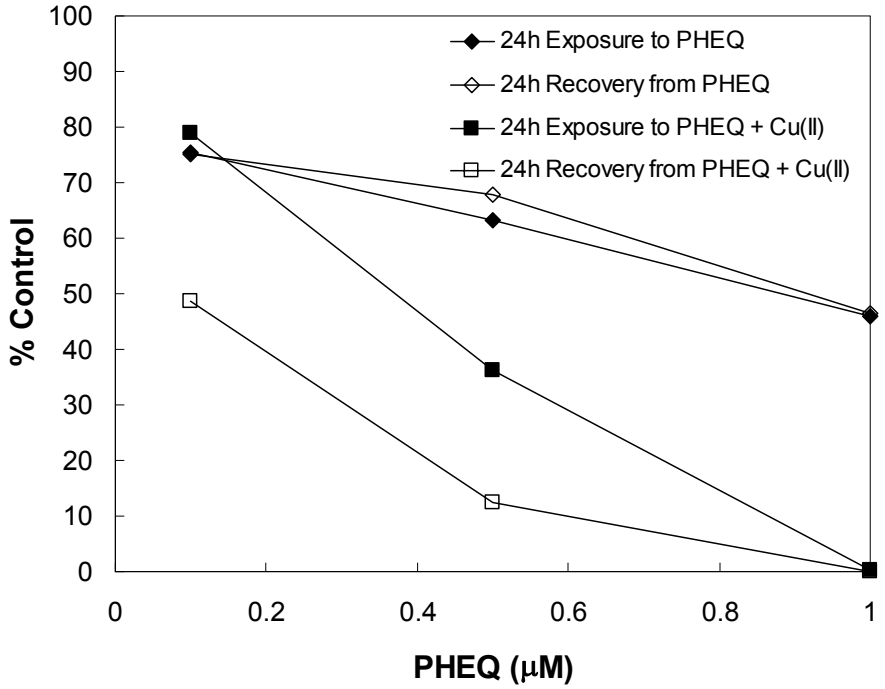

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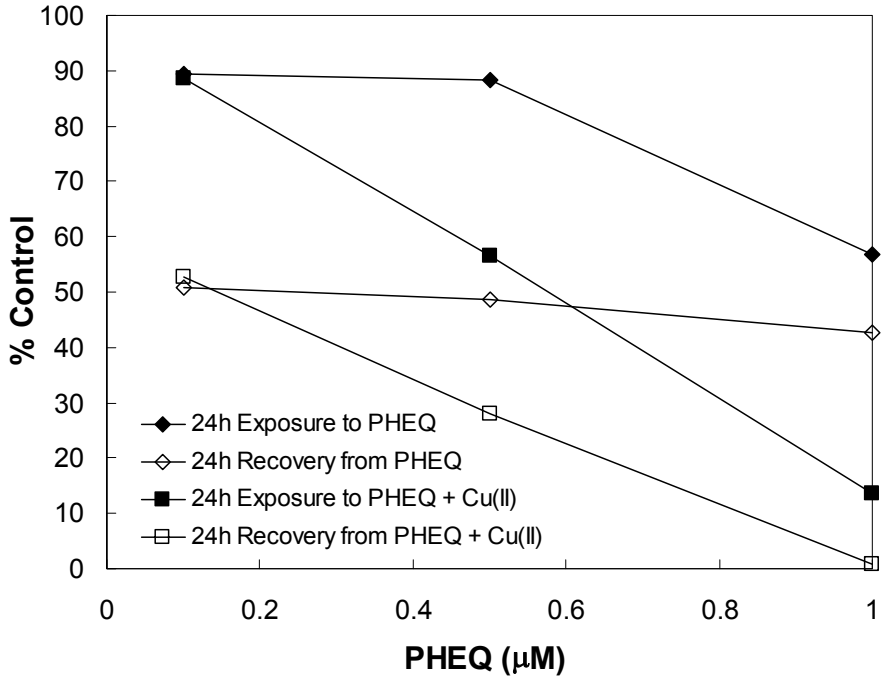

Figure 4.8 Ability of JEG-3 cells to recover after PHEQ +/- 100 μM Cu^{2+} exposure. Graphs depict MA (A) and MI (B) for cells that were exposed to PHEQ for 24 hours, then allowed to recover for 24 hours, cells exposed to PHEQ + Cu^{2+} for 24 hours, and then allowed to recover for 24 hours. Cells exposed to PHEQ only do not appear to have any further decline in either MA or MI, however, cells treated with PHEQ + Cu^{2+} continue to experience decreases in both MA and MI after toxicants are removed. Data represents n=1.

the initial exposure and the cells allowed to recover. However, when cells were incubated with both PHEQ and Cu^{2+} and then allowed to recover, viability continued to decrease compared to the initial toxicity (Figure 4.8). This data illustrates that regardless of treatment (PHEQ alone or PHEQ + 100 mM Cu^{2+}) JEG-3 cells could not recover from the initial toxic event once the toxicants had been removed.

Morphological differences were observed under a phase contrast microscope in the JEG-3 cells due to PHEQ and Cu^{2+} treatments. Previous studies describe JEG-3 cells as multinucleated cell clusters with prominent nucleoli (Lin et al., 1997). Untreated cells had a uniform, monolayer appearance with clear nuclei and distinct membranes (Figure 4.9A). At 0.5 μM PHEQ, the cells appeared to shrink and become rounder in shape but retained visible nuclei, were still distinct and relatively viable (Figure 4.9C). When PHEQ was increased to 5.0 μM, the cells became more diffuse without visible nuclei (Figure 4.9E). Increasing the concentration of PHEQ also caused numerous cells to detach from the plate and large clear sections were observed (Figure 4.9E). When treated with 100 μM Cu^{2+}, JEG-3 cells appeared rounder with slightly less distinct nucleoli, but also did not appear as diffuse as 5.0 μM PHEQ treated cells (Figure 4.9B). As concentrations of PHEQ increased in the presence of 100 μM Cu^{2+}, the cells appeared to shrink until they became ghost-like structures with only the cell membrane visible (Figure 4.9D and F). In addition, large clear patches with floating cellular debris were observed in the treatments containing 5.0 μM PHEQ (Figure 4.9F).

The effect of PHEQ and Cu^{2+} on progesterone production in JEG-3 cells was tested. While PHEQ and PHEQ + 100 μM Cu^{2+} both inhibited progesterone production, the

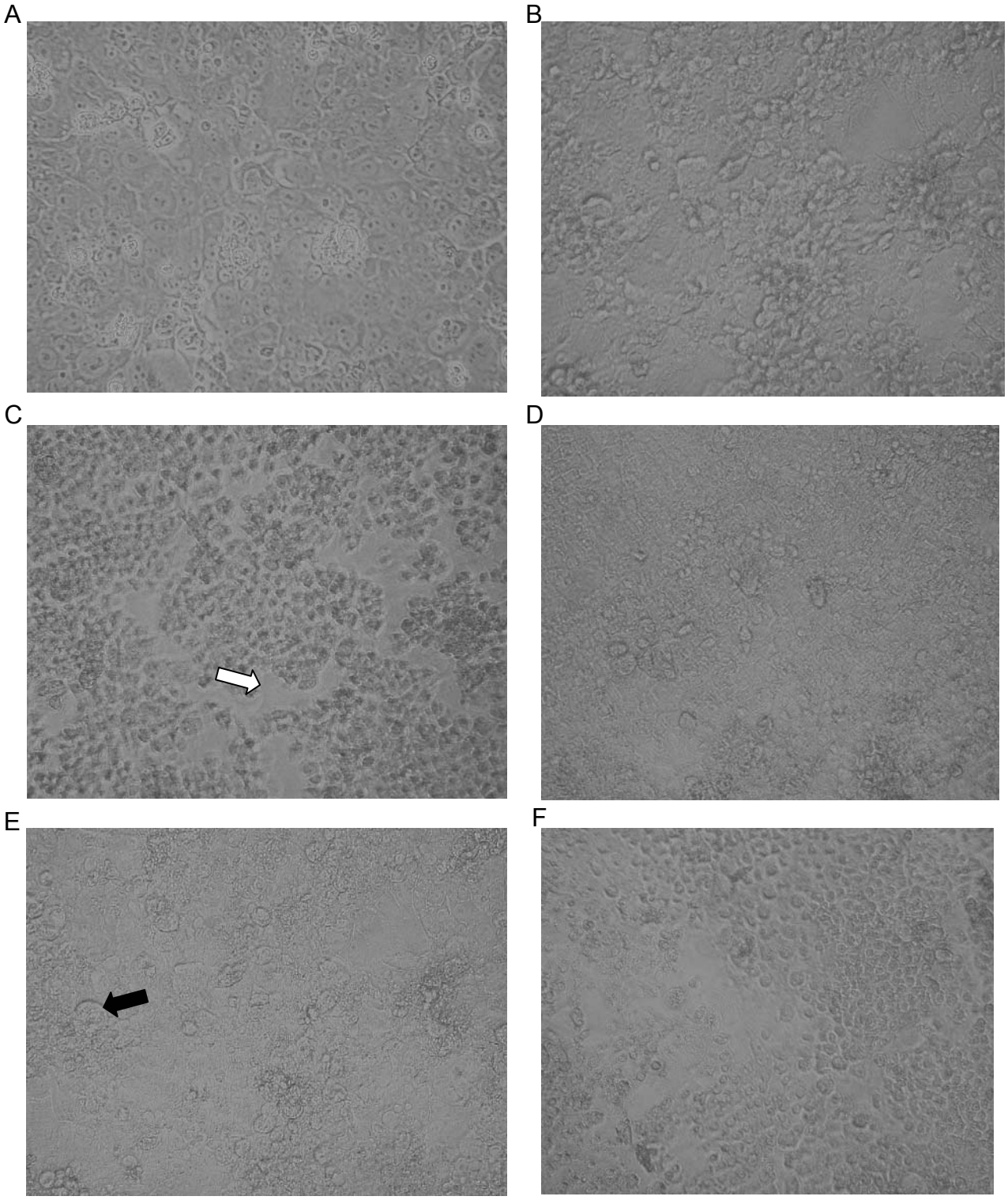

Figure 4.9 Pictures of JEG-3 cells treated with PHEQ ± 100 μM Cu^{2+}. Pictures were taken using phase contrast microscope at 100 x magnification and represent untreated JEG-3 cells (A), cells treated with 0.5 μM PHEQ (C), 5.0 μM PHEQ (E), 100 μM Cu^{2+} (B), 0.5 μM PHEQ + Cu^{2+} (D), or 5.0 μM PHEQ + Cu^{2+} (F). As concentrations of PHEQ +/- Cu^{2+} increase, clearing patches (⇨) and ghost-like structures (➡) become visible.

addition of Cu^{2+} did not cause further inhibition over PHEQ alone (Figure 4.10). The inhibition of progesterone production due to PHEQ and PHEQ + 100 μM Cu^{2+} exposure had EC50s of 2.8 μM and 1.2 μM, respectively. The PHEQ EC50s for MA (2.0 μM) and progesterone production (2.8 μM) were roughly equivalent. However, the PHEQ + 100 μM Cu^{2+} EC50 for MA (0.1 μM) was 10 times lower than the EC50 for progesterone production (1.2 μM). When a low concentration of PHEQ (0.5 μM) was added to various concentrations of Cu^{2+}, the effect on progesterone production was similar to cell viability. The incubation of JEG-3 cells with Cu^{2+} alone had no effect on progesterone production however, adding 0.5 μM PHEQ inhibited progesterone but only at the highest concentrations of Cu^{2+} added (data not shown).

4.3.3 Effect of antioxidants and Cu^{2+} chelators on PHEQ and Cu^{2+} toxicity

Glutathione (GSH), a common intracellular antioxidant, was added to JEG-3 cells to determine its effect on PHEQ and Cu^{2+} induced toxicity. For both MA and MI, GSH did not appear to be toxic on its own (Figure 4.11). Interestingly, when GSH was added to cells treated with PHEQ or Cu^{2+} alone or in combination not only did GSH not appear to mitigate toxicity from either toxicant and toxicity actually appeared to increase.

Two copper chelators were used in combination with Cu^{2+} treatment. Both membrane permeable neocuproine (NC) and membrane impermeable bathocuproine (BC) have been used as selective Cu(I) chelators (Göçmen et al., 2004, Kadiiska and Mason, 2002, Kagan et al., 2001). NC was tested at a constant 10 μM concentration

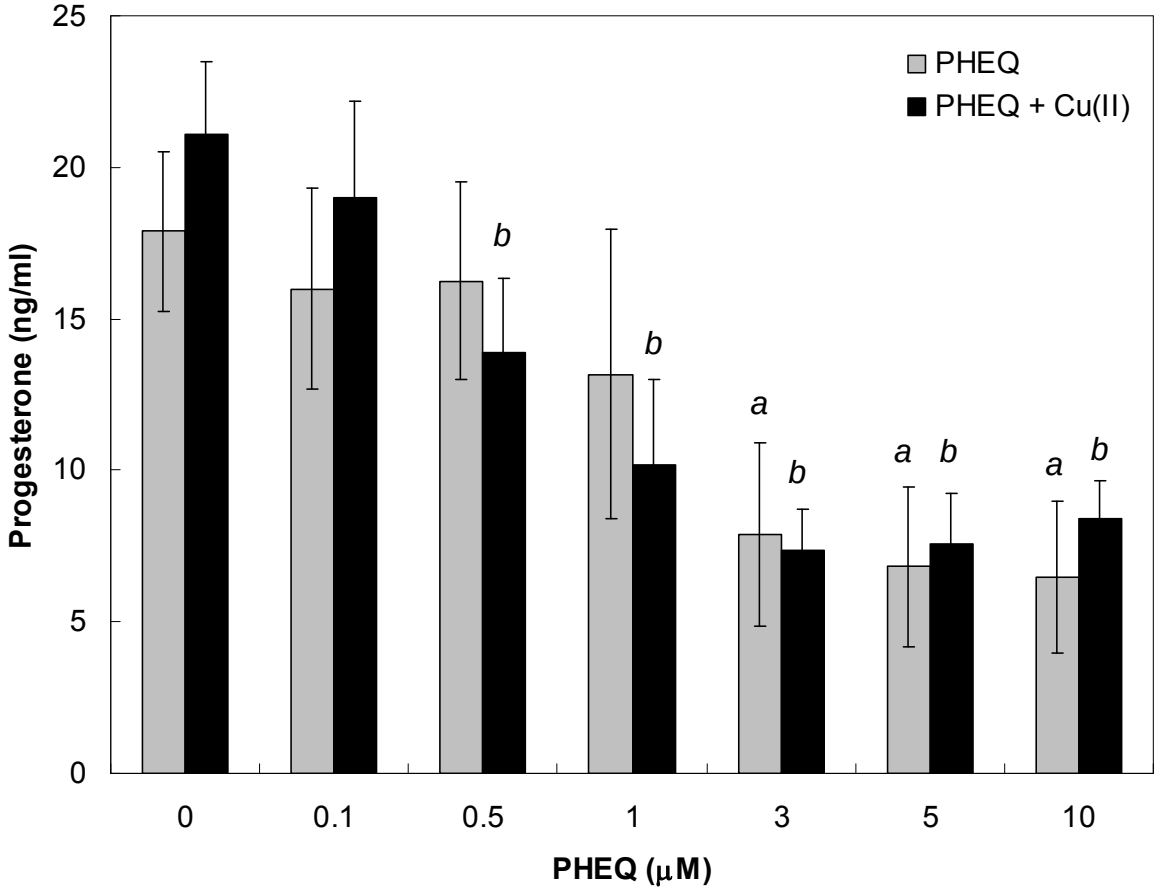

Figure 4.10 Effect of PHEQ + 100 □M Cu^{2+} after 24 hour exposure on progesterone secretion from JEG-3 cells. Addition of PHEQ caused a significant concentration dependent decrease in progesterone secretion in JEG-3 cells. No further inhibition of progesterone production was observed when cells were exposed to both PHEQ and 100 □M Cu2+. a Significantly different from 0 μM PHEQ. b Significantly different from 0 μM PHEQ + 100 □M Cu2+. Data represents mean of n=4 ± S.E. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

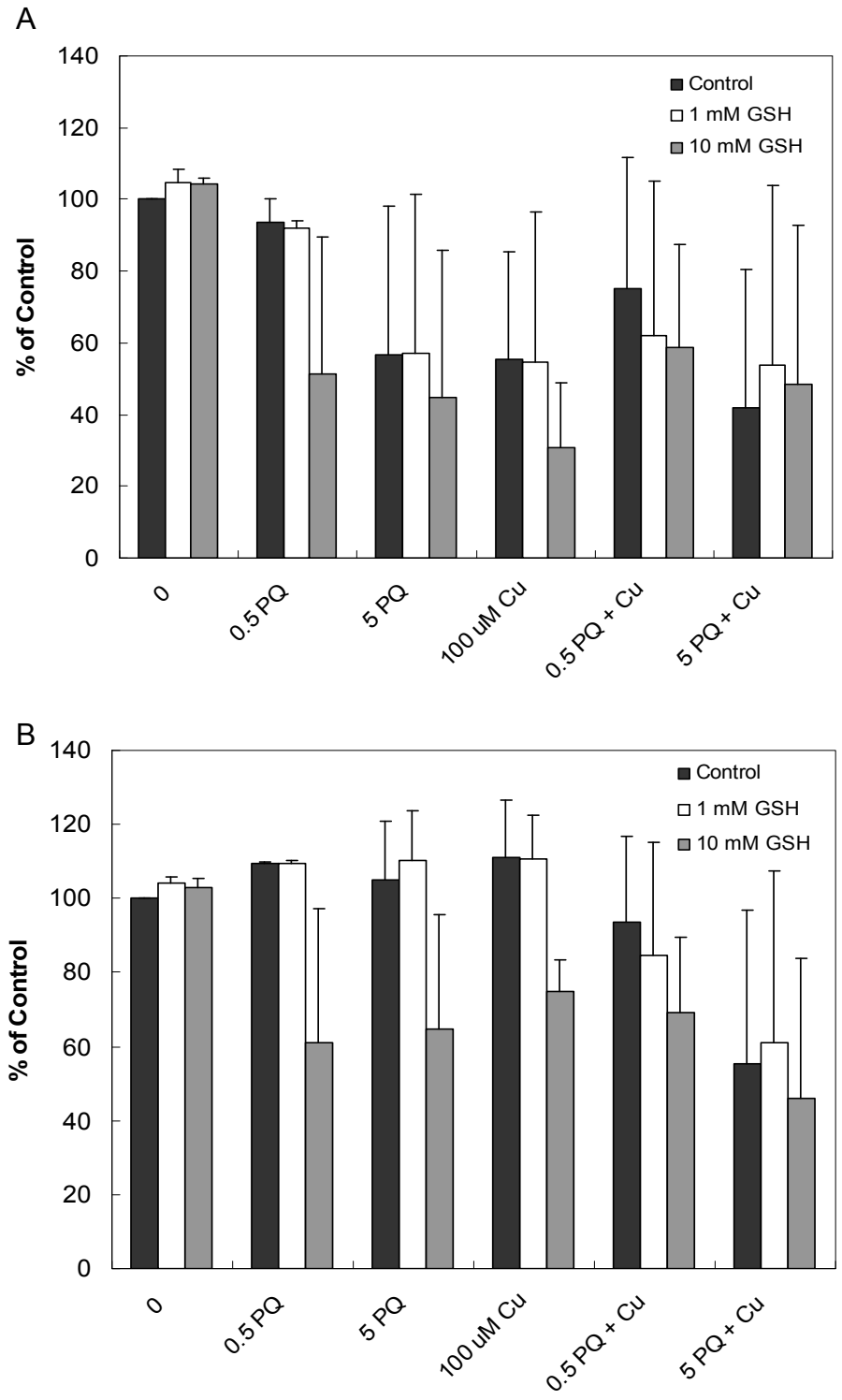

Figure 4.11 Effect of GSH on PHEQ + 100 μM Cu^{2+} toxicity in JEG-3 cells. Addition of 10 mM GSH appears to enhance 100 μM Cu^{2+} alone and PHEQ alone induced toxicity in both (A) MA and (B) MI of JEG-3 cells. Data represents n=2.

