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Dose-Response of Low Dose Co-exposures to Arsenic and Benzo[a]pyrene in Mice

A thesis submitted to the
Division of Research and Advanced Studies
at the University of Cincinnati

In partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

In the Department of Environmental Health
of the College of Medicine

2004

by

Brian Arthur Meier

B. S., United States Coast Guard Academy 1996

Committee Members:

Glenn Talaska, Ph.D., CIH

Kathleen Dixon, Ph.D

Paul Succop, Ph.D.

ABSTRACT

Workers are simultaneously exposed to benzo[a]pyrene (BaP) and arsenic in many industrial settings. Independent epidemiologic studies have reported varying effects—from additive to multiplicative—among such populations. *In vitro* experiments have shown evidence of synergism between the two exposures, and an *in vivo* experiment has recently supported this finding. Our goal was to better characterize this relationship using a mouse model with DNA adducts as a measure of effect. Eighty-four female mice were divided into nine treatment groups: +100 nM BaP/- As; +100 nM BaP/+ 2.0 mg As; +100 nM BaP/+ 4.0 mg As; +100 nM BaP/+ 8.0 mg As; +200 nM BaP/- As; +200 nM BaP/+ 2.0 mg As; +200 nM BaP/+ 4.0 mg As; +200 nM BaP/+ 8.0 mg As; and control. Arsenic was provided *ad libitum* for four weeks in order to reach steady state levels. BaP was topically applied to shaven dorsal skin the four consecutive days prior to sacrifice. Lung and skin tissues were analyzed for DNA adducts using ³²P-postlabeling. Inductively Coupled Plasma Mass Spectrometry was used to determine arsenic concentrations in these same tissues. Our results were inconclusive. Relative adduct levels (RAL) in the 100 nM BaP treatment groups were extremely variable. Data for the 200 nM BaP treatment groups were in better agreement with earlier studies, but none of these relationships were found to be statistically significant. Working with such low doses may have been problematic. Actual arsenic concentrations among the 2.0 mg and 4.0 mg treatment groups were very similar, and a greater difference in dose should be used in the future to observe truly heterogeneous treatment groups. Additionally, the 100 nM BaP treatments may have been too low to see a uniform effect—a 200 nM BaP dose should be used as an initial treatment group in future experiments.

ACKNOWLEDGEMENTS

I offer my sincere thanks to my advisor, Dr. Glenn Talaska, for his guidance and patience throughout this whole process. I owe a debt of gratitude to Brenda Schumann who graciously attempted to teach me how to perform postlabelling experiments, and to Kathy LaDow who did the same with the animal treatment part of this study. I would like to thank Jay Vietas for providing an ear for both my coherent and (more often) incoherent ramblings. This study was made possible by grant number P42 ES04908 from the National Institute of Environmental Health Sciences, NIH.

On a more personal note, I'd like to thank my children, Josie and Ben, for always giving me a reason to smile. And last but not least, I'd like to thank my wife, Mary—without her inspiration and support none of this would have been possible.

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List of Abbreviations

100 nM BaP, 0 mg As	A1
100 nM BaP, 2 mg As	A2
100 nM BaP, 4 mg As	A3
100 nM BaP, 8 mg As	A4
200 nM BaP, 0 mg As	B1
200 nM BaP, 2 mg As	B2
200 nM BaP, 4 mg As	B3
200 nM BaP, 8 mg As	B4
American Conference of Governmental Industrial Hygienists	ACGIH
Analysis of Variance	ANOVA
Benzo[a]pyrene	BaP
Benzo[a]pyrene Diol Epoxide	BPDE
Biological Exposure Index	BEI
Centimeters	cm
Comprehensive Environmental Response, Compensation, and Liability Act	CERCLA
Control	C
Counts per Minute	cpm
Deoxyribonucleic Acid	DNA
Dimethylarsonic acid	DMA