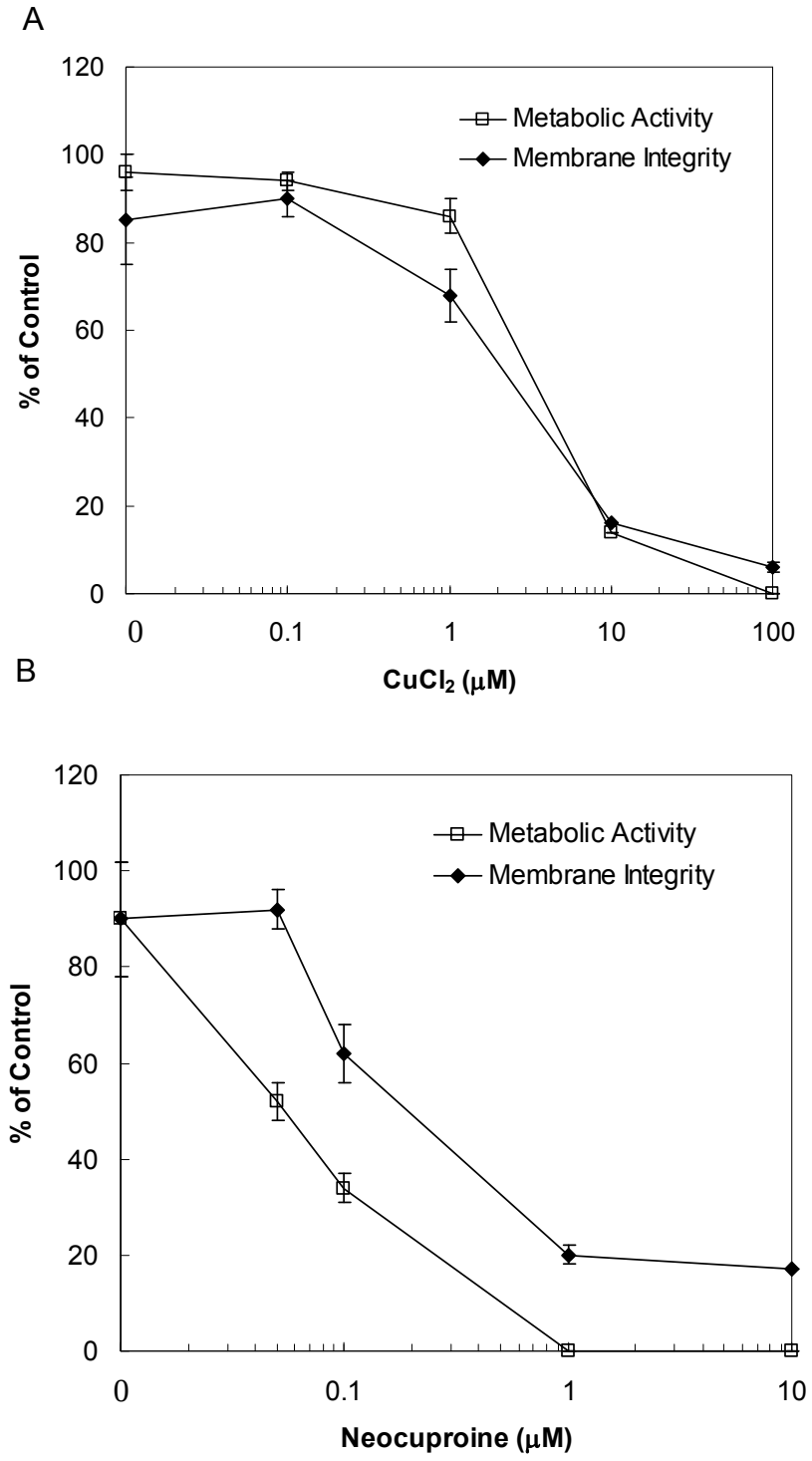

Figure 4.12 Effect of Cu^{2+} and neocuproine (NC) together on viability of 4 day old JEG-3 cells immediately after termination of 24 h exposures. In (A) NC was kept constant at 10 μM and copper was varied. In (B) copper was at 100 μM and NC was varied. Cu^{2+} while not toxic alone, was toxic at all concentrations of NC. Data represents n=6 +/- SD.

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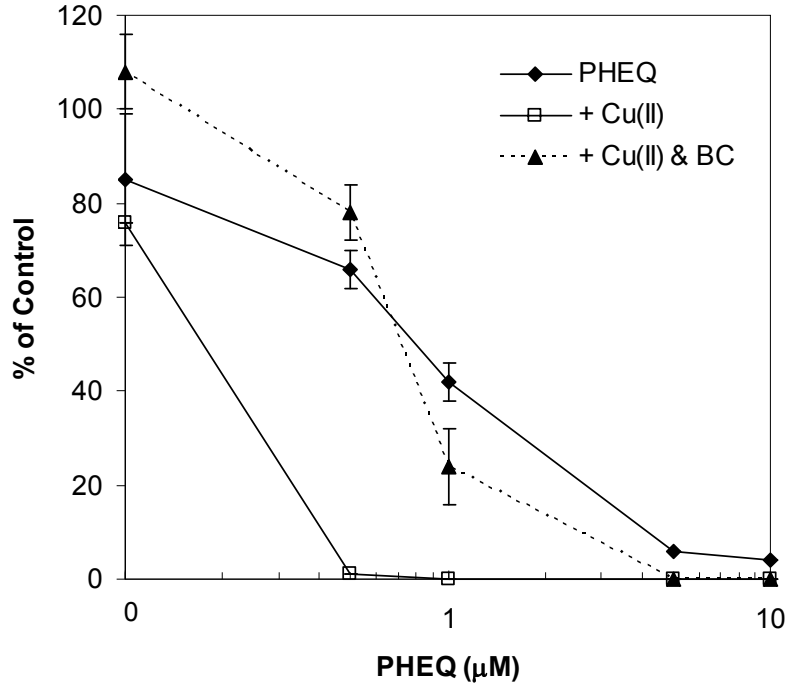

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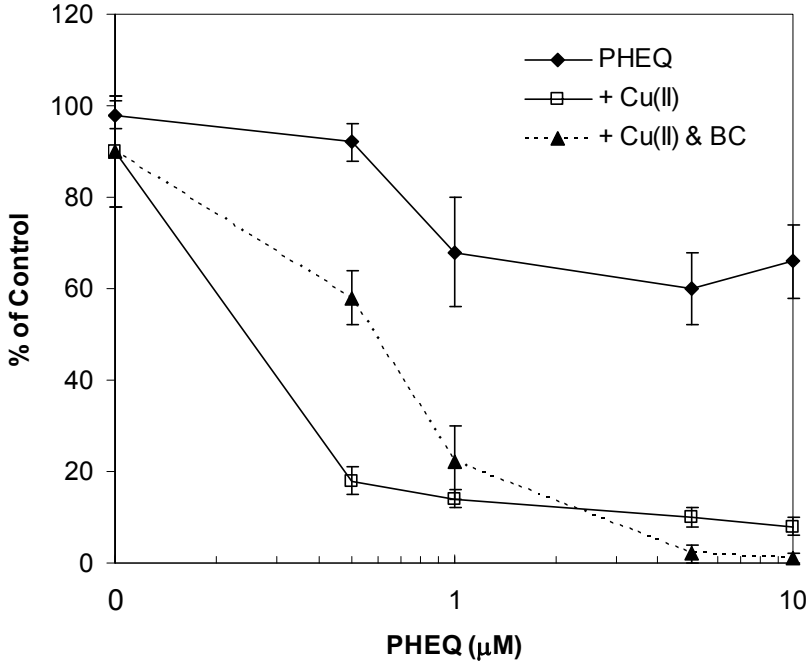

Figure 4.13 Effect of 100 μM Cu^{2+} and 200 μM bathocuproine (BC) on viability of 4 day old JEG-3 cells exposed to increasing concentrations of PHEQ. Metabolic activity (A) and MI (B) of PHEQ + 100 μM Cu^{2+} treated JEG-3 cells diminished less in presence of BC. Data represents n=6 +/- SD.

with varying Cu^{2+} (Figure 4.12A) and with a varying NC concentration with constant 100 μM Cu^{2+} (Figure 4.12B). NC did not appear to mitigate Cu^{2+} toxicity as Cu^{2+} levels increased nor did increasing concentrations of NC appear to decrease toxicity at 100 μM Cu^{2+}. Conversely, BC did appear to decrease the synergistic toxicity observed when both PHEQ and 100 μM Cu^{2+} were added to JEG-3 cells (Figure 4.13). Both MA and MI increased upon addition of BC to PHEQ and 100 μM Cu^{2+} treated cells.

4.3.4 Effect of Hg^{2+}, ANT and 1,2-dhATQ on JEG-3 toxicity

Hg^{2+} was substituted for Cu^{2+} in toxicity testing. Alone, Hg^{2+} caused little decrease in either MA or MI until the highest concentration (50 μM) was tested, which caused a sharp decline in both (Figure 4.14). The EC50s for Hg^{2+} were calculated at 20.0 μM and 23.0 μM for MA and MI, respectively (Table 4.1). When Hg^{2+} was combined with 0.1 μM PHEQ (EC50 = 7.0 μM – Table 4.1), MA exhibited a slight decline with a shallower slope compared to the abrupt decrease observed with Hg^{2+} alone (Figure 4.14). However, when treated with Hg^{2+} + 0.1 μM PHEQ (EC50 = 17.0 μM – Table 4.1), the shape of the curve for MI retained the abrupt decrease observed with Hg^{2+} alone (Figure 4.14).

A second PAH/oxyPAH pair was tested in JEG-3 cells. 1,2-dhATQ and its parent compound ANT were incubated with JEG-3 cells under the same conditions used with PHEQ and PHE. ANT and 1,2-dhATQ were tested alone and in combination with Cu^{2+} or Hg^{2+}. ANT alone was non-toxic up to the highest concentrations tested (Figure 4.15). When co-incubated with 100 μM Cu^{2+}, toxicity remained unchanged from ANT alone (Figure 4.15). Exposing JEG-3 cells to 1,2-dhATQ appeared to have no effect on either MA or MI at any concentration used (Figure 4.16). Addition of 100 μM Cu^{2+} in

combination with 1,2-dhATQ caused a dramatic decrease in both MA and MI compared to 1,2-dhATQ treatment alone (Figure 4.16). The EC50s for 1,2-dhATQ + 100 μM Cu^{2+} were 18.6 and 55.0 μM for MA and MI, respectively (Table 4.1). JEG-3 cells also were exposed to Hg^{2+} in combination with 1,2-dhATQ. The addition of 1,2-dhATQ to Hg^{2+} did not appear to alter Hg^{2+} toxicity in either MA or MI (Figure 4.17) with Hg^{2+} + 1,2-dhATQ EC50s remaining the same as Hg^{2+} (Table 4.1).

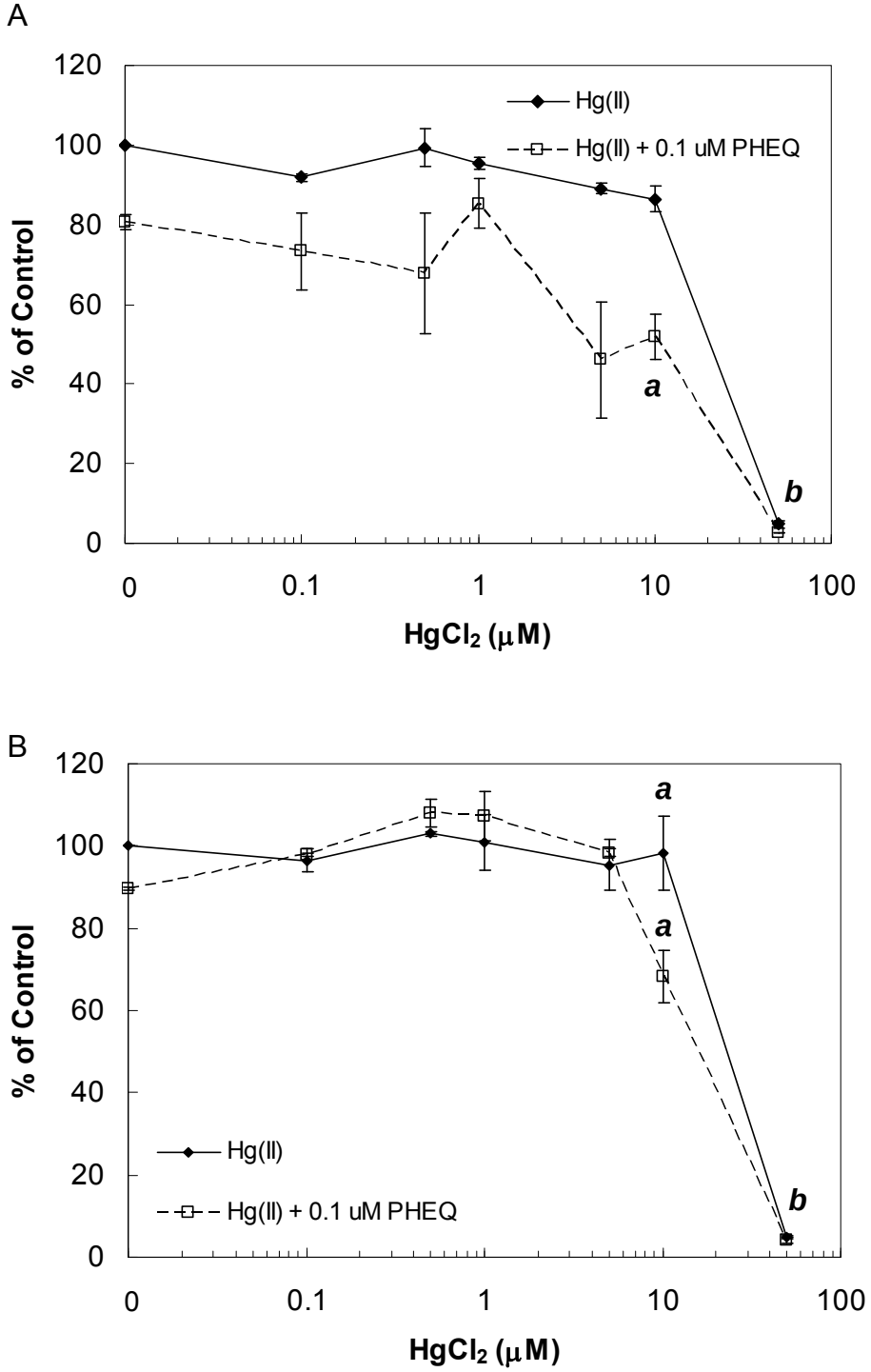

Figure 4.14 Effect of Hg^{2+} and 0.1 μM PHEQ on JEG-3 viability. Addition of Hg^{2+} alone caused significant inhibition of MA (A) and MI (B) at 50 μM. Co-exposure to both 0.1 μM PHEQ and Hg^{2+} resulted in significant decrease in MA at 5 μM Hg^{2+} and MI at 10 μM Hg^{2+}. [a] Significantly different from 0 Hg^{2+}. [b] All points significantly different from 0 Hg^{2+}. Data represents mean of n=3 ± S.E. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

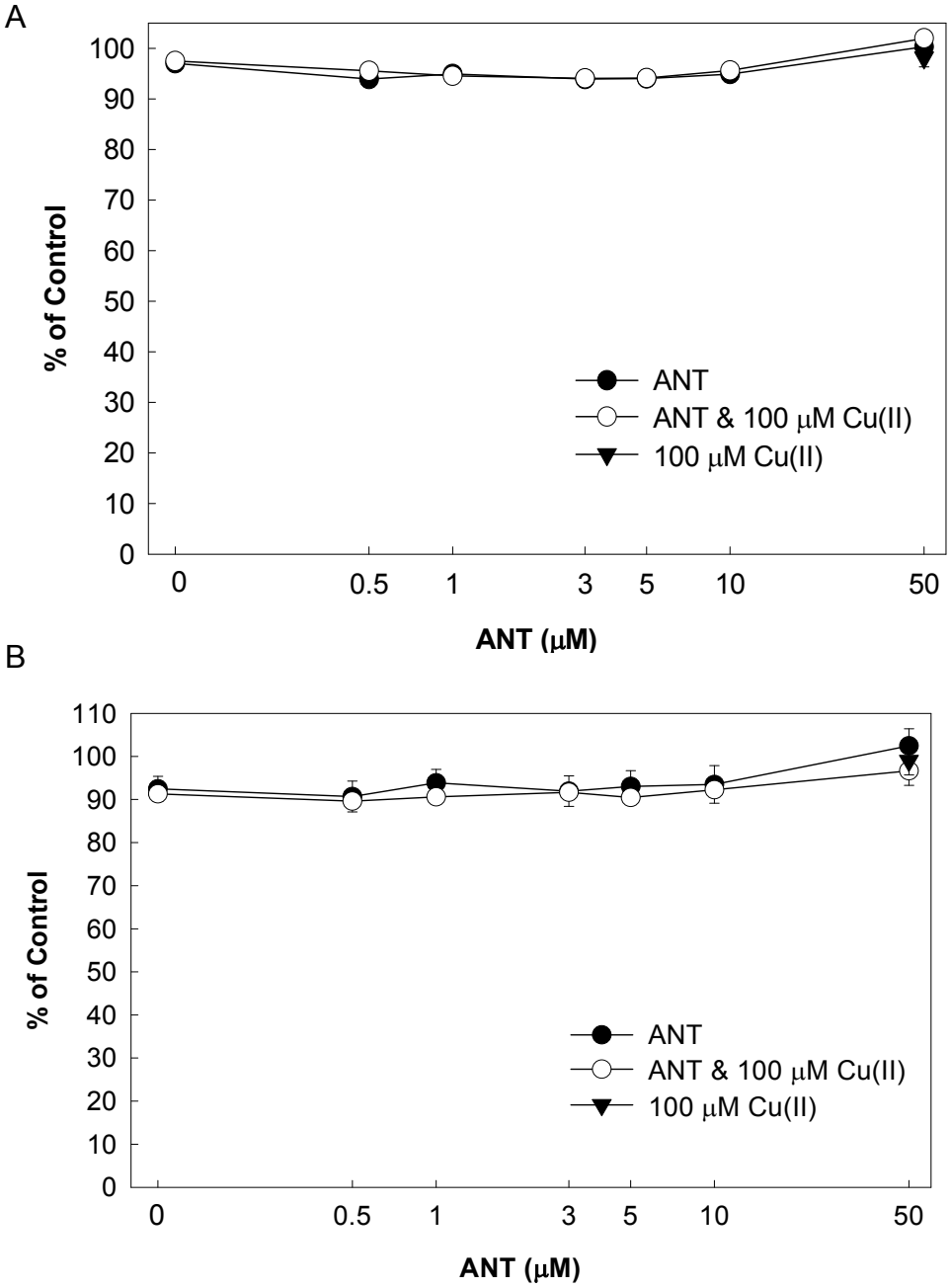

Figure 4.15 Effect of ANT and 100 μM Cu^{2+} on JEG-3 cell viability. Addition of ANT with or without 100 μM Cu^{2+} did not affect either MA (A) or MI (B). Alone, 100 μM Cu^{2+} did not have an effect on either MA or MI. Data represents mean of n=3 ± S.E. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

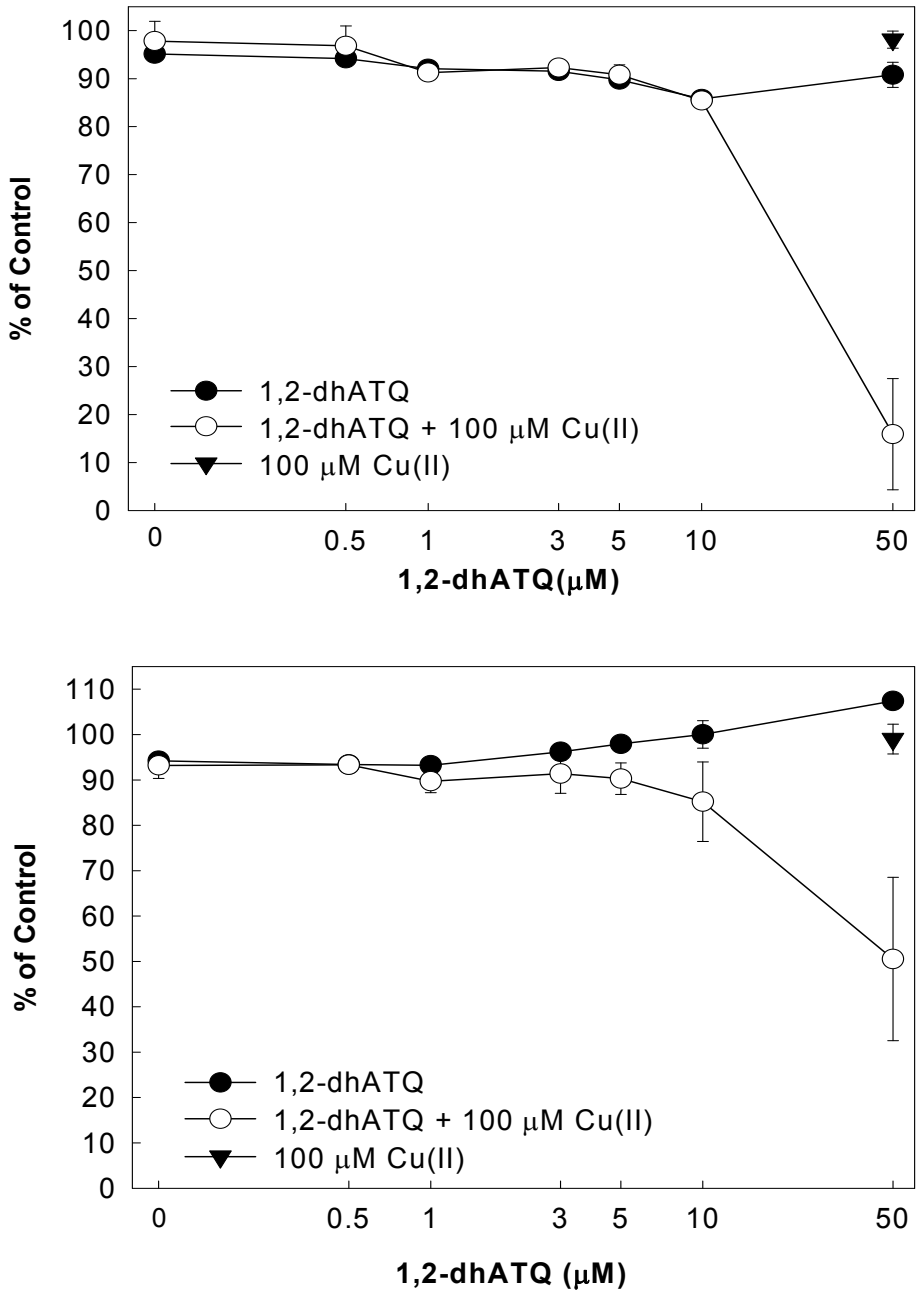

Figure 4.16 Effect of 1,2-dhATQ +/- 100 μM Cu^{2+} on JEG-3 viability after 24 hour exposure. Alone, 1,2-dhATQ did not affect either MA (A) or MI (B). Addition of 100 μM Cu^{2+} did decrease both MA and MI at 50 μM 1,2-dhATQ. Data represents average ± SE. For 1,2-dhATQ alone, n=3 while n=2 for 1,2-dhATQ + Cu^{2+}. For data with n=3, results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

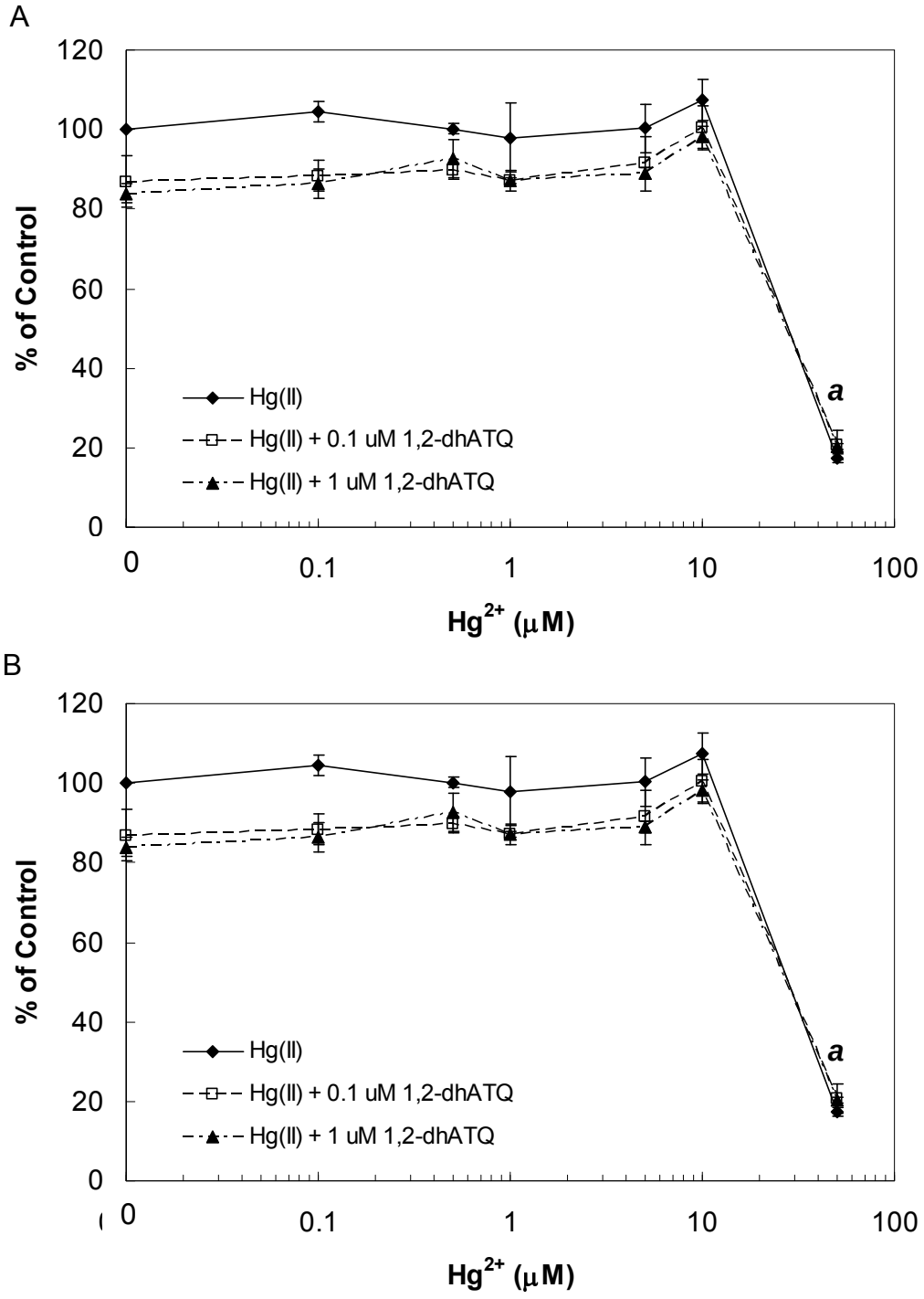

Figure 4.17 Effect of Hg^{2+} and 1,2-dhATQ on JEG-3 cell viability. Exposure of JEG-3 cells to Hg^{2+} alone caused significant decreases in both MA (A) and MI (B). Addition of 1,2-dhATQ to Hg^{2+}-treated cells did not significantly alter Hg^{2+}-mediated toxicity. [a] Significantly different from 0 µM 1,2-dhATQ. Data represents mean of n=3 ± S.E. Results analysed using two-way ANOVA with Fisher's LSD post-hoc test ($p < 0.05$).

Table 4.1 Summary of EC50s for JEG-3 cell treatments.

Treatment	Metabolic Activity (μM)	Membrane Integrity (μM)
PHE	NT	NT
PHE + 100 μM Cu^{2+}	NT	NT
PHEQ	2.0 ± 0.5	110.0
PHEQ + 100 μM Cu^{2+}	0.1	0.5
ATH	NT	NT
ATH + 100 μM Cu^{2+}	NT	NT
1,2-dhATQ	NT	NT
1,2-dhATQ + 100 μM Cu^{2+}	18.6	55.0
Cu^{2+} + 0.5 μM PHEQ	64.8 ± 8.0	74.5 ± 4.2
Hg^{2+}	20.0	23.0
Hg^{2+} + 0.1 μM PHEQ	7.0	17.0
Hg^{2+} + 0.1 μM 1,2-dhATQ	29.0	26.0
Hg^{2+} + 1.0 μM 1,2-dhATQ	29.0	24.0

NT = not toxic.

4.4 Discussion

The purpose of this study was to determine the toxicity to choriocarcinoma cells (JEG-3) of two PAHs and their oxygenated derivatives as well as two metals. Toxicity was assessed using MA and MI as parameters. In general, MA appeared to be more sensitive to toxic insult than MI. Neither parent PAH, PHE or ANT, were toxic to JEG-3 cells, however, the oxyPAHs, PHEQ and 1,2-dhATQ, were toxic. Of the two metals tested, Cu^{2+} was less toxic than Hg^{2+} to JEG-3 cells. When the metals were combined with PHE or ANT, no change was observed in toxicity. The same finding occurred when Hg^{2+} was coupled with the oxyPAHs, but a much greater toxicity was observed when combining Cu^{2+} with PHEQ but not 1,2-dhATQ. Progesterone secretion was inhibited only in cells treated with PHEQ or PHEQ + Cu^{2+}. Interestingly, pictures of JEG-3 cells treated with Cu^{2+} showed alterations in cell morphology that were not detected by viability assay.

4.4.1 The Effect of Contaminants on JEG-3 Viability

In this study, the efficacy of various contaminants was tested using JEG-3 viability as an endpoint. Two tests were used to assess viability: MA and MI as measured by alamarBlue™ and CFDA-AM, respectively. The ability of PHEQ but not PHE to decrease MA suggests that the quinone moiety is the reactive structure of the chemical. Previous work has shown that PHEQ generates ROS while PHE does not (Jarabak et al., 1996, Nykamp et al., 2001). PHEQ and PHE do not bind the aryl hydrocarbon receptor (AhR – Schirmer et al., 1998a, Schirmer et al., 1998b), therefore the mode of action for PHEQ toxicity is not AhR mediated. Some studies suggest that PHEQ toxicity may be

mediated through ROS production (Jarabak et al., 1996, Jarabak et al., 1997, Nykamp et al., 2001). The ability of PHEQ to enter futile redox cycling appears to require NAD(P)H which is found predominantly in the mitochondria (Hasspieler et al., 1995, Flowers-Geary et al., 1996, Flowers et al., 1997). Interestingly, MI was not compromised even at high concentrations of PHEQ. When taken together, these results suggest that MA, which resides predominantly in the mitochondrion, is a more sensitive target to PHEQ toxicity than the cell membrane. Additionally, with all of the machinery available in the mitochondrion for redox cycling, the opportunity to create ROS is more likely for PHEQ at this organelle than at the cell membrane.

Exposing JEG-3 cells to Cu^{2+} did not appear to have an effect on viability; neither MA nor cell MI showed any signs of damage up to the highest concentrations tested. The complex nature of the media used for culturing the cells as well as the cells themselves contained abundant sources of compounds that could have chelated copper thus lowering the effective concentration so that Cu^{2+} became non-toxic. However, when Cu^{2+} was combined with PHEQ, the toxicity observed for both MA and MI was greater than for either compound alone. This may be due to the localisation of Cu^{2+} and the proximity of PHEQ to Cu^{2+} and the site of toxic action. According to Babu et al (Babu et al., 2001), a quinone moiety may be sequestered in the mitochondrial electron transport chain of plants due to similarity of chemical structure. Even small amounts of Cu^{2+} present in the mitochondrion at the same time may divert electron flow from the electron transport chain via the quinone and thus set the stage for a futile redox cycling that would produce abundant ROS. This mechanism might explain the high level of

metabolic toxicity observed at low concentrations of toxicants but not the susceptibility of the cell membrane to combined Cu^{2+} and PHEQ toxicity.

Interestingly, a comparison of EC50s involving MA between PHEQ treatments alone or with 100 µM Cu^{2+} showed a decrease from 2 µM to 0.5 µM, whereas, the decrease from 110 µM to 0.5 µM in EC50s involving MI for the same treatments was observed. It appears that maximal toxicity for MA occurred regardless of the presence of Cu^{2+}. However, MI was compromised at much lower concentrations of PHEQ upon addition of Cu^{2+}. This suggests a different mechanism than the one responsible for MA toxicity.

Treating JEG-3 cells with PHEQ +/- Cu not only caused changes in metabolism, but morphological differences were observed as well under a phase contrast microscope. Untreated cells exhibited the characteristic appearance of JEG-3 cells with distinct multinucleated clusters with prominent nucleoli as described by Lin et al (Lin et al., 1997). The changes observed with PHEQ treatment included cell shrinkage, rounding of the cell shape and at 5.0 µM PHEQ, presence of ghost-like structures and floating cells detached from the plate. Lin et al (Lin et al., 1997) also described these types of changes with Cd treatment in JEG-3 cells. When 100 µM Cu^{2+} was added to PHEQ treated cells, these differences became more prominent. The changes observed in the present study suggest that cell death may have occurred through apoptosis. Using a phase contrast microscope, Rello et al (2005) described morphological changes that accompanied apoptosis including cell rounding, shrinkage and blebbing of the plasma membrane. Interestingly, while viability remained unaltered with the addition of 100 µM Cu^{2+} alone, the cells appeared rounder with slightly less distinct nucleoli. This too was observed by Lin et al (Lin et al., 1997) at low concentrations of Cd. Cell shape

alterations may be the result of cytoskeletal reorganization in response to toxic insult needed to protect the cytoskeleton (Li et al., 1994).

4.4.2 Variables in cell culturing and their effect on PHEQ and Cu^{2+} toxicity

Different incubation periods before and after exposure to toxicants were tested. The time period between plating and exposure altered the toxicity of the compounds tested. As the time period increased, toxicity decreased. This reciprocal relationship may indicate the protection conferred by culture age that makes the cells more resistant to toxic insult than newly plated cells. As a cell culture ages, more cells are present, cell to cell communication is better established, and the cellular matrix that facilitates attachment is formed.

The presence of more cells could dilute the effective concentration while cell to cell communication may initiate adjoining cells to produce protective mechanisms to ward off toxic insult. The presence of a mature cellular matrix may give a false impression of increased viability compared to newly plated cells. The tests for viability rely on the presence of oxidizing conditions found in cells. If, upon exposure to toxicants, the younger cells detach and are washed out as a part of the testing protocol, then little of the reagents are oxidized resulting in a low viability assessment. In contrast, older cells with a better developed matrix may stay attached to the plate and thus provide some oxidizing power for the reagents that could be included in the viability assessment.

Another variable tested was the ability of JEG-3 cells to recover from toxic exposure. The cells were not able to recover from PHEQ exposure but removal of PHEQ appeared to halt the decline in viability. However, with the addition of PHEQ and Cu^{2+}, the cells

were not only unable to recover, but viability still decreased after the toxicants were removed. The most likely explanation to these results involves toxicant retention. The removal of media containing PHEQ and replacement with fresh media likely caused the PHEQ to diffuse out of the cell. This situation resulted in the cells not recovering from initial exposure to PHEQ. Alternatively, with the replacement of media after PHEQ and Cu^{2+} exposure, the PHEQ may have diffused out but the Cu^{2+} may have remained inside the cell where redox cycling previously initiated by the presence of PHEQ could continue thus affecting cellular viability.

PHEQ toxicity was both concentration and time dependent. Kinetic experiments demonstrated that moderate and high concentrations of PHEQ significantly inhibited MA immediately and continued in a time-dependent manner. MI was not as sensitive. This finding has shown that PHEQ appears to be a fast-acting toxicant capable of initiating toxicity in a short time span and that toxicity increases with exposure time. When combined with the recovery experiment, this data suggests that the mechanism of action for PHEQ may be the production of ROS that can in turn damage a multitude of cellular targets (Figure 4.23). Specifically, PHEQ can be reduced via a one-electron reduction to its corresponding semiquinone which can in turn reduce molecular oxygen producing superoxide and regenerating the quinone (Jarabak et al., 1997, Boelsterli, 2003).

Another interesting characteristic of the PHEQ and Cu(II) toxicity to JEG-3 cells appeared when different components of the culturing media were altered. When a minimal media was used instead of the more complex media, the apparent synergy between PHEQ and Cu(II) toxicity was abolished. By adding components present in the

complex media one-by-one to the minimal media, synergy was restored with the presence of glucose in the minimal media.

4.4.3 Effect of antioxidants and Cu^{2+} chelators on PHEQ and Cu^{2+} toxicity

With the proposed mechanism of toxicity involving ROS, experiments were conducted to determine if antioxidants could mitigate PHEQ and Cu^{2+} toxicity. The antioxidant glutathione (GSH) was chosen for its hydrophilicity as well as its ability to cross cell membranes. In cells not exposed to toxicants, MA increased slightly upon incubation with GSH. However, in all cases of toxicant exposure, cells with added GSH exhibited lower MA and MI than without GSH added. While this result would seem to be counter-intuitive, studies have shown that antioxidants can become prooxidants at high concentrations (Tafazoli et al., 2005, Chen et al., 2007). Antioxidants can generate ROS in the presence of metals such as Cu^{2+} (Bhat et al., 2006).