Environmental Protection Agency	EPA
Glutathione	GSH
Grams	GSH
Inductively Coupled Argon Plasma, Atomic Emission Spectroscopy	ICP
Liters	L
Methylarsonic acid	MMA
Micrococcal endonuclease/spleen phosphodiesterase	MNSPD
Micrograms	µg
Microliters	µl
Milligrams	mg
Milliliters	ml
Molar	M
nanograms	ng
Nanomoles	nM
National Institute for Occupational Safety and Health	NIOSH
National Occupational Research Agenda	NORA
Nuclease P1	NP1
Nucleotide Excision Repair	NER
Occupational Safety and Health Administration	OSHA
Permissible Exposure Limit	PEL
Polycyclic Aromatic Hydrocarbon	PAH

Polynucleotide kinase	PNK
Reactive Oxygen Species	ROS
Recommended Exposure Limit	REL
Relative Adduct Levels	RAL
Threshold Limit Value	TLV

Background

Arsenic—Environmental/Occupational Exposures

Arsenic is a ubiquitous element, naturally occurring in rock, soil, food and water. The most prominent route of exposure among the general population is thought to be through ingestion of various foodstuffs and beverages. The average daily intake of inorganic arsenic from food is estimated to be 50 µg. Exposures from drinking water vary greatly worldwide—ranging from less than 1 µg/L in relatively inert geothermal areas to 3.6 mg/L in areas of high geothermal activities. Smokers are also exposed to arsenic at higher levels (between a few and 20 µg a day), but these concentrations have likely declined due to the disuse of arsenic-based pesticides [1]. Safe drinking water standards are still under debate, but in 2001 the EPA lowered maximum level of arsenic permitted in drinking water from 50 µg/L to 10 µg/L [2].

In the industrial setting, copper and lead smelters, workers sawing and handling arsenic treated wood, agricultural workers and hazardous waste workers may all be at risk for elevated exposures [3]. NIOSH and the ACGIH both list arsenic as a known carcinogen and recommend occupational exposure limits of 0.002 mg/m³ and 0.01 mg/m³ respectively [4, 5].

Due to the widespread nature of the contaminant and its potential effects, arsenic ranked number one on the 2003 CERCLA Priority List of Hazardous Substances.

BaP—Environmental/Occupational Exposures

Polycyclic aromatic hydrocarbons (PAHs) are also common in the environment and in industry. Among this large group of compounds, benzo[a]pyrene (BaP) is commonly used in the laboratory setting. Among the general population, PAHs are mainly inhaled—having been released by the combustion of fossil fuels and wood. Exposure also occurs from the consumption of charred or grilled foods. Cigarette smoke and urban air also contain PAHs and

are sources of exposure. In industrial settings PAHs are released during coking operations and during the production of coal tar and asphalt. Road paving crews and incinerator workers are also likely to have high exposures [6]. ACGIH has listed BaP as an A2, “suspected human,” carcinogen, but has not established a TLV. As with any chemical with such designations, exposures should be carefully controlled and kept as low as possible [7].

Co-mutagenesis of Mixed Exposures to Arsenic and BaP

With respect to co-exposures to arsenic and PAHs, the most well studied groups are smokers with occupational exposures to arsenic. Independent epidemiologic studies have reported varying effects, from additive to multiplicative, among such populations. The earliest study reviewed here reported a multiplicative effect with respect to lung cancer among Swedish copper smelter workers with arsenic exposure and heavy smoking habits [8]. Another group of Swedish smelters were examined and the data from that study fit both additive and multiplicative models of interaction [9]. A review of similar studies also came to the conclusion that a greater than additive, or synergistic, effect between the two exposures existed [10].

In vitro experiments have shown evidence of synergism between the arsenic exposures and other known mutagens. Inorganic arsenic has been shown to be both co-mutagenic and co-carcinogenic with ultraviolet light [11]. Of particular relevance to this study is evidence of co-mutagenesis among cell cultures treated with BaP and arsenic [12]. An *in vivo* experiment using BaP and arsenic in mice with DNA adducts as a measure of effect has also supported this finding [13].