Experiments using Cu^{2+} chelating agents were done to determine their effect on PHEQ + Cu^{2+} toxicity. The presence of the membrane permeable chelator, NC, was found to enhance Cu^{2+} cytotoxicity in JEG-3 cells. This finding was supported by studies demonstrating NC enhanced Cu^{2+} toxicity to bacterial and human tumour cells (Byrnes et al., 1992; Mohindru et al., 1983; Zhu and Chevion, 2000). Conversely, the membrane impermeable chelator, BC, was not cytotoxic either itself or in the presence of Cu^{2+}. These results suggested that the NC/Cu^{2+} complexes were forming intracellularly and thus initiated damage inside the cell by two potential mechanisms. The first could have increased the intracellular Cu^{2+} concentration by enhanced Cu^{2+} uptake (Byrnes et al., 1992, Mohindru et al., 1983, Zhu and Chevion, 2000). The other could have been

enhanced ROS production through NC-Cu^{2+} complexes which have been found to be a more potent oxidizer than either Cu^{2+} or Cu^{1+} (Apak et al., 2005).

4.4.4 Comparison of different metals and/or PAHs on JEG-3 toxicity

The other PAH/oxyPAH pair tested, ANT and 1,2-dhATQ, demonstrated no apparent cytotoxicity to JEG-3 MA or MI. Toxicity of these compounds appears to vary widely in the literature from highly toxic (Huang et al., 1995, Mallakin et al., 1999, Babu et al., 2001) to non-toxic (Choi and Oris, 2003) depending on the organism studied. Interestingly, one study demonstrated that toxicity of ANT and its photoproducts depended greatly on the organism studied and the endpoint used (Brack et al., 2003). With the wide variety of responses stated in the literature, the non-toxic finding of ANT and 1,2-dhATQ in JEG-3 cells was not surprising.

The addition of Cu^{2+} to ANT treated cells did not cause any change in toxicity, however, co-incubation of Cu^{2+} with 1,2-dhATQ did appear to decrease both MA and MI. Previous work with the higher plant *Lemna gibba* postulated that the co-exposure of 1,2-dhATQ with Cu^{2+} resulted in continuous ROS generation in the photosynthetic electron transport chain causing damage (Babu et al., 2001). Specifically, 1,2-dhATQ appeared to block electron flow from the cytochrome b_6/f complex resulting in an accumulation of reduced plastoquinone. The added Cu^{2+} was able to accept electrons from the reduced plastoquinone and pass the electrons to O_2 to form ROS. This experiment can be used to compare and contrast to the JEG-3 results. Since MA was unaffected, it is unlikely that 1,2dhATQ blocked electron flow in the mitochondrial electron transport chain. However, the presence of Cu^{2+} did increase toxicity to both MA and MI suggesting that

there were multiple targets not just the mitochondria. ROS generation has not been ruled out as a mechanism, but the 1,2-dhATQ/Cu^{2+} redox cycling probably was not limited to the mitochondria. The high concentrations of both 1,2-dhATQ and Cu^{2+} required to elicit an effect would seem to support this argument.

While other metals were tested in CL cells for toxicity (Nykamp, 2007 – Chapter 3) and only Hg^{2+} was tested in JEG-3 cells. The trend observed with CL cells was repeated with JEG-3 cells exposed to Hg^{2+}: little change in MA occurred until an abrupt decrease at the highest concentrations. Since Hg^{2+} does not possess the ability to produce ROS directly by redox-cycling, the most likely mechanism of toxicity would have involved Hg^{2+}-mediated inactivation of antioxidants indirectly resulting in ROS level increases. For instance, Hg^{2+}-mediated ROS production has been suggested in lymphoma cells (Kim and Sharma, 2003), kidney (Lund et al., 1993), testis, and epididymus (Mahboob et al., 2001) but the increased ROS levels may have been the result of antioxidant inactivation rather than redox-cycling. Hg^{2+} can accomplish this through inactivation of proteins containing thiol groups such as glutathione, cysteine, and metallothionein due to a high affinity for reduced sulphur atoms. At high concentrations of thiol-containing compounds, Hg^{2+} can bind to two thiol groups at once (Boelsterli, 2003). Interestingly, in whole animal studies of nephrotoxicity, dose-response curves associated with Hg^{2+} were very steep, possibly due to the presence of protective thiols such as glutathione binding Hg^{2+} until their detoxification capacity was exceeded (Lash et al., 1999, Zalups, 2000). The decline in antioxidant proteins like glutathione would seem a likely explanation for the results observed in the current study. Furthermore, the mitochondrion has been shown as a specific Hg^{2+} target through

enhancement of ROS production by ubiquinone-cytochrome b and NADH hydrogenase regions of the mitochondrial electron transport chain (Lund et al., 1991). However, the slight difference between MA and MI would suggest that Hg^{2+} toxicity observed in the current study was most likely due to the overall increase in ROS levels arising from antioxidant inactivation by Hg^{2+}.

Co-incubation of Hg^{2+} and PHEQ appeared to cause synergistic toxicity at mid-range concentrations of Hg^{2+}. It is unlikely that Hg^{2+} and PHEQ can redox cycle through the same mechanism as Cu^{2+} and PHEQ. However, the greater than additive toxicity observed suggests that some kind of interaction between Hg^{2+} and PHEQ may have occurred. The enhanced generation of ROS by PHEQ coupled with decreased antioxidant activity could account for the observed toxicity. This synergistic toxicity was not observed when JEG-3 cells were exposed to Hg^{2+} and 1,2-dhATQ at the same time. Since 1,2-dhATQ does not appear to redox-cycle as does PHEQ, these results support the proposed mechanism of toxicity.

Non-modified PAHs, PHE and ANT, did not appear cytotoxic to JEG-3 cells. However, of the two oxyPAHs tested, only PHEQ was cytotoxic on its own. Cu^{2+} was not immediately toxic but the co-exposure of PHEQ and Cu^{2+} caused a synergistic cytotoxicity to JEG-3 cells compared to either chemical alone. Hg^{2+} did cause cytotoxicity alone but synergistic cytotoxicity was also observed in cells treated with Hg^{2+} and PHEQ. The most probable mechanism behind the toxicity involves ROS generation mediated by redox cycling of PHEQ. Interactions with the metals tested involved either a catalytic mechanism of ROS generation through metal/oxyPAH redox cycling (Cu^{2+}) or co-ordinated increases in ROS by PHEQ redox cycling and metal inactivation of

antioxidants (Hg^{2+}). The placenta is a particularly sensitive target to oxidative stress and these results highlight the need to further examine metal/PAH combinations since these occur both in the environment and through lifestyle exposure.

Chapter 5 – Initial Assessment of 9,10-Phenanthrenequinone Induced Changes in Gene Expression in a Placental Cell Line, JEG-3

5.1 Introduction

Molecular toxicology tests impacts of contaminants and has been a recent help for researchers to elucidate some of the mechanisms behind lethal and non-lethal effects of various toxic compounds (Lee et al., 2005, Merrick and Madenspacher, 2005). According to the US Environmental Protection Agency's (US EPA) Interim Policy of Genomics (July 26, 2002), the "EPA believes that genomic analyses will significantly impact many areas of scientific research and human and ecological health assessments" (EPA, 2002). While exploring single toxicant – single gene interactions has occurred for many years, the advent of differential display reverse transcription polymerase chain reaction (ddRT-PCR) and DNA microarray technologies has provided a global view of gene expression in response to toxicant exposure.

The strategy behind these approaches has been to compare gene expression patterns between two or more samples with one control and one or more exposed samples (Snell et al., 2003). Exposure to toxicants has been shown to transiently alter gene expression in yeast for protection and repair (Causton et al., 2001). What portion of the genome can be activated through toxicant induced stress is unknown, but each pathway likely signifies a unique response of the organism to the stressor. For toxicologists, the key to using these technologies begins with identifying differentially regulated genes in relevant model organisms to known environmental contaminants. Particular attention has been paid to toxicants such as metals, dioxins, and

benzo[a]pyrene (Chang et al, 2004, Abe et al., 2006, Fong et al., 2005). However, little data has been collected thus far regarding the effect of other environmental polycyclic aromatic hydrocarbons (PAHs) on global gene expression.

Phenanthrene (PHE) is one of the most common PAHs found in the environment. Through anthropogenic sources such as diesel exhaust, most PHE has been released into the air. This results in exposure of organisms by either direct routes such as inhalation or deposition on surfaces, or indirect routes such as ingestion of contaminated sources. Though not considered acutely toxic or highly carcinogenic, PHE can be transformed into more reactive compounds by photo-oxidation or Phase I/II metabolism (McConkey et al., 1997, Mallakin et al., 1999, Slawson et al., 1996). Kadry et al, (Kadry et al., 1995) demonstrated that 9,10-phenanthrenequinone (PHEQ) was one of the major metabolites of PHE through phase I/II metabolism, while others have shown that photo-oxidation of PHE produces mainly PHEQ (McConkey et al., 1997, Mallakin et al., 1999).

In general, many carcinogenic PAHs have been shown to mediate their function through the binding of the aryl hydrocarbon receptor (AhR) (Machala et al., 2001a, Machala et al., 2001b). Interestingly, Bols et al., (Bols et al., 1999) found that PHE did not bind AhR. Additionally, PHE did not appear to induce mRNA expression of the phase II enzymes UDP-glucuronosyltrasferase and sulfotransferase (Lampen et al., 2004). A study by Kim et al (Kim et al., 2007) also found that PHE did not alter expression of various stress genes in *E. coli*. In contrast, exposure of *Arabidopsis* to PHE appeared to induce physical signs of oxidative stress as well as an increase in genes responsive to oxidative stress (Alkio et al., 2005).

PHEQ has been shown to be acutely toxic to bacteria, invertebrates, plants, and animals (El-Alawi et al., 2001, Xie et al., 2006, McConkey et al., 1997, Nykamp et al., 2001). Reactive oxygen species (ROS) production has been suggested as one of the mechanisms of action behind the acute toxicity observed in such a wide variety of organisms. PHEQ also has been shown to cause DNA strand breaks through ROS generation which may lead to mutations (Hasspieler et al., 1995, Bolton et al., 2000, Park et al., 2005). In addition, a large body of work now supports ROS as a major regulator of gene expression (reviewed by Bauer et al., 1999 and Dröge, 2002). However, little work has been done to examine the effect of PHEQ/ROS-induced changes on gene expression.

There are a number of ways to probe for genes expressed in response to contaminant exposure. These include suppression subtractive hydbridization, representational differences analysis, serial analysis of gene expression, and DNA microarrays (Stein and Liang, 2002a). Difficulties with these procedures include limited number of sample comparisons, one-way expression detection (up or down) amount of RNA needed, and high detection threshold (Stein and Liang, 2002a). Differential display reverse transcription PCR (ddPCR) was first described by Liang and Pardee (Liang and Pardee, 1992) as a means to visualize and compare mRNA expression between two or more samples (Figure 5.1) and is a very flexible technique to assay for gene expression differences due to a series of treatments. While newer techniques have been developed since that time, the popularity of ddPCR has remained due to the simplicity of the basic reactions involved; reverse transcription of mRNA, PCR amplification, and

polyacrylamide gel electrophoresis (Stein and Liang, 2002a). Also, the amount of data generated is manageable and there are relatively few false positives.

ddPCR begins with reverse transcription of RNA to its complementary DNA (cDNA) using oligo(dT) primers to anneal to the polyadenylated (polyA) tails of mRNA (Stein and Liang, 2002a) (Figure 5.1). The cDNA fragments are amplified by PCR using random primers 18 base pairs long. The conditions used in the thermocycler are not stringent allowing for multiple, non-specific annealing sites for the primers (Stein and Liang, 2002a). Estimates have suggested that almost all of the expressed mRNA in a cell can be surveyed with approximately 200 different primer combinations (Liang and Pardee, 1992). The randomly generated cDNA fragments are labelled either radioactively or fluorescently and then separated by size using denaturing polyacrylamide gel electrophoresis (Stein and Liang, 2002a, Diener et al., 2004, Akhtar et al., 2005, Lees, 2005). The resulting banding patterns between samples can be compared side-by-side as the differentially expressed DNA fragments are visualized. Bands of interest can be excised from the gel and the DNA extracted. Further amplification can then be accomplished by cloning in bacteria (Diener et al., 2004, Akhtar et al., 2005, Lees, 2005). After retrieving the DNA from the bacteria, the fragment can be sequenced and the results compared with the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST). Ideally, differential expression of sequences would then be confirmed by Northern blotting or quantitative PCR (qPCR) (Stein and Liang, 2002b, Akhtar et al., 2005, Lees, 2005).

As with any technique, problems exist when using ddPCR. The selection of differentially expressed bands is subjective. Experience and replication can help in

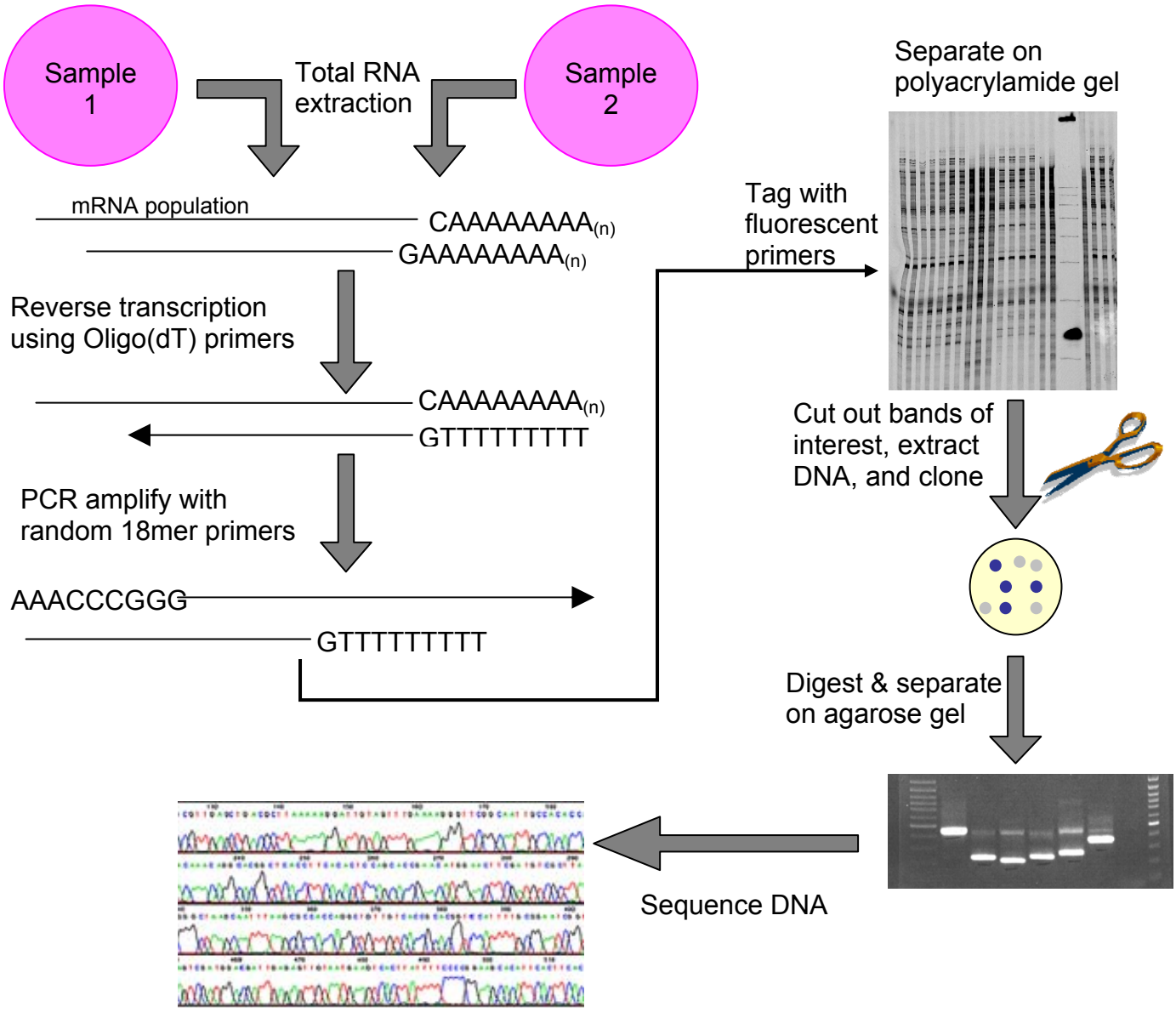

Figure 5.1 Schematic representation of ddPCR. In general, total RNA was reversed transcribed to cDNA, amplified by PCR, then separated on a polyacrylamide gel. Bands containing DNA were excised, the DNA extracted from the gel and the DNA cloned in JM109 competent cells. The DNA of interest was removed from the bacteria and was then sent for sequencing.

distinguishing differential regulation from artefacts; however, many bands may be missed due to the number of bands observed on a gel. Additionally, more than one band can migrate together (Akhtar, 2004). This can be monitored through cloning and selecting multiple colonies per band where identities of the colonies should match. Insensitivity to low copy transcripts has also been of concern for many researchers.

Generally, ddPCR is biased toward transcripts in higher abundance (Bertioli et al., 1995), however, several researchers have discovered variations on the ddPCR protocol to allow for detection of genes expressed in low abundance (Wan et al., 1996, Jurecic, 1996, Liao and Freedman, 1998). Another problem concerns the threshold needed to detect differential expression, whether twofold changes or less can be detected (Liao and Freedman, 1998).

Advent of the GenBank genome database and the BLAST search engine has revolutionized genomics. The ability to find identities for sequences through nucleotide-nucleotide sequences, translated nucleotide-protein sequences and many other variations has allowed researchers to analyze interactions between treatments and expression of known genes as well as identifying novel genes (Altschul et al., 1997). From the information given, the researcher can determine the quality of the alignment (Altschul et al., 1997). One such measure, the bit score, calculates the alignment of identical and/or similar residues as well as accounting for any gaps that may occur in the sequence (Altschul et al., 1997). The other measure of alignment, the E-value, is similar to the P-value in statistics (Altschul et al., 1997). Factors such as parwise alignment, the size of database used and the scoring system are used to calculate the E-value

(Altschul et al., 1997). As with the P-value, the lower the E-value is the more significant the alignment.