These observed relationships pose a seemingly endless series of questions for epidemiologists, toxicologists and industrial hygienists alike. Three of these questions are posed here. First and foremost, the validity of these observations is still in doubt. The smoking vs.

non-smoking model used by epidemiologists has some inherent confounders. Cigarette smoke is a complex mixture in and of itself, consisting of much more than arsenic and BaP.

Occupationally exposed groups are also not without confounding variables. If these confounders are controlled for, then a relationship does seem to exist between PAHs and arsenic. The specific relationship is still under debate. *In vitro* models provide some of the most compelling data, but it is difficult to extrapolate these findings to an actual living system. Efforts to extrapolate these *in vitro* models to *in vivo* studies in mice have not had the same degree of success, but that is—in many ways—a typical result. Even beyond such animal models, the human implications of this relationship need to be further developed.

Assuming the validity of the proposed relationship between BaP and arsenic, a second and perhaps more obvious question is why does this synergistic relationship exist? This question is very difficult to answer, but much work has been done to solve the quandary. Arsenic and BaP have both been shown to induce cancer [2, 6, 7], but are thought to do so in different ways. BaP follows a classic chemical carcinogen model. Once inside the body it is metabolized to benzo[a]pyrene-r-7,t-8-diol-t-9,10-epoxide (BPDE), which binds to guanine bases in DNA causing the formation of stable DNA adducts [7, 14]—the measured endpoint of this study. Arsenic's role in carcinogenesis is much more complex. Arsenic is thought to inhibit DNA repair—thus potentiating the effects of carcinogen-induced adducts, such as BPDE-adducts (reviewed in [11, 15]). The repair pathway of interest for chemical carcinogenicity is the nucleotide excision repair (NER) pathway, a complex pathway involving over 20 proteins that has been shown to be affected in the presence of arsenic [16]. The metabolism and biotransformation of arsenic may also produce more reactive form of arsenic and reactive oxygen species (ROS) thereby increasing cytotoxicity [17, 18]. Arsenic, its metabolites and

ROS produced by its metabolism may also be scavenged by glutathione and in turn be competing with BaP for that detoxification outlet [19]. Simply stated, arsenic may affect detoxification and repair pathways, thus enhancing the effects of BPDE-adducts (Figure 1).

Figure 1: Mechanisms of BaP adduct formation and possible interactions of As

A third and final question to pose here, has to do with the field of occupational health. More specifically—and personally—why is an industrial hygiene student interested in this relationship? As previously discussed, exposures to both arsenic and BaP are commonplace. To protect the worker, the industrial hygienist must have, at the minimum, a basic understanding as to how these two chemicals interact within the human body. For that matter, the industrial hygienist must take the possible synergistic effect of all metals into account when exposures to chemical carcinogens are present. Merely evaluating exposures to arsenic (alone) and PAHs (alone) will not necessarily be protective enough given the possible synergism between the two chemicals. These two agents, if present in the same work environment, must be evaluated as a mixed exposure in order to fully understand their co-carcinogenic potential.

Approaches in Evaluating Mixed Exposures

Traditional methods of industrial hygiene rely on comparing air samples or personal samples to occupational exposures limits—usually Permissible Exposure Limits (PELs) set by OSHA, Recommended Exposure Limits (RELs) published by NIOSH or Threshold Limit Values (TLVs) published by ACGIH. These standards or recommendations are usually based on the evaluation of controlled experiments using a single chemical of exposure [20].

Traditional industrial hygiene practices do little to address complex mixed exposures. A common approach is to evaluate each hazardous substance separately, unless those substances are known to act on similar target organs. If similar affinities do exist—and if no documentation exists to the contrary—the compounds in question are assumed to be additive. The ratios of observed concentrations to exposure limits are added up and compared to unity (or 1). A sum greater than unity would represent overexposure [5]. This approach may be valid when dealing with multiple solvents or other similar materials, and these types of mixed exposures may be representative of the most common scenarios presented to a practicing industrial hygienist. However, such an approach would overlook the interactions between dissimilar substances, such as arsenic and BaP.

Alternatives to this approach are not readily available. Best practice would be to study the literature and use professional judgment when evaluating mixed exposures. With respect to community risk assessments, guidelines have been established for the EPA which have attempted to outline a consistent approach for their toxicologists [21]. Clearly the evaluation of mixtures is very complex, and the EPA acknowledges that no single approach can be used in all scenarios. The agency has recently expounded upon its original guidance document—introducing pharmacokinetic modeling and giving more specific information of common/well-

studied chemical interactions [22]. NIOSH has also recognized the need to further examine the health effects of mixed exposures having placed the topic on the National Occupational Research Agenda (NORA). Future research directions call for studying the mechanisms responsible for causing synergistic effects, for using improved field techniques to better characterize simultaneous exposures and for improving statistical methods used to analyze mixed exposures [23].

Role of Biomarkers and ^{32}P -Postlabeling in Evaluating Mixed Exposures

Biomonitoring will play an important role in further evaluating mixed exposures. Figure 2 shows the place of biological monitoring within the continuum from exposure to disease. As seen in the figure, air sampling only estimates possible exposure to a certain chemical.

Figure 2: Continuum between exposure and disease (Adapted from [19])

Furthermore, air sampling, though well established in the field of industrial hygiene, does not adequately account for mixtures. Classical epidemiology may account for the effects of mixtures, but they do so by evaluating a final outcome thus limiting their use with respect to the

promotion of worker health. Only biological monitoring can provide specific insight into what's actually occurring within the human body.

This specific insight is crucial in addressing the question of mixtures. Measurements of internal dose are much more useful than measurements of exposure because they integrate for various routes of exposure. Measures of effective dose and early effect (such as DNA adducts) may strengthen epidemiologic arguments because they help demonstrate biological plausibility [19].

By using biomarkers, studies have further examined the problem illustrated in Figure 1. These studies have subsequently strengthened the relationships first investigated by classical epidemiology. Maier *et al* [12], for example, came to three important conclusions by using ³²P postlabelling analysis of mouse hepatoma Hepa-1 cells. First, cell lines were treated with BaP and a metabolite of BaP. DNA adducts were compared between the two treatments. No significant differences were observed showing that the interaction of arsenic occurs downstream of initial CYP1A1 mediated metabolism of BaP. Secondly, this experiment showed that GSH depleted cells had higher adduct levels than did normal cells suggesting that arsenic and BaP do compete for the same detoxification pathway. Thirdly, a time course experiment investigating adduct removal showed that NER was somehow affected, presumably by arsenic.

The same types of relationships need to be examined integrating for the complexities of an *in vivo* model. Evans *et al* [13], used DNA adducts as a marker of effective dose and ICP arsenic concentrations as a biomarker of internal dose in a mouse model. Revisiting Figure 1, these two markers compared together can give insight into the most basic of questions: what effect does arsenic have on BaP DNA adduct levels? Evans found a significant increase (twofold) of adducts in the lung tissues of arsenic treated animals when compared to the lung tissues of non-

arsenic treated animals. This study hopes to better characterize this basic relationship using the same type of murine/DNA adduct model. Furthermore, the interactions between arsenic and GSH or NER can and will be examined using transgenic mice.

Ultimately, human studies will be augmented by the use of biomarkers. The ACGIH has validated a biologic exposure index (BEI) for urinary arsenic and one will shortly be approved for PAHs. These measurements offer a better way to evaluate human exposure so that epidemiologists no longer have to rely solely on questionnaires to define groups at risk. DNA adduct levels have the extra advantage of providing a link between exposure and biological effect. Several studies have investigated the relationships between ambient BaP concentrations and adduct levels in the blood (reviewed in [20]). The same can and should be done in the occupational environment.