The mammalian placenta is a fundamental organ necessary for gas, nutrient and waste exchange with the fetus as well as production and secretion of various hormones (Hadley, 1996). The placenta is the sole link between mother and fetus. With the myriad of demands placed on one organ, the importance of this organ is obvious. Therefore, determining the vulnerability of the placenta to toxicant exposure has also become of great importance. There are many model systems available for experimentation including whole animal, human placental tissues, and placental cell lines (Andrews et al., 2001). The reproducibility, low cost, ease of use, and ability to perform mechanistic studies has popularized the use of placental cell lines in both environmental and pharmaceutical toxicity testing (Zhang and Shiverick, 1997, Letcher et al., 2000). One such cell line, JEG-3 a choriocarcinoma cell line of human epithelial origin, has been used in endocrinology, reproductive biology, toxicology and genomic research (Burnside et al., 1985, Zygmunt et al., 1998, Li et al., 1998). Because of its importance, investigating the ability of environmental toxicants to alter gene expression in the mammalian placenta has taken on a sense of urgency. Not only are humans at risk, but so too are all other mammals both domestic and wildlife. The objective of this study was to make an initial assessment of toxicant induced changes in gene expression for human JEG-3 cells.

5.2 Materials and Methods

5.2.1 Reagents

Most reagents were purchased from Sigma (St. Louis, MO) including TEMED, TriReagent, L-15/ex media, Minimal Essential Medium Eagle (MEM), sodium pyruvate, gentamycin, trypsin, PHEQ and $CuSO_4$. Fetal Bovine Serum (FBS), random primer sets as well as primers T7 and SP6 (Table 5.1) were ordered from Sigma Canada (Oakville, ON). Versene and MEM non-essential amino acids were obtained from Life Technologies (Burlington, ON). Stock solutions of PHEQ were prepared in dimethyl sulfoxide (DMSO) as described previously (Nykamp et al., 2001). Stock solutions of $CuSO_4$ were prepared in sterile distilled, deionized H_2O. All microcentrifuge tubes used in cell culture and molecular work were sterile and DNase/RNase free (Diamed, Mississauga, ON). Ready-To-Go PCR beads were purchased from Amersham Pharmacia (Baie d'Urfe, QC) and contained all reagents necessary for a PCR reaction.

5.2.2 Cell Culture and Treatment

JEG-3 cells were obtained from the American Tissue Culture Collection (ATCC). Cells were grown in 75 cm^2 tissue culture flasks (Becton-Dickinson, Oakville, ON) using MEM supplemented with 10% FBS, 0.2 mM MEM non-essential amino acids, 2 mM L-glutamine, 0.5 mM sodium pyruvate, and 1 µl/ml gentamycin. Cells were incubated in a humidified atmosphere supplemented with 5% CO_2 at 37°C. Once cell growth was confluent, cultures were divided by first aspirating the old media followed by washing with versene twice followed by the addition of 1 ml trypsin (2.5 g/L). Cells were re-

suspended in MEM with 10% FBS and plated at a cell density of 2×10^5 cells/ml in 6 well Falcon plates (Becton-Dickinson, Oakville, ON) to a total volume of 3.0 ml. Prior to treatment with the chemicals of interest, cells were assessed for viability and attachment to the plate using a phase contrast microscope. After removing media containing FBS, chemical toxicants were added to the wells containing MEM without added FBS. PHEQ was diluted in DMSO to a working concentration from stock before addition to the wells (2.5 µl/well). Working solutions of $CuSO_4$ were prepared in sterile, purified water and 5 µl of each concentration was added per well. All experiments included a DMSO control. Cells were incubated for 24 hours under the same conditions as stated in Chapter 4 (Nykamp, 2007). At the end of the incubation, media was removed from each well, cells were washed twice with ice-cold L-15/ex media and then 1 ml of Tri Reagent added (Chomczynski and Mackey, 1995). The lysate was removed from the plate well and placed in an RNase-free microcentrifuge tube and frozen on dry ice before storage at -80°C.

5.2.3 RNA Isolation

This procedure was adapted from Diener et al., (Diener et al., 2004) and Akhtar et al., (Akhtar et al., 2005). All equipment were either purchased RNase/DNase free or baked at 280°C overnight prior to use. Samples stored at -80°C were thawed on ice then incubated at room temperature for 5 minutes to allow disassociation of nucleoprotein complexes. To each tube, 200 µl of chloroform was added, the tubes shaken vigorously for 15 seconds and then left at room temperature for 15 minutes. Samples were then centrifuged at 12 000 x g at 4°C for 15 minutes to promote phase

separation. The upper aqueous phase was transferred to a new microfuge tube where RNA was precipitated by addition of 500 μl isopropanol and incubated at room temperature for 10 minutes. The RNA was pelleted by centrifugation at 12 000 x g for 8 minutes at 4°C. The supernatant was removed and the pellet washed for 15 seconds by 1 ml of 75% ethanol followed by centrifugation at 7500 x g for 5 minutes. The supernatant was removed and the pellet allowed to air dry for 10 minutes at room temperature. The RNA was dissolved in 30 μl of DEPC-treated water and frozen at -80°C until further analysis.

RNA concentration was determined spectrophotometrically at absorbance 260 nm using a Cary50 spectrophotometer (Varian, Palo Alto, CA). RNA purity was estimated by calculating the RNA-DNA Abs_{260}/protein Abs_{280} ratio. Samples with values between 1.6 – 2.0 were used for differential display PCR.

5.2.4 RNA Denaturing Gel Electrphoresis

RNA integrity was verified prior to further processing using denaturing gel electrophoresis (Sambrook et al, 1989). Briefly, 3 μg of total RNA was dissolved in 10 μl of loading buffer (50% formamide [v/v], 17% formaldehyde [v/v], 1X MOPS buffer [0.02 M morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0], 10% glycerol [v/v], 0.05% bromophenol blue [w/v]) and incubated for 10 minutes at 65°C. Samples were cooled on ice for 5 minutes prior to loading on a 1.2% [w/v] agarose gel containing 18% formaldehyde [v/v], 1X MOPS buffer [v/v], and 0.7mg ml^{-1} ethidium bromide. RNA was separated for 2 hours at 80 volts with 1X MOPS as the running

buffer. The RNA was visualized under UV light (Fluorchem 8000, Alpha Innotech, San Leandro, CA).

5.2.5 Reverse Transcription

First strand cDNA synthesis was performed using total RNA and the First Strand cDNA Synthesis kit (Fermentas, Burlington, ON) as per product instructions. Briefly, 3 μg of total RNA was added to a 0.5 ml microfuge tube along with 1 μl of Oligo$(dT)_{18}$ primer and the volume brought up to 11 μl with DEPC-treated water. The mixture was heated to 70°C for 5 minutes and then quickly cooled on ice before condensation was collected with a brief centrifugation. To each tube, 4 μl of 5X reaction buffer, 1 ml of ribonuclease inhibitor (20 u/μl) and 2 μl of 10 mM dNTP mix was added. The tube was gently mixed and incubated at 37°C for 5 minutes before 2 μl of M-MuLV reverse transcriptase (20 u μl) was added. The mixture was incubated at 37°C for 60 minutes. The reaction was stopped by heating at 70°C for 10 minutes and then the sample was cooled on ice. The cDNA was then ready for PCR amplification.

5.2.6 Polymerase Chain Reaction

Ready-To-Go PCR beads (Amersham Phamacia Biotech, Baie d'Urfe, QC) were used in 0.5 ml microfuge tubes containing 200 μM dNTP, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, and 2.5 units of puRe Taq DNA polymerase at 25 μl final volume. Amplification of cDNA following reverse transcription was accomplished using 5 μl of 1:10 diluted cDNA from control and treated samples, 1μl of each primer at 25 μM (A3

and A5 - Table 5.1), and 18 μl of sterile water. A negative control containing all components except template cDNA was included in each set of reactions. The cycling conditions used for the PTC-200 Thermocycler (MJ Research Products, Waltham, MA) were: 94°C for 1 minute, 35°C for 5 minutes, 72°C for 2 minutes followed by 39 cycles of 94°C for 2 minutes, 50°C for 2 minutes, and 70°C for 2 minutes (Diener et al., 2004), Akhtar et al., 2005). All PCR reactions were stored at -20°C until further use.

5.2.7 Differential Display PCR Gel Electrophoresis

The PCR products were labelled with a fluorescent Cy5 primer (AGCT Inc., Mississauga, ON) that was complementary to the conserved regions of the arbitrary primers A3 and A5 used in the PCR reaction. Briefly, 1 μl of Cy5 primer (0.2 μM ml^{-1}) was added to each microfuge tube containing the entire PCR reaction then incubated for 2 minutes at 95°C. The tubes were cooled on ice for 5 minutes and 1 μl of dNTPs (25 μM), 3 μl of 10X Klenow reaction buffer, and 1 μl of Klenow polymerase, exo- (5 U ml^{-1}) (MBI Fermentas, Burlington, ON) were added to each tube. Each tube was mixed gently and incubated at room temperature for 30 minutes followed by 2 hours at 37°C. Aliquots of the fluorescently tagged product were mixed with loading buffer containing 80% formamide [v/v], 50 mM Tris HCl pH 6.8, 1 mM EDTA, and 0.25% bromocresol green [v/v] then incubated for 2 minutes at 80°C. The entire mixture was cooled on ice and loaded onto a denaturing polyacrylamide sequencing gel containing 6% acrylamide (19:1 acrylamide:*bis* acrylamide; BioShop, Burlington, ON), 7 M urea and 1X Tris-borate-EDTA buffer. A Cy5-labelled DNA ladder of 50 – 500 base pairs was included on the gel for size comparison. Electrophoresis was carried out for 2 hours at 1600 V and

55 W. Empty lanes in the gel were loaded with loading dye every 30 minutes to act as a visible marker for later recovery of fragments of interest from the gel. PCR products were visualized on the Storm 860 Imaging System (Molecular Dynamics Corp., Sunnyvale, CA) using the red fluorescence mode.

5.2.8 Isolation of ddPCR Fragments

A full size print out of the polyacrylamide gel was produced from the image provided by the Storm Imaging system. This print out was carefully overlaid by the gel and the sites marked by loading buffer were aligned. The fragments of interest were excised from the gel using fresh sterile razor blades and the gel slice placed in a 1.5 ml microfuge tube. The gel was rescanned to confirm the removal of the desired bands. To each tube, 200 μl of diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS pH 8.0) was added and incubated for 30 minutes at 50°C. DNA was recovered using the QIAGEN gel extraction kit (QIAGEN Inc, Mississauga, ON) as per product insert for polyacrylamide gel extraction. The eluted cDNA was dissolved in 20 μl of sterile water and an aliquot was re-amplified using the same primers and conditions as previously stated. The re-amplified cDNA was purified using a 1% [w/v] agarose gel electrophoresis containing 0.7 mg ml^{-1} ethidium bromide. The products and GeneRuler 100bp DNA Ladder Plus (MBI Fermentas, Burlington, ON) were electrophoresed on the agarose gel for approximately 45 minutes at 100 V. Any DNA present in the gel was visualized under UV light. Bands corresponding in size to the ones excised from the polyacrylmide gel were cut out of the agarose gel using sterile razor blades and placed in 1.5 ml microfuge tubes. The DNA was extracted from the

agarose using the QIAGEN gel extraction kit as per product insert for agarose gels. The eluted DNA was dissolved in 20 μl of sterile water before the concentration was determined spectrophotometrically at 280 nm. The isolated DNA was stored at -20°C until further use.

5.2.9 Cloning of Recovered cDNAs

Amplification of recovered cDNA was accomplished through cloning. An aliquot (3.5 μl) of the recovered DNA band was ligated into the pGEM T-easy vector (Promega, Madison, WI) in a 10 ml reaction containing 5 μl of 2X ligation buffer (100 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 20 mM DTT), 1 μl of vector (50 ng μl^{-1}), and 1 μl of T4 DNA ligase (2 Weiss units ml^{-1}). The samples were incubated at 4°C for 24 hours. From the ligation reaction, an aliquot was used to transform *E. coli* JM109 high efficiency competent cells (Promega, Madison, WI). Briefly, 2 μl of ligation reaction product was transferred to a 1 ml microfuge tube whereby 50 μl of competent cells was added and incubated on ice for 20 minutes followed by a brief heat shock at 42°C for 45 seconds. Tubes were immediately placed on ice for 3 minutes and 500 μl of LB broth (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl) added. The samples were agitated at 150 rpm for 1.5 hours at 37°C. From each, 100 μl was spread over the surface of LB agar plates containing ampicillin (100 μg ml^{-1}) (MBI Fermentas, Burlington, ON), 0.5 mM IPTG, and 80 μg ml^{-1} X-Gal (Promega, Madison, WI). The transformed bacteria were allowed to adsorb to the agar surface for 20 minutes at room temperature prior to incubation at 37°C for 16 hours.

5.2.10 Isolation of Plasmid DNA

Bacteria containing the pGEM T-easy vector (Promega, Madison, WI) with an insert produced white colonies while those that did not contain the vector were blue. Employing the method first described by Sambrook et al (Sambrook et al., 1989), a single colony was selected and used to inoculate 5 ml of LB broth containing 100 mg ml-1 ampicillin. The culture tubes were agitated at 200 rpm for 16 hours at 37°C. From each tube, 1.5 ml of culture was transferred to a 1.5 ml microfuge tube and centrifuged at 13 000 x g for 1 minute at 4°C. The supernatant was removed and another 1.5 ml of culture added to each corresponding tube for a second centrifugation as stated above. Again, the supernatant was removed and the pellet was resuspended in 200 μl of ice-cold solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8) with vigorous vortexing. The cells were lysed using 200 μl of freshly prepared solution 2 (0.2 M NaOH, 1% SDS [w/v]) and the suspension mixed gently by inversion prior to placing on ice for 4 minutes. The lysate was neutralized using 200 μl of solution 3 (3 M potassium acetate, 5 M glacial acetic acid) and inverted 5 times before placing on ice for 5 minutes. The lysed cells were pelleted by centrifugation at 13 000 x g for 10 minutes at 4°C. The supernatant was transferred to a fresh 1.5 ml microfuge tube where any RNA present was destroyed through incubation for 25 minutes at 37°C with 10 μl of pancreatic RNase A (10 mg ml^{-1}) (BioShop, Burlington, ON). DNA was extracted from this solution with the addition of 600 μl of phenol:chloroform (1:1) followed by vortexing for 30 seconds followed by centrifugation at 13 000 x g for 2 minutes at 4°C. The aqueous phase containing the DNA was removed to a fresh 1.5 ml microfuge tube and the process repeated to remove any residual proteins or lipids from the sample. Again the

supernatant was transferred to a fresh microfuge tube where 420 μl of ice-cold 100 % isopropanol was added to precipitate the DNA. After incubating at room temperature for 10 minutes, the samples were centrifuged at 13 000 x g for 3 minutes at 4°C. The supernatant was discarded and the pellet allowed to air dry for 10 minutes. The pellet was dissolved in 25 μl of sterile water and stored at -20°C until further use.

5.2.11 Restriction Digest of Purified Plasmids

To release the inserts from the plasmids, restriction enzyme *Eco* RI (Promega, Madison, WI) was used (Diener et al., 2004, Akhtar et al., 2005). A 1.5 μg aliquot of plasmid was added to a 0.5 ml microfuge tube and digested in a 20 μl reaction using 2 μl of 10 X reaction buffer H (90 mM Tris-HCl pH 7.5, 500 mM NaCl, 100 mM $MgCl_2$), 0.5 μl bovine serum albumin (0.1 mg ml^{-1}), and 0.5 μl of Eco RI (10 U ml^{-1}). The mixture was incubated for 2 hours at 37°C then analyzed on a 1% {w/v] agarose gel containing 0.7 mg ml^{-1} ethidium bromide that had been electrophoresed for 1.5 hours at 95 volts. A GeneRuler 100 bp DNA Ladder Plus was added to the gel for size comparison.

5.2.12 Sequence Analysis

Plasmids with inserts of confirmed size were sequenced using an Opengene DNA sequencer (Visible Genetics, Inc, Toronto, ON) at the Molecular Core Facility (Dept. of Biology, University of Waterloo, Waterloo, ON). Inserts were sequenced in both the forward and reverse directions using primers Sp6 and T7 (Table 5.1). Sequences were compared to data in Genbank using the NCBI (National Centre for Biotechnology

Information) network BLAST software version 1.8 (Altschul et al., 1997). Both nucleotide and predicted amino acid sequences were analyzed using blastn and blastx databases, respectively.

Table 5.1 List of primers and corresponding sequences used for molecular experiments as stated in Section 5.2.

Primer	Sequence
Arbitrary primer A3	AAT CTA GAG CTC CAG CAG
Arbitrary primer A4	AAT CTA GAG CTC TCC TGG
Arbitrary primer A5	AAT CTA GAG CTC TCC AGC
Cy5 fluorescent primer	AAT CTA GAG CTC
Sp6	ATT TAG GTG ACA CTA TAG AAT AC
T7	TAA TAC GAC TCA CTA TAG GG

5.3 Results

The contaminant PHEQ was tested for its effect on gene expression in the human choriocarcinoma cell line, JEG-3. Although PHEQ can produce ROS and that ROS are now acknowledged as an important regulator of gene expression, PHEQ has not been tested for its ability to alter gene expression therefore a general survey was initiated to investigate the potential up or down regulation of genes in response to PHEQ exposure.

5.3.1 Optimization of ddPCR protocol

The ddPCR protocol followed was adapted from Diener et al., 2004) and Akhtar et al., 2005); however the organisms used in those studies were significantly different from the model used in the current study. Fortunately, no alterations in the protocol were necessary except for the collection of RNA for isolation. After testing different methods for treating the JEG-3 cells to maintain RNA integrity, the protocol followed produced acceptable RNA for ddPCR (Figure 5.2A). To minimize exposure to RNases, media containing PHEQ was aspirated from each well of the cell culture plate and each well gently washed twice with ice-cold minimal media (L-15/ex) to remove any dead cells. Additionally, Tri Reagent containing RNase inhibitors was added directly to each well and the resultant lysate frozen immediately on dry ice in RNase/DNase free micro centrifuge tubes.

PHEQ was tested singly and in combination with 100 μM Cu^{2+}, however, the co-exposure of the two chemicals at any concentration of PHEQ caused widespread cell

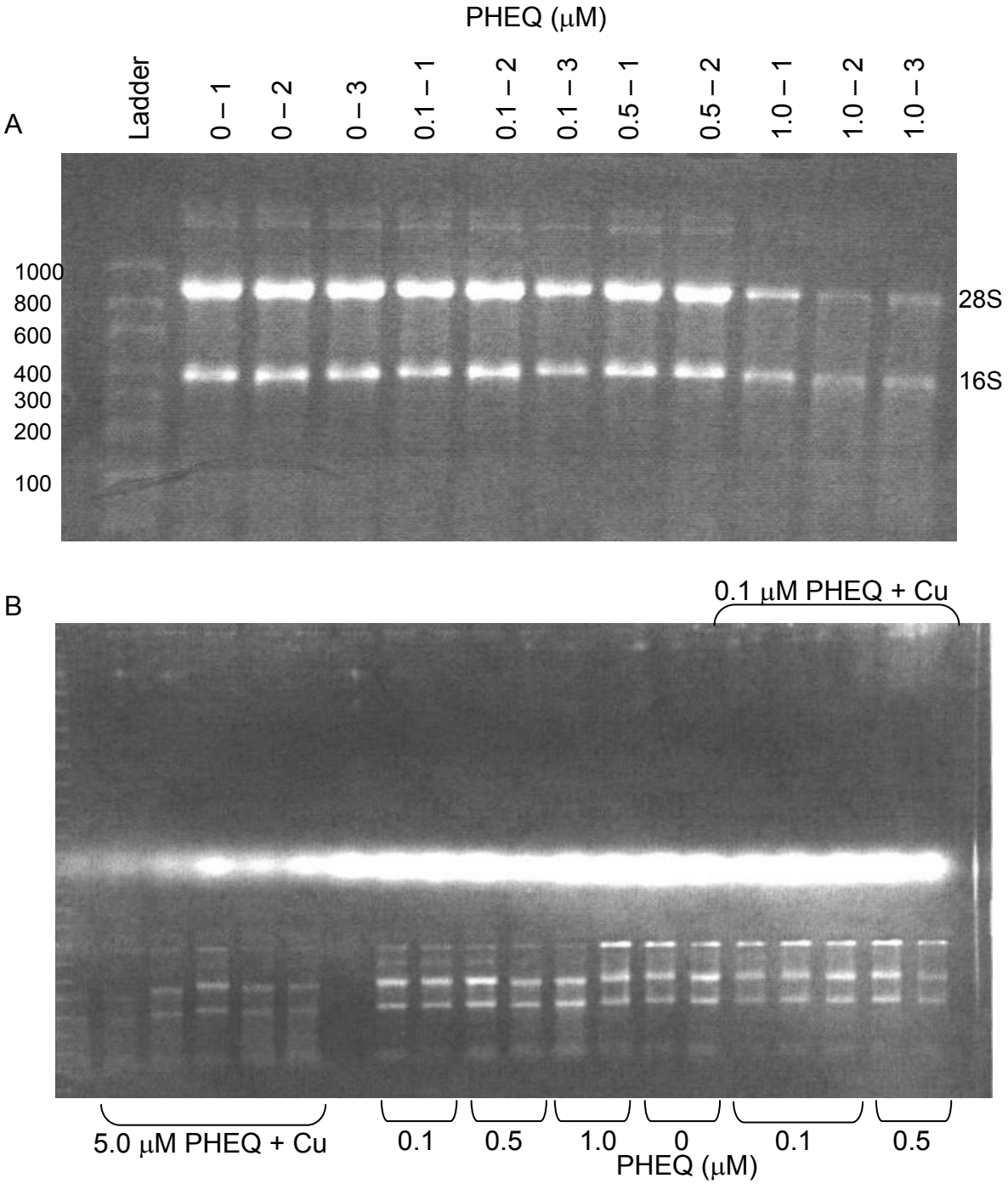

Figure 5.2 RNA denaturing agarose gel to test RNA integrity. Total RNA with acceptable integrity should appear as a smear with two bright bands corresponding to the 28S and 18S ribosomal RNA (rRNA) subunits present in human cells. Single-stranded RNA ruler provided on left side for size comparison. Samples shown (A) were exposed to PHEQ alone or (B) PHEQ + 100 μM Cu^{2+} for 24 hours after which the media was removed, the cells washed and then Tri Reagent added. The cell lysates were placed in an RNase free microfuge tubes and frozen on dry ice prior to storage at -80°C. Results show that increasing PHEQ or addition of 100 μM Cu^{2+} degrades RNA.

death and RNA degradation (Figure 5.2B). Therefore, the current study involved exposing JEG-3 cells only to PHEQ. Further experimentation found that RNA degradation also occurred with high concentrations of PHEQ. JEG-3 cells were treated with 0.1, 0.5, or 1.0 μM PHEQ since exposure to 5 and 10 μM PHEQ for 24 hours resulted in RNA degradation (Figure 5.2B).

5.3.2 PHEQ–induced alterations in gene expression as detected by ddPCR

Previously, PHEQ had been shown to produce ROS in CL cells and that much of the acute toxicity observed may have been due to ROS induced damage (Nykamp et al., 2001). However, the ability of PHEQ to induce changes in gene expression through either direct or indirect means has not been explored. To study PHEQ genotoxicity, differential display PCR (ddPCR) was chosen as the experimental method. ddPCR is able to produce a pattern of bands representing expressed genes that can be visualized through polyacrylamide gel electrophoresis. When the banding patterns of samples with varying treatments are compared side by side, differences in gene expression can be determined. By exploiting the presence of polyadenylated (poly-A) tails on mRNA, Oligo(dT) primers used during reverse transcription can select for mRNAs out of the total RNA extraction performed (Stein and Liang, 2002a).

The first ddPCR experiment tested the effect of low concentrations of PHEQ on JEG-3 cell gene expression. The cells were exposed to PHEQ for 24 hours before RNA was collected, processed and the resulting cDNA separated using a polyacrylamide gel. The resulting banding patterns showed distinct changes between treatments (Figure 5.3).

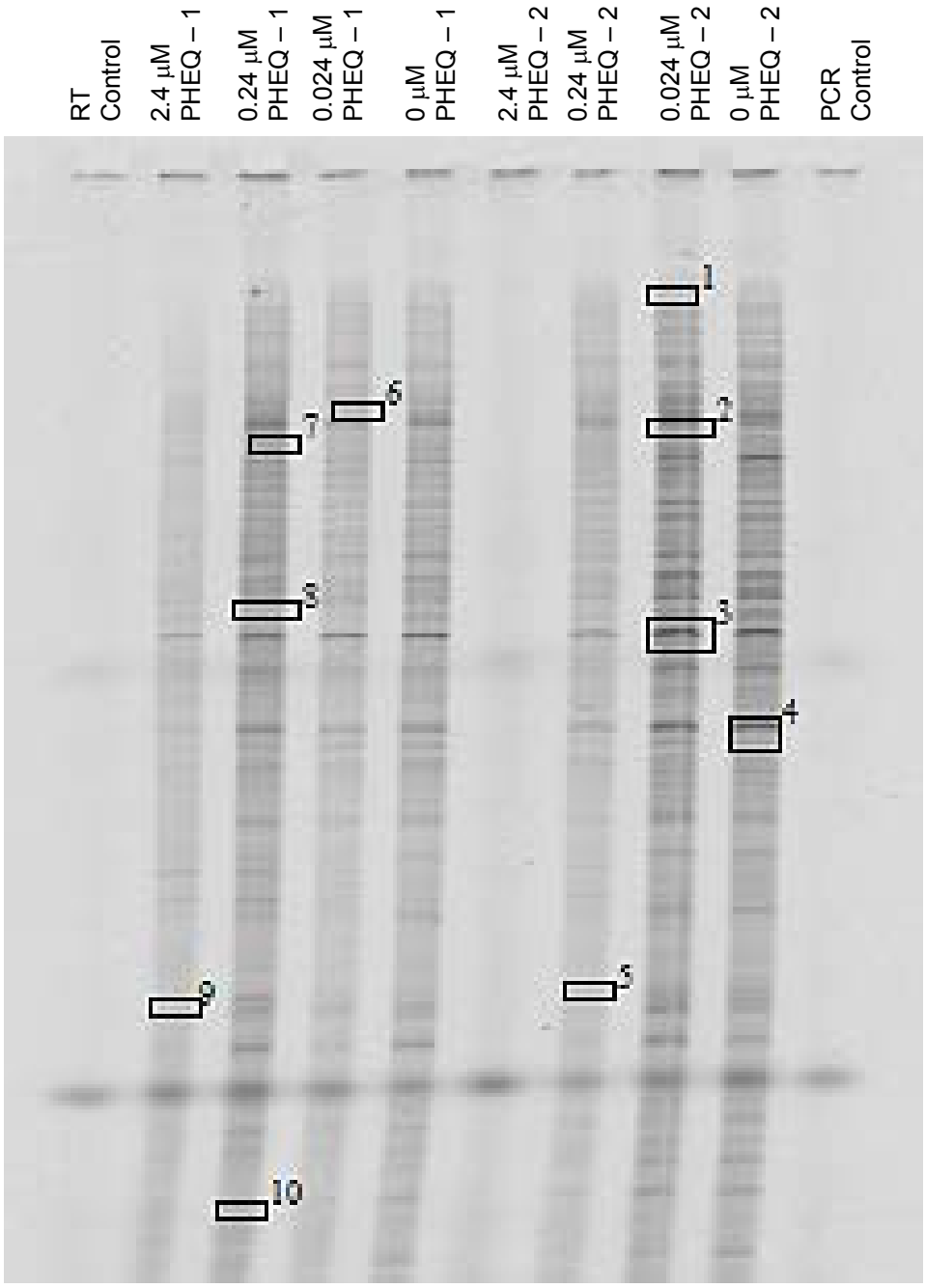

Figure 5.3 First ddPCR gel of JEG-3 cells treated with PHEQ on Sept 20/01. RNA quality as measured spectrophotometrically (A260/A280) was not as high in samples treated with 2.4 mM PHEQ compared to the treatments with lower concentrations of PHEQ. RNA reverse transcribed using mixture of random primers (see Table 5.1) prior to PCR amplification with primers A3/A5. No ladder was included since nothing was commercially available at the time. Bands 1 through 10 excised and DNA extracted. Sequence results in Table 5.2.

Table 5.2 Sequences from bands excised from ddPCR polyacrylamide gel of JEG-3 cells treated with PHEQ Figure 5.1. The letters in the sequence ID refer to the following; J – JEG-3 cells, B1 – band 1, C1 – colony 1, P – the relative [PHEQ] 0, low and medium. The size refers to the length in number of base pairs of the band cut out from the gel. The E value given by the NCBI BLAST search refers to the statistical significance of the hit, the smaller the E value, the more significant the match. The score signifies the quality of the alignment between the sequences, the higher the score, the better the alignment.

Sequence ID	Size (bp)	Name	Species	E value	Score	Gene Regulation
JB2C2PL	466	Proteasome	*Mus musculis*	$1e^{-56}$	179	Up
JB4C1P0	529	Alu	*Homo sapiens*	$4e^{-11}$	59	Up
JB4C2P0	603	Spectrin, β chain	*Rattus norvegicus*	$1e^{-80}$	241	Up
JB6C2PL	901	Heterogeneous ribonuclear ribonucleoprotein C (hnRNP C)	*Homo sapiens*	$2e^{-07}$	59	Up
JB7C1PM	1194	Similar to eukaryotic translation elongation factor 1α1 (eEF1α1)	*Homo sapiens*	$6e^{-52}$	188	Up
JB7C2PM	472	Similar to eukaryotic translation elongation factor 1α1 (eEF1α1)	*Homo sapiens*	$9e^{-49}$	196	Up
JB8C1PM	364	WD repeat & FYVE domain, similar to ALFY	*Homo sapiens*	$5e^{-52}$	204	Up
JB8C1PM	988	Heterogeneous ribonuclear ribonucleoprotein C (HNRNP C)	*Homo sapiens*	$2e^{-50}$	135	Up

Most bands that appeared changed increased intensity indicating up-regulation due to PHEQ treatment.

From this gel, 10 bands were excised for further analysis. The DNA from the excised bands was cloned and sequenced. The sequences obtained were compared to the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST) algorithms (Altschul et al., 1997). The data output of the BLAST search yields not only potential identities but also the "expect" value or E-value, which is a reflection of the statistical confidence of the match between the two sequences. The more significant a match between the two sequences is the lower the E-value is with 0 being a perfect match (Altschul et al., 1997). The other pertinent value given by the BLAST search is the Score. This data reflects the alignment of identical and/or similar residues as well as accounting for any gaps that may occur in the sequence.

Not all of the DNA sequences revealed matches to the NCBI database. Of the ones whose identity could be obtained, all of the excised bands containing DNA appeared to be up-regulated with PHEQ treatment. The sequences were identified as proteasome, Alu, spectrin (β chain), heterogeneous ribonuclear ribonucleoprotein C (hnRNP-C), eukaryotic translation elongation factor 1α1 (eEF1α1), and autophagy-linked FYVE domain (Alfy) (Table 5.2). All sequence matches were highly specific as indicated by their low E values. Bands JB2C2PL and JB6C2PL were 466 and 901 base pairs in size, respectively, and yielded proteasome and hnRNP-C genes. These appeared to be up-regulated in response to concentrations of PHEQ as low as 0.024 μM. Bands JB4C1P0, JB4C2P0, JB8C1PM, and JB8C2PM corresponding to Alu, spectrin (β-chain), Alfy, and

hnRNP-C also appeared to be up-regulated in response to PHEQ at low concentrations (0.024 μM). The sizes of the bands were 529, 603, 364, and 988 base pairs, respectively. Only bands JB7C1PM and JB7C2PM both identified as eEF1α1 seemed to be up-regulated with moderate (0.24 μM) concentrations of PHEQ. The size of the bands excised were 1194 and 472 base pairs, respectively. The discrepancy in the size of the DNA fragments extracted from the same bands may have been due to the presence of restriction sites within the gene. When the template DNA was cut out of the plasmid for sequencing, the restriction enzyme used could have digested the template DNA as well. With this problem in mind, all subsequent sequencing reactions were performed on undigested DNA after isolation from bacteria.

A second set of experiments involved exposing JEG-3 cells to a tighter concentration range (0.1 to 1.0 μM) of PHEQ than was previously done (0.024 to 2.4 μM) (Figure 5.4). Primers A3 and A4 were used to amplify the cDNA fragments after reverse transcription with Oligo(dT)s. Three independent experiments (conducted on three separate days) with each performed in triplicate were compared on one gel. Not all triplicate samples were included due to insufficient or degraded RNA. When replicates were compared (i.e. 0 – 1, 0 – 2, etc), little variability was observed between samples obtained during the same experiment. Unfortunately, variability between the independent experiments at each concentration was higher. However, the same general banding patterns were still observed despite the variability.

Six bands of interest were excised from the gel, the DNA extracted, cloned and sequenced (Figure 5.4). One band of approximately 450 base pairs in size appeared to be up-regulated with exposure to 0.5 μM PHEQ. This band (BI) was cloned and then

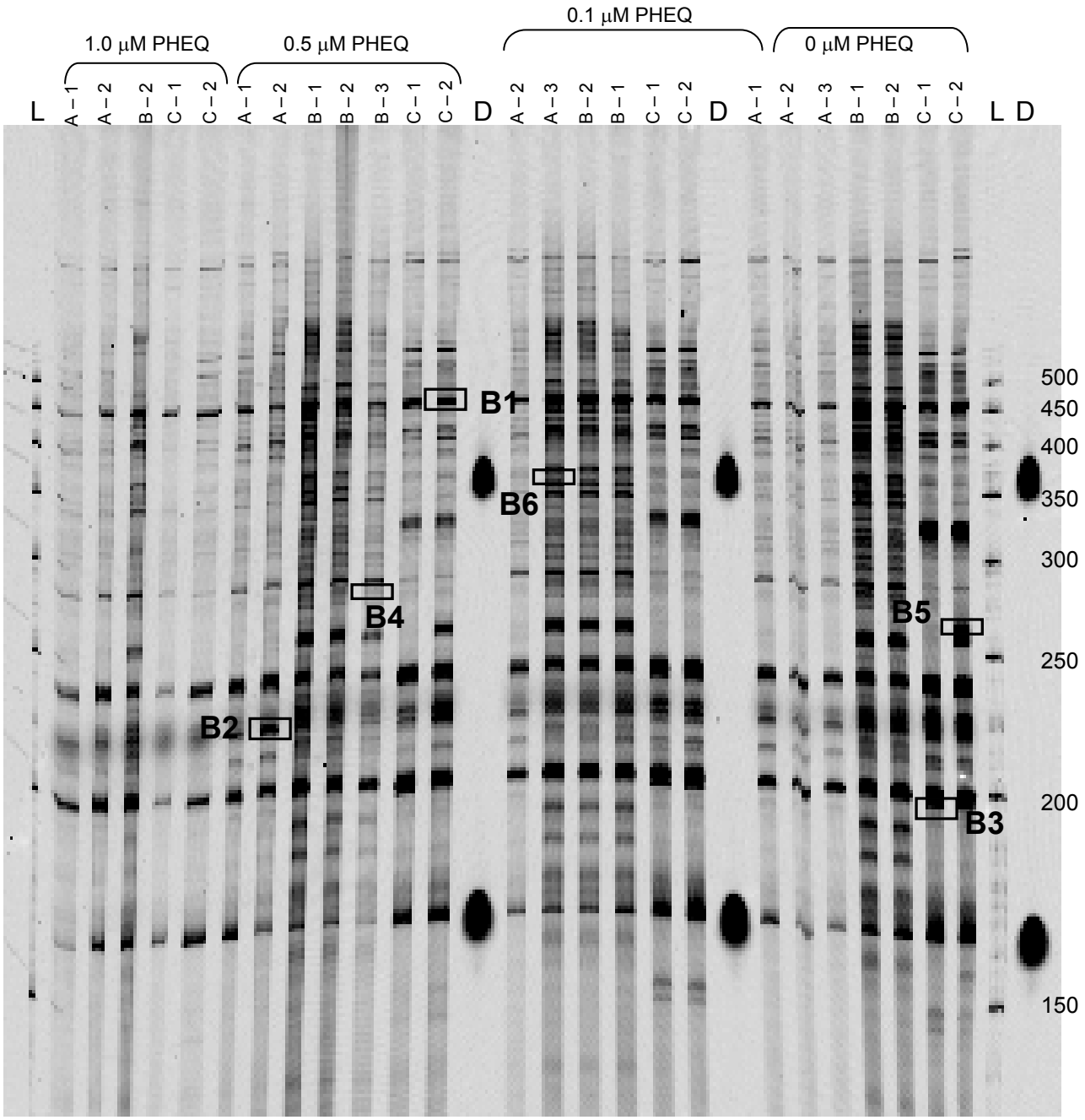

Figure 5.4 Differential display PCR polyacrylamide gel (050526). Experiments conducted on three different dates (A – May 10/05, B – May 5/05, C – April 14/05) with JEG-3 cells exposed to varying concentrations of PHEQ. Experiments were run in triplicate, however not all samples yielded usable RNA. RNA suitable for ddPCR reverse transcribed using Oligo(dT) primers and A3/A4 primers for PCR reaction. Lanes labelled for ladder (L) and loading dye (D). Dye was used as a visual cue to match printout of fluorescently labelled products with gel. This helped to facilitate excision of bands from gel. Bands excised for cloning and sequencing labelled B1 through B6. DNA ladder is shown on both sides of gel for size reference. DNA fragments smaller than 200 bp were not used because the small size would not give specific sequence results. See Table 5.3 for sequence identities.