Hypotheses

The following null hypotheses will be tested:

1. For the 100 nM BaP treatment groups, Skin RAL and Lung RAL in non-arsenic treated animals (group A1) will not be different at the 0.05 level of statistical significance from the arsenic treated animals (A2, A3 and A4).
2. For the 200 nM BaP treatment groups, Skin RAL and Lung RAL in non-arsenic treated animals (group B1) will not be different at the 0.05 level of statistical significance from the arsenic treated animals (B2, B3 and B4).
3. For both treatment groups, skin RAL will not be correlated with ICP arsenic concentration in the skin at the 0.05 level of statistical significance.
4. For both treatment groups, lung RAL will not be correlated with ICP arsenic concentration in the lung at the 0.05 level of statistical significance.
5. For both treatment groups, skin RAL will not be correlated with Lung RAL at the 0.05 level of statistical significance.

Materials & Methods

Materials

Sodium arsenite, BaP, phosphodiesterase II, apyrase, ribonuclease A, micrococcal endonuclease/spleen phosphodiesterase, adenosine 5'-triphosphate, nuclease P1, lithium hydroxide, urea, trizma® hydrochloride, bicine and sodium hydroxide solution, spermidine, dithiothreitol, magnesium chloride and sodium succinate were purchased from Sigma Co. (St Louis, MO). ³²P was acquired from Perkin-Elmer, NEN Life Science (Boston, MA). T4 polynucleotide kinase was obtained from United States Biochemical Corporation (Cleveland, OH). Lithium chloride, sodium phosphate monobasic, calcium chloride and 90% laboratory grade formic acid were obtained from Fisher-Scientific (Pittsburgh, PA). The Wizard® Genomic DNA Purification Kit was manufactured by Promega (Madison, WI). Cellulose polyethyleneimine plates were purchased from Scientific Adsorbents (Atlanta, GA). Fuji, Super RX, medical X-ray film was purchased from Philips Medical (Highland Heights, OH).

Animal Treatment

The animal study was carried out in accordance with University of Cincinnati Institutional Animal Care and Use Committee Protocol #01-10-18-01. Eighty-four (84) C57BL/6 female mice, aged 4 to 6 weeks, were acquired from Jackson Laboratories in Bar Harbor, Maine. The mice were divided into nine treatment groups: A1, A2, A3, A4, B1, B2, B3, B4 and C. The dosing regimen for each of these groups can be found in Table 1.

Table 1: Animal dosing regimen

Animal Group	Number of Animals	As Dose (mg/L)	BaP Dose (nM)
A1	10	.	100
A2	10	2	100
A3	10	4	100
A4	10	8	100
B1	10	.	200
B2	10	2	200
B3	10	4	200
B4	10	8	200
C	4	.	.

The mice were separated into treatment groups and housed in clear plastic cages in the Kettering Laboratory animal housing facility. Each cage housed 5 mice with one exception; the four mice in control group C were kept in one cage. After arrival, the animals were given 6 days to acclimatize before treatments began. Temperature and relative humidity remained constant—at 72°F and 42% respectively. Twelve (12) hour light cycles were maintained, with the light cycle beginning at 6:00 a.m. and ending at 6:00 p.m.

Arsenic was provided *ad libitum* for four weeks in order for the mice to reach steady state levels. The 8 mg/L dose was made first. Eight (8) milligrams of sodium arsenite were added for each liter of milli-Q water. From this master solution two more dilutions were made—giving the 4.0 mg/L and the 2.0 mg/L doses. Water intake was measured. At the beginning of each week, 250 milliliters of the appropriate water treatments were placed into plastic water bottles.

Leftover water for each cage was measured and recorded at the end of each week. Cage weights were also taken each week.

Two different BaP doses were made: a 100 nM of BaP in acetone solution and a 200 nM of BaP in acetone solution. The two solutions were wrapped in foil and stored in ice at -20°C in order to prevent degradation. Twenty-five (25 µl) of the appropriate dose was topically applied by pipette to shaven dorsal skin the four consecutive days (24 hours apart) prior to sacrifice. Control animals in group C were treated with 25 µl of acetone via pipette.