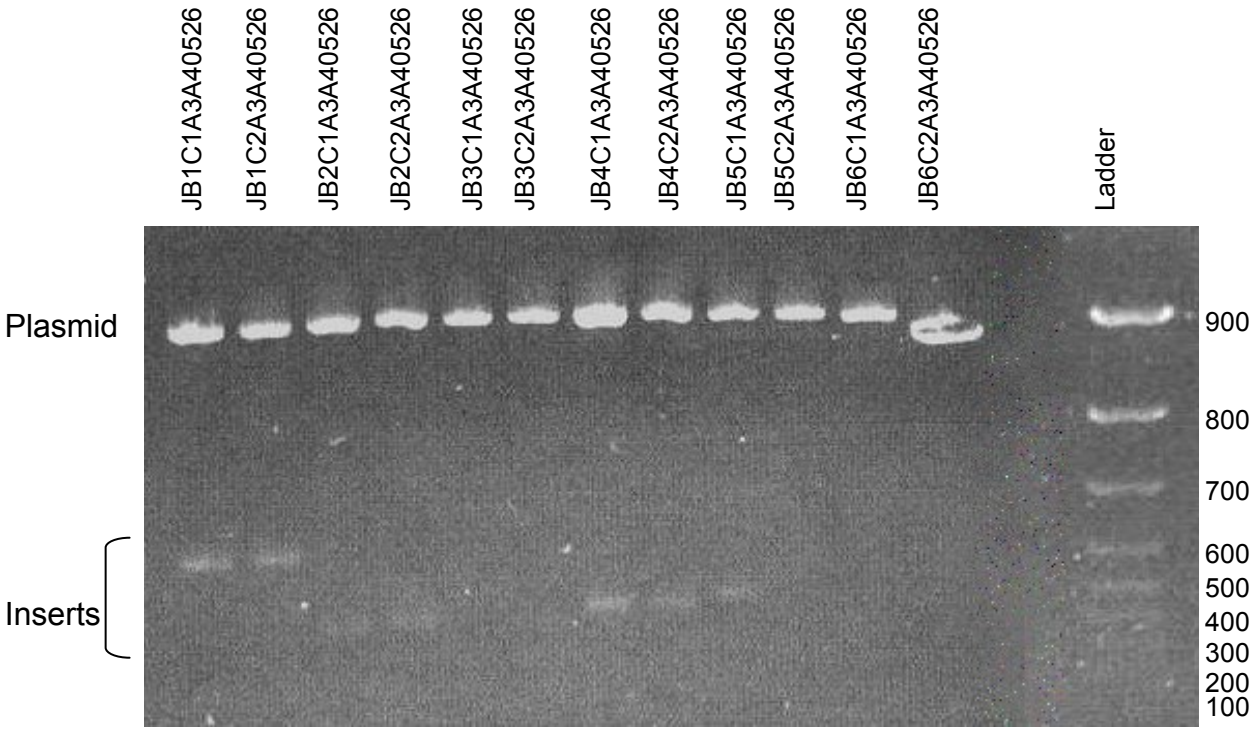

Figure 5.5 Agarose gel electrophoresis of cloned inserts from bands B1 to B6. Cloned inserts were excised from plasmids using ECORI in the restriction digest and separated on 1% agarose gel. The identification code refers to the following; J – JEG-3 cells, B – band #, C – colony #, followed by the primer set used and the date the cells were exposed to PHEQ. Successful ligation reactions occurred for JB1C1A3A40526, JB1C2A3A40526, JB2C1A3A40526, JB2C2A3A40526, JB3C2A3A40526, JB4C1A3A40526, JB4C2A3A40526, and JB5C1A3A40526. All bands of the same approximate size as observed on the polyacrylamide gel were excised and the DNA extracted from the agarose gel. See Table 5.3 for sequence identities.

Table 5.3 Sequence identities of DNA bands excised from ddPCR polyacrylamide gel Figure 5.6. The letters in the sequence ID refer to the following; B1 – band 1, C1 – colony 1, A3A4 – primers used in PCR, 0526 – date cells exposed to PHEQ. The size refers to the length in number of base pairs of the band cut out from the gel. The E value given by the NCBI BLAST search refers to the statistical significance of the hit, the smaller the E value, the more significant the match. The score signifies the quality of the alignment between the sequences, the higher the score, the better the alignment.

Sequence ID	Size (bp)	Name	Species	E value	Score	Gene Regulation
B1C1A3A40526	481	Eukaryotic translation elongation factor 1α1 (eEF1α1)	*Homo sapiens*	0	864	Up
B1C2 A3A40526	480	Eukaryotic translation elongation factor 1α1	*Homo sapiens*	0	864	Up
B3C1 A3A40526	225	Poly(ADP-ribose) polymerase family (PARP10)	*Homo sapiens*	4.0	38.2	Down
B5C1 A3A40526	286	Eukaryotic translation initiation factor 5A (eIF5A)	*Homo sapiens*	0.33	42.1	Down

two colonies selected for DNA isolation and sequencing (Figure 5.5). When sequenced, both clones (JB1C1A3A40526 and JB1C2A3A40526) were identified as eukaryotic translation elongation factor 1α1 with very high specificity (Table 5.3). With apparent down-regulation with PHEQ treatment, bands 3 and 5 (Figure 5.4), approximately 250 base pairs in length, were excised from the gel. Cloning and sequencing of the DNA from bands 3 (JB3C1A3A40526) and 5 (JB5C1A3A40526) were identified as poly(ADP-ribose) polymerase (PARP10) and eukaryotic translation initiation factor 5A (eIF5A) (Table 5.3). Unfortunately, with high E-values, these identities can not be stated with any great certainty.

While 6 bands were excised from the gel, only three of the bands (B1, B3, and B5 - see Figure 5.5) yielded DNA that eventually produced sequence information. When analyzed on a 1% agarose gel stained with ethidium bromide, B3C1, B5C2, B6C1 and B6C2 did not contain the inserted DNA (see Figure 5.5). The presence of the plasmid on the gel confirmed that the bacteria did take up the plasmid. Also, all of the colonies chosen for further processing were white indicating that a successful ligation of the insert had occurred. Therefore, either the restriction digest freeing the insert from the plasmid did not work or there was a restriction site within the DNA fragment that produced fragments smaller than expected and ran off the end of the agarose gel during electrophoresis. The agarose gel (see Figure 5.5) did indicate the presence of DNA from bands B2C1, B2C2, B4C1, and B4C2; however no specific identity could be obtained when the sequence was submitted to GenBank for comparison.

A second ddPCR analysis using RNA extracted from the same three experiments as stated above was performed except primers A3 and A5 were used in the amplification of

cDNA (Figure 5.6). When visualized on the polyacrylamide gel, less variability between samples was observed compared to the previous experiment (Figure 5.4). One band approximately 400 base pairs in size that showed no variation with PHEQ treatment was excised, cloned, and sequenced. Band 1 (JB1C1A3A50526) was identified as aconitase with a high degree of specificity (Table 5.4). Band 2 intensified with increasing PHEQ (Figure 5.6). With an approximate size of 350 base pairs, band 2 appeared to contain two sequences that were identified as eukaryotic translation initiation factor 3 (eIF3 – JB2C1A3A50526) and nucleoporin-like protein that binds HIV-1 reverse transcriptase (JB2C2A3A50526). Band 4 also intensified with 0.5 μM PHEQ treatment (Figure 5.6). At approximately 380 base pairs in length, two sequences identities were found from the DNA extracted from band 4 with eIF3 (JB4C1A3A50526) and serine protease inhibitor (JB4C2A3A50526). Bands 3 and 5 decreased in intensity with PHEQ treatment (Figure 5.6). Band 3 (JB3C1A3A50526) appeared to be down-regulated with 0.5 μM PHEQ. Once sequenced, the DNA fragment was identified as keratin with high specificity (Table 5.4). Band 5 (JB5C1A3A50526) also appeared to be down-regulated when exposed to as little as 0.1 μM PHEQ (Figure 5.6). When sequenced, the DNA fragment was identified as polyglutamic binding protein (Table 5.4). In total, seven bands were excised from the gel and the DNA extracted and cloned. As with the previous gel (Figure 5.7), not all of the bands excised from the gel yielded DNA to produce sequence information.

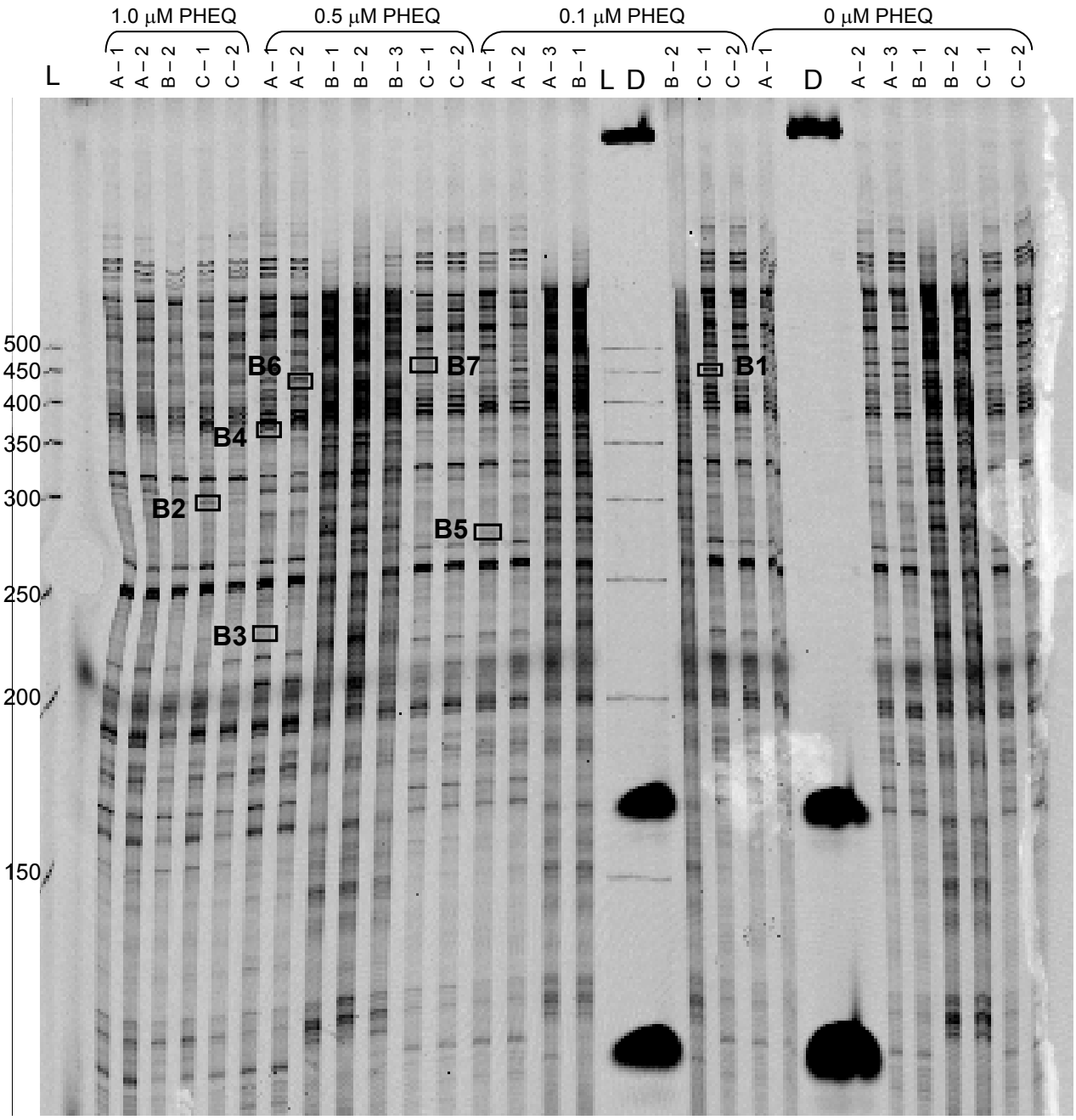

Figure 5.6 Differential display PCR polyacrylamide gel of PHEQ exposed JEG-3 cells from three different experiments (see Figure 5.5). Oligo(dT) primers used for reverse transcription of RNA to cDNA, but primers A3 and A5 used for PCR. Lanes labelled for ladder (L) and loading dye (D). Dye was used as a visual cue to match printout of fluorescently labelled products with gel. This helped to facilitate excision of bands from gel. Excised cDNA bands shown as boxes numbered 1 through 7. DNA ladder is shown on both sides of gel for size reference.

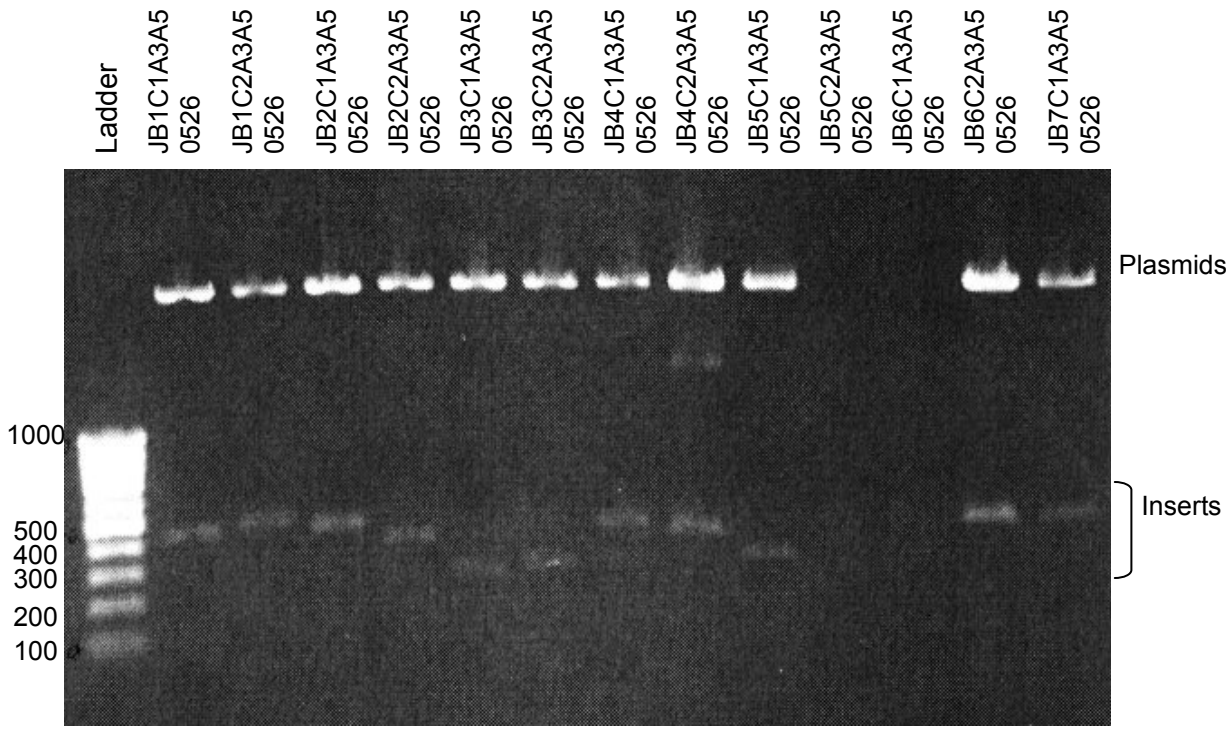

Figure 5.7 Agarose gel electrophoresis of cloned inserts from bands JB1C1A3A50526 to JB7C1A3A50526. Cloned inserts were excised from plasmids using ECORI in the restriction digest and separated on 1% agarose gel. The identification code refers to the following; J – JEG-3 cells, B – band #, C – colony #, followed by the primer set used and the date the cells were exposed to PHEQ. Successful ligation reactions occurred for all bands except JB5C2A3A50526 and JB6C1A3A50526. All bands of the same approximate size as observed on the polyacrylamide gel were excised and the DNA extracted from the agarose gel. See Table 5.4 for sequence identities.

Table 5.4 Sequence identities of DNA bands excised from ddPCR polyacrylamide gel Figure 5.8. The letters in the sequence ID refer to the following; B1 – band 1, C1 – colony 1, A3A5 – primers used in PCR, 0526 – date cells exposed to PHEQ. The size refers to the length in number of base pairs of the band cut out from the gel. The E value given by the NCBI BLAST search refers to the statistical significance of the hit, the smaller the E value, the more significant the match. The score signifies the quality of the alignment between the sequences, the higher the score, the better the alignment.

Sequence ID	Size (bp)	Name	Species	E value	Score	Gene Regulation
B1C1A3A50526	408	Aconitase	*Homo sapiens*	$7e^{-86}$		Unchanged
B2C2A3A50526	315	Nucleoporin-like protein, binds HIV-Rev	*Homo sapiens*	$6e^{-49}$	161	Up
B2C1 A3A50526	379	Eukaryotic translation initiation factor 3	*Homo sapiens*	$5e^{-79}$	299	Up
B3C1 A3A50526	204	Keratin	*Homo sapiens*	$5e^{-36}$	153	Down
B4C1 A3A50526	380	Eukaryotic translation initiation factor 3	*Homo sapiens*	$5e^{-79}$	299	Up
B4C2 A3A50526	370	Similar to *D. melanogaster* serine protease inhibitor	*D. melanogaster*	$2e^{-71}$	151	Up
B5C1 A3A50526	499	Polyglutamic binding protein	*Homo sapiens*	$7e^{-34}$	149	Down

5.4 Discussion

The objective of this study was to survey alterations in gene expression in JEG-3 cells in response to 24 hour PHEQ exposures. ddPCR was the method chosen for its ability to characterize genes responding to toxicant stress; in other words an unbiased approach to toxicogenomics. The presence of PHEQ appeared to both up and down regulate gene expression in JEG-3 cells. The use of a human cell line was advantageous in that retrieving identities of sequenced genes was straightforward due to the large database of known human genes.

Initial efforts with ddPCR in this study resulted in important range finding data. The toxicity of PHEQ was such that moderate concentrations (5 and 10 μM) caused widespread RNA degradation (Figure 5.2A). This also occurred with the addition of 100 μM Cu^{2+} to any concentration of PHEQ, therefore further experiments were conducted with PHEQ only (Figure 5.2B). The reason behind the degradation could have been threefold; (1) cell death from toxicant exposure leads to release of RNases that degraded the cellular RNA, (2) reactive oxygen species (ROS) produced from PHEQ and PHEQ + Cu^{2+} redox cycling could have degraded the RNA, and (3) induction of apoptosis which is characterized by RNA/DNA degradation (Bellacosa and Moss, 2003, Rolo et al., 2004). While ROS generation was probably the main cause of cell death through either necrosis or apoptosis and thus RNA degradation, ROS could have directly attacked RNA as well.

Some concerns regarding ddPCR were raised during the current study. After DNA fragments were cloned into competent cells, the growth of bacterial colonies was observed. With the pGEM plasmid, a successful ligation of the desired DNA split the

gene coding for β-galactosidase in the plasmid. Colonies with the β-galactosidase gene intact produced a blue colour while those that did not were white. From each plate, 2 colonies were picked to inoculate fresh media. The bacteria was grown and then later lysed to retrieve the plasmid. Ideally, sequencing the DNA obtained from duplicate colonies would have been the same, but this was not always the case. For instance, 2 colonies containing DNA from Band 4 (Table 5.2) revealed sequences of the *Alu* elements and spectrin, β-chain. The co-migration of DNA fragments of similar size through the polyacrylamide gel remains one of the unresolved issues regarding ddPCR and reinforces the need for confirmation.

It is estimated that each cell expresses approximately 15 000 mRNAs at any one time (Jorgensen et al., 1999). According to Diener et al.,(Diener et al., 2004), optimal primer sets should generate 50 – 100 bands per gel. Both primer sets used in this study, A3/A4 and A3/A5, appeared to generate the appropriate number of bands. As expected (Diener et al., 2004), using different primer sets with the same starting material in the PCR amplification generated different banding patterns. While numerous genes were observed as up- or down-regulated, no more than 10 bands/gel were excised due to the volume of work required to resolve the identity of each gene. All of the gene fragments isolated for sequencing were matched to some gene found in GenBank, however, most of the sequences did not have specific identities after extensive BLAST searches. With the JEG-3 cells being of human origin, this finding was not surprising. In total, 11 bands containing cDNA were up-regulated with PHEQ treatment while 4 bands appeared to be down-regulated.

5.4.1 Up-Regulated Genes

The genes whose expression appeared to be up-regulated with PHEQ exposure were serine protease inhibitor, Alu repeat sequence, heterogeneous ribonuclear ribonucleoprotein C (hnRNP C), eukaryotic translation initiation factor 3 (eIF3), nucleoporin-like protein, eukaryotic translation elongation factor 1α1 (eEF1 α 1), autophagy-linked FYVE domain (Alfy), spectrin, and proteasome. Most of these genes can be grouped into 2 classes: transcription/translation regulators and cell cytoskeleton components. A schematic of the interactions of the various gene products altered by PHEQ treatment can be found in Figure 5.8.

The *Alu* sequences are the most abundant repetitive elements in the human genome representing approximately 10% of the whole genome mass. These elements are part of a family of non-autonomous retrotransposons called short interspersed nuclear elements (SINEs). Once thought to be junk DNA, evidence now suggests that their insertion into the genome might have brought about rapid evolutionary change (Häsler and Strub, 2006). *Alu* insertions also have been reported as effectors of gene transcription by providing new enhancers, promoters and polyadenylation signals to neighbouring genes (Brosius, 1999). *Alu* elements contain components of the RNA polymerase III promoter from the 7SL RNA gene and appear to have multifunctional roles in regulating DNA replication and recombination, transcription and translation (Vansant and Reynolds, 1995, Chu et al., 1998, Weichenreider et al., 2001). *Alu* RNAs transcribed from *Alu* elements are present in low amounts during normal growth conditions; however, their levels are transiently increased upon stress conditions before rapidly decreasing on recovery (Liu et al., 1995). The up-regulation of the *Alu* elements

in response to PHEQ exposure may indicate the increased transcription/translation of various proteins used by the cells to either repair the cell or induce apoptosis.

Expression of both the heterogeneous ribonuclear ribonucleoprotein C (hnRNP C) and nucleoporin-like protein (binds HIV-1 Reverse transcriptase) genes appeared to be up-regulated with PHEQ treatment. hnRNP C binds and condenses RNA for splicing and other processing prior to translation (Huang and Miller, 2004, Chen et al., 2006, Lee et al., 2006). Nucleoporin-like protein is a transmembrane protein that facilitates the transport of RNA from the nucleus to the cytosol (Hallberg et al., 1993, Fritz et al., 1995, Farjot et al., 1999). Increased transcription and translation coincides with up-regulation of both hnRNP C and nucleoporin-like protein genes (Ruhl et al., 1993 , Schatz et al., 1998, Kim et al., 2003, Whitson et al., 2005). In the current study, up-regulation of both hnRNP C and nucleoporin-like protein with PHEQ treatment may signify an increase in protein synthesis by the cell as a response to manage toxic stress.

Up-regulated sequences also involved in regulating gene expression were the eIF3 and eEF1α1. Translation initiation occurs in a multi-step process whereby a large number of translation initiation factors stimulate the partial reactions involved in the process (Thornton et al., 2003, Russell and Zomerdijk, 2005). One of the largest in size of the translation initiation factors, eIF3, binds to the 40S ribosome along with eIF1A, eIF2, GTP and $tRNA^{Met}$ to form the 43S ribosome complex (Thornton et al., 2003). The up-regulation of eIF3 with PHEQ exposure may have indicated increased protein synthesis as a response to the toxic insult. Ivanov et al., (Ivanov et al., 2003) discovered that eIF3 was involved in stress granule formation. During times of stress, untranslated mRNAs accumulate and eIF3 transcripts transiently increase. eIF3 has

been shown to bind to microtubules and untranslated mRNAs to facilitate stress granule formation (Ivanov et al., 2003). When the cell has recovered, the stress granules disappear and the mRNAs are available to be translated. The stress induced by PHEQ exposure may have required increased gene expression of the eIF3 gene.

Along with aminoacyl-tRNA synthetase (ARS), eEF1α1 has been found to advance the translation elongation cycle. Petrushenko et al (Petrushenko et al., 2002) found that eEF1A could bind to the cytoskeleton as well forming a complex with other translation components demonstrating protein synthesis compartmentalization. Recently, when determining the molecular mechanisms behind the carcinogenicity of Cd^{2+}, a variant of eEF1α1, TEF-1δ, showed increased expression in response to Cd^{2+} exposure (Joseph et al., 2002). Increased protein production is a hallmark of transformation, however, PHEQ treatment may increase the need for proteins due to ROS-induced damage and therefore a need to up-regulate expression of eEF1α1 to compensate.

While many of the genes sequenced have some function related to protein production, several gene products were found to have a primary role in the mechanical framework of the cell. Some of the up-regulated sequences identified included autophagy-linked FYVE protein (Alfy) containing a WD repeat, spectrin β-chain, and proteasome. The sequence for spectrin, β-chain was found in the same band as the *Alu* elements. Spectrin has been established as a major cytoskeleton protein that modulates the structural integrity of cell membranes including the plasma membrane and golgi bodies (Chakrabarti et al., 2006). Spectrin has also been implicated in secretory protein trafficking and endocytosis (De Matteis and Morrow, 2000, Phillips and Thomas, 2006). These abilities have lent spectrin a chaperone-like role in the cell.

However, Nath et al (Nath et al., 1998) discovered that induction of apoptosis in neuronal cells could be characterized by the breakdown of spectrin. Further studies by Cumming et al (Cumming et al., 2004) determined that spectrin was a redox-sensitive protein that could form mixed disulfide bonds with heat shock protein 70 (HSP70). The production of ROS in the JEG-3 cells due to PHEQ exposure would likely have activated the spectrin protein for its roles in cellular protein trafficking. Therefore, an up-regulation in the gene expression of spectrin would not be unexpected.

Both proteasome and Alfy have roles in degradation of proteins. Proteasomal degradation of peptides requires ubiquitination to mark the proteins committed for destruction while Alfy plays a role in autophagic break down of denatured proteins. Autophagy involves degradation of denatured protein aggregates and organelles in lysosomes, and does not require ubiquitin tagging for identification. Recently, a new protein has been shown to bind to protein aggregates and shuttle them to the autophagic machinery (Simonsen et al., 2004). The degradation of proteins by the proteasome begins with the identification of a misfolded or denatured protein by the cell. A ubiquinating enzyme tags the protein for destruction whereby the tagged protein is transported to the proteasome. Interestingly, regulation of various proteasome genes appears to be controlled by antioxidant response elements (Takabe et al., 2006). The prospect of the redox state influencing the expression and production of organelles such as the proteasome makes the exposure of cells to redox-cycling compounds such as PHEQ of critical importance.

The serine protease inhibitor protein or serpin gene was apparently up-regulated with PHEQ treatment. In general, serpins are proteins that inhibit serine proteases, although

not all serpins have inhibitory properties such as storage proteins (i.e. ovalbumin), tumour suppressor proteins (i.e. maspin), and hormone-binding proteins (i.e. thyroxine and cortisol binding proteins) (Gettins, 2002, Gao et al., 2004, Azukizawa et al., 2005). Inhibitory serpins bind to a serine residue in a serine protease which induces a conformational change in the serpin and a concomitant shape change in the protease. This action obliterates the enzymatic active site of the protease (Gettins, 2002, Whisstock and Bottomley, 2006). Inactivation only occurs while the serpin is bound to the protease, thus the serpin is consumed in the inhibitory reaction (Huntington et al., 2000, Gettins, 2002, Whisstock and Bottomley, 2006). Most serpins function extracellularly, however, some are found intracellularly as well (Liu et al., 2004, Azukizawa et al., 2005, Papp et al., 2006). Concentrations of serine proteases are known to increase with induction of apoptosis (Bird, 1999, Liu et al., 2004). Liu et al (Liu et al., 2004) demonstrated that serine protease inhibitor 2A was able to inhibit cathepsin B, a protein used in caspase-independent apoptosis triggered by ROS. It has been suggested that the induction of caspase-independent apoptosis may represent a back-up mechanism for cell death that may be used in pathological situations where caspase activity is absent. Therefore, serpins may represent regulatory control of pro-apoptotic agents such as cathepsin B. The up-regulation of serpins with increasing concentrations of PHEQ up to 0.5 μM may represent one point of control in the balance between pro- and anti-apoptotic signals in the JEG-3 cell.

5.4.2 Genes down-regulated with PHEQ exposure

Interestingly, studies have found that nucleoporin-like protein (binds HIV-1 Reverse transcriptase) and eIF-5A appear to interact. As with nucleoporin-like protein, eIF5A also is involved in mRNA trafficking from the nucleus to the ribosome for translation (Ruhl et al., 1993, Caraglia et al., 2001). Studies have demonstrated the interaction between HIV-1 Rev protein and eIF-5A in mediating transport of unspliced or incompletely spliced viral mRNA's across the nuclear envelope (Ruhl et al., 1993, Schatz et al., 1998). Although expression of nucleoporin-like protein appeared to be up-regulated, eIF5A gene expression appeared to be down-regulated with PHEQ treatment. eIF5A is an interesting translation initiation factor in that it possess the unique amino acid hypusine. Hypusine is the result of post-translational modification of lysine via spermidine (Park et al., 1982, Abbruzzese et al., 1988). This modification is required for eIF5A activity and interaction with ribosomes, and is correlated with cell proliferation inducing a reversible arrest at the G1-S boundary of the cell cycle (Park, 1987). Furthermore, induction of apoptosis has been found to correlate with decreased formation of hypusine-containing eIF5A (Tome and Gerner, 1997). The dual role of eIF5A suggests a balance between proliferation and apoptosis implicit in the production of proteins in the cell. The apparent down-regulation of eIF5A with exposure to PHEQ may represent a shift in that balance and be one of the early steps down the apoptotic pathway.

Also active inside the nucleus is the newly discovered protein poly(ADP-ribose)polymerase 10 (PARP10). Although PARP10 can be found in both the cytosol and the nucleus, the active phosphorylated form of PARP10 is localized to the nucleus.

As an emerging subfamily to the PARP family, their endogenous substrates have yet to be determined. However, PARP10 may have interactions with RNA Polymerase I in a differentiation-stage manner. Down-regulation of PARP10 interferes with proper G_1/S progression of the cell cycle and cell viability. The apparent down-regulation of PARP10 in response to PHEQ exposure may have revealed a so far unknown characteristic of this protein. Sul et al (Sul et al., 2007) demonstrated that PARP1 expression was down-regulated in the presence of formaldehyde. Since formaldehyde can produce ROS, gene expression of PARP1 may be redox-dependent. This also may hold true for PARP10, but remains unproven.

Another nuclear protein involved in transcription with apparent down-regulation in gene expression upon treatment with PHEQ was PQBP. This protein has been found localized predominantly in the nucleus where it binds to polyglutamine tracts found in many transcription factors. Recent evidence has suggested that PQBP may form a link between transcription factors and spliceosomes (Waragai et al., 2000, Okazawa et al., 2001). Interestingly, overexpression of PQBP has been shown to suppress cell growth and making the cell vulnerable to stress (Waragai et al., 1999). The down-regulation of PQBP in response to PHEQ exposure may have been an attempt by the cell to defend against PHEQ-induced stress.

Keratins or cytokeratins are part of the intermediate filament (IF) network found mainly in epithelial cells. Keratin often occurs as heteropolymers of type 1 and type 2 keratins in differentiated epithelial cells (Fuchs and Weber, 1994, Steinert et al., 1994). In vivo experiments showed that keratin degradation is slow (Denk et al., 1987); however, in vitro experiments demonstrated that monomers of keratin can be degraded

quickly through ubiquitination (Ku and Omary, 2000). They are major contributors to the mechanical integrity of cells and the keratin IF network provides a link between the cytosol and surface membrane receptors some of which are involved in cell – cell and cell – extracellular matrix (ECM) interactions. Keratins are not just limited to the cytosol. Davie et al. (Davie et al., 1999) found that keratins bind nuclear DNA with the prospect of participating in chromatin organization through the nuclear scaffold. Historically, cytokeratins were only considered as part of the skeletal proteins that provided mechanical stability for the cell; however, mechanical signals at the cell surface may trigger changes in cell shape and alter nuclear function mediated by the keratin IF network (Marceau et al., 2001). For instance, spreading cells on an ECM can switch off the default differentiation program and promote the G1 – S cell cycle transitions in hepatocyte monolayer cultures (Hansen et al., 1994).

New evidence has suggested multiple roles for cytokeratins during apoptosis. Caulin et al., (Caulin et al., 2000) found that cytokeratins may transmit apoptic signals under stress conditions. Furthermore, hepatocytes exposed to high levels of oxidative stress show blebs on the cell surface, a sign of keratin degradation (Chen et al., 2000, Kishimoto et al., 2000). However, Gilbert et al., (Gilbert et al., 2001) observed that the presence of keratin appears to oppose Fas-induced apoptosis while recent findings by Gonsebatt et al., (Gonsebatt et al., 2007) determined that increased oxidative stress due to arsenite exposure can lead to up-regulation of cytokeratin 18. This finding is opposite to the down-regulation observed with PHEQ treatment which would occur if the cell was becoming apoptotic.

The use of toxicogenomics has added a new dimension to the study of environmental toxicology. Through the use of ddPCR, preliminary data has suggested new potential targets in gene expression for oxyPAHs such as PHEQ. Most gene expression changes induced by PHEQ as detected by ddPCR have a function in transcription and translation. While the effect of PHEQ on the gene expression profiles presented in this study requires confirmation, in most cases the observed changes in gene expression could be the result of oxidative stress and thus are reasonable considering the redox-cycling ability of PHEQ.

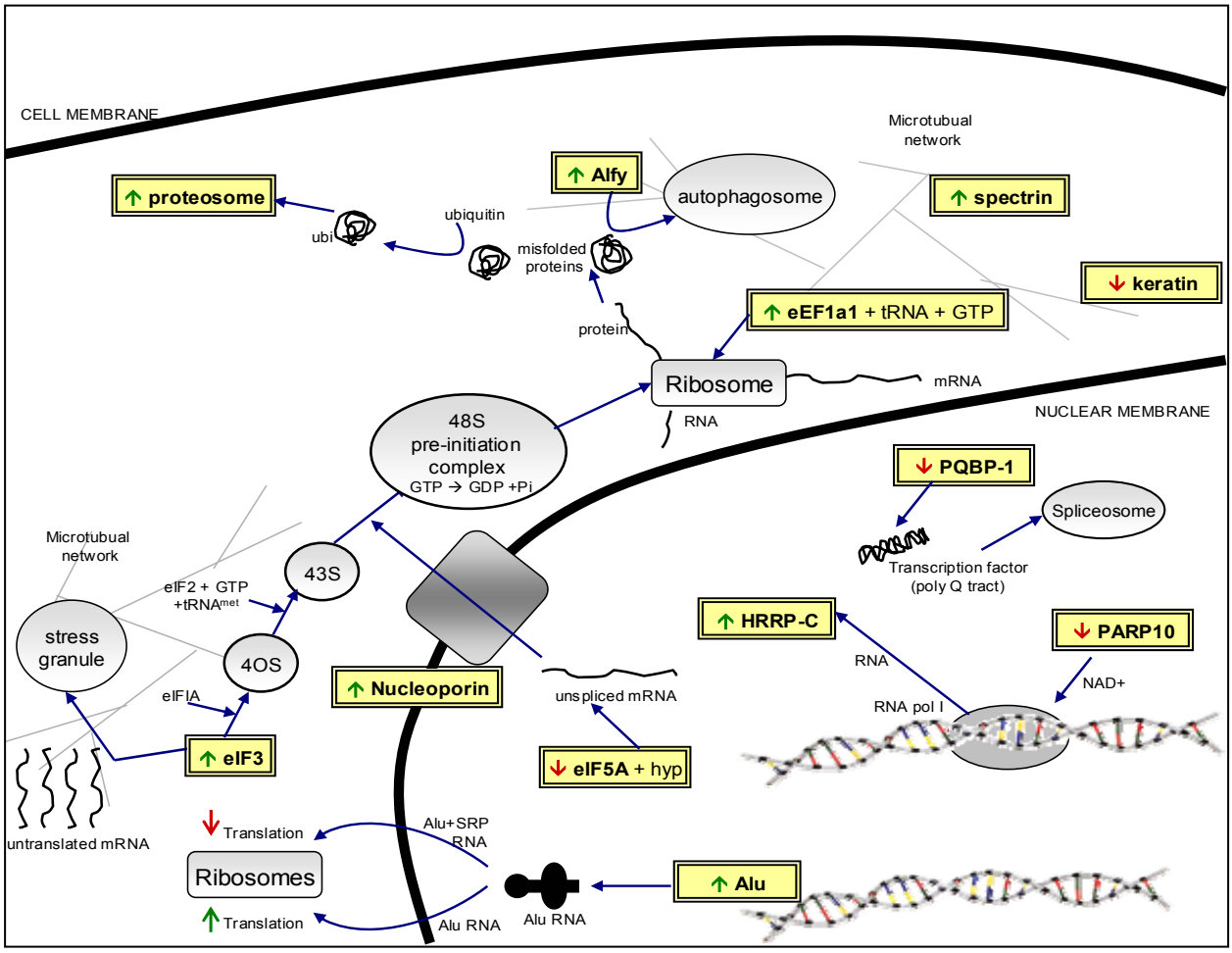
CELL MEMBRANE
Microtubual network
↑ proteosome
ubi
ubiquitin
misfolded proteins
↑ Alfy
autophagosome
↑ spectrin
↓ keratin
protein
↑ eEF1a1 + tRNA + GTP
Ribosome
mRNA
RNA
NUCLEAR MEMBRANE
48S pre-initiation complex
GTP → GDP +Pi
↓ PQBP-1
Spliceosome
Transcription factor (poly Q tract)
Microtubual network
43S
eIF2 + GTP +tRNAmet
stress granule
4OS
eIFIA
↑ Nucleoporin
unspliced mRNA
↑ HRRP-C
↓ PARP10
RNA
NAD+
RNA pol I
↑ eIF3
↓ eIF5A + hyp
untranslated mRNA
↓ Translation
Alu+SRP RNA
Ribosomes
↑ Translation
Alu RNA
Alu RNA
↑ Alu

Figure 5.8 Schematic representation of gene product levels altered through PHEQ exposure in JEG-3 cells. The up-regulation of the gene is highlighted by the green up arrow while down-regulation is represented by the red down arrow. The following are the abbreviations used: PARP10 – poly(ADP-ribose) polymerase 10, PQBP-1 – polyglutamine binding protein-1, hnRNP C – heterogeneous ribonuclear ribonucleoprotein C, eIF5A – eukaryotic translation initiation factor 5A, SRP – signal recognition particle, eIF3 – eukaryotic translation initiation factor 3, eEF1a1 – eukaryotic translation elongation factor 1a1, Alfy – autophagy-linked FYVE domain protein.

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