One day after the final BaP treatment, all animals were sacrificed by asphyxiation with carbon dioxide. Treated skin, distal skin, lung and urinary bladder tissues were removed from each animal. Tissues were kept at -80°C until DNA isolation was performed.

DNA Extraction

Prior to DNA extraction the skin and lung samples were randomized, and anyone involved with extractions or experiments was blinded through ³²P-postlabeling assays. Each skin sample was given a 6 digit random number generated by Microsoft Excel. The samples were sorted by random number and were relabeled accordingly. The same process was done with the lung samples.

DNA extraction was done using the Wizard[®] Genomic DNA Purification Kit in accordance with the procedures outlined in the kit's technical manual. A Beckman DU 640 spectrophotometer was used to measure the concentration of DNA in the final solution. The extracted DNA samples were stored at -20°C until post-labeling experiments were conducted.

DNA Adduct Analysis

With exception of minor laboratory modifications, ³²P-postlabeling was completed in accordance to the method initially reported by Reddy, Gupta and Randerath [24]. Thirty (30)

total ^{32}P -postlabeling experiments were performed. Eight (8) extracted DNA samples were analyzed per experiment. These samples were split to ensure reproducibility. The split samples were numbered 1A, 1B, 2A, 2B, 3A, 3B, and so on. If the split samples were not reproducible, then the labeling of that particular sample was repeated. The experiments were numbered BAM1 through BAM30. Experiment BAM1 was an unsuccessful attempt to use the contact transfer method—a method used previously in the laboratory [25]. The following method was used for all other experiments.

The samples were first hydrolyzed. A hydrolysis cocktail was prepared consisting of 36 μl of milli-Q water, 36 μl of 5x salts and 36 μl of MNSPD. In microcentrifuge tubes, 6 μl of hydrolysis cocktail were added to 4 μl of sample per split sample. The samples were mixed, spun down and incubated for three hours at 37°C . After incubation, the hydrolyzed samples were stored at -80°C until the postlabelling assay.

NP1 was used to enhance the sensitivity of the postlabelling assay [26]. A NP1 cocktail was made consisting of 21.6 μl of NP1, 32.4 μl of zinc chloride (0.3 mM) and 54 μl of sodium acetate (0.25 M, pH 5). Six (6) μl of NP1 cocktail were added to the hydrolyzed samples. The samples were mixed, spun down and incubated for forty minutes at 37°C .

In order for the DNA adducts to be effectively labeled, ^{32}P had to undergo ATP synthesis [27]. A ^{32}P cocktail was made consisting of 27 μl of PNK buffer, a calculated amount of the synthesized ^{32}P -ATP, bicene and 5.4 μl of PNK. Four (4) μl of ^{32}P cocktail were added to the samples. The samples were mixed, spun down and incubated for thirty minutes at 37°C .

Two (2) μl of apyrase were then added to each sample. The samples were once again mixed, spun down and incubated for thirty minutes at 37°C .

Eighteen (18) μ l of each sample was pipetted, or spotted, onto 20 cm x 20 cm cellulose PEI plates. Two samples (the split A and B) were spotted on each 20x20 plate or chromatogram. A filter paper wick was stapled to each 20x20 plate. The plates were then placed into a tank containing D1 solvent (0.65 M sodium phosphate, pH 6.0) and allowed to run up overnight.

The following day the 20x20 chromatograms were then cut into 10x10 chromatograms—each one containing one sample. The 10x10 chromatograms were then washed and air-dried. For the first nine experiments completed (BAM1-BAM9), washing was conducted by rapidly moving the chromatograms back and forth in a tub of tap water for approximately 2 minutes. Due to problems with excessive background, this procedure was modified. For the last twenty-one experiments (BAM10-BAM30), washing was conducted by soaking the chromatograms in de-ionized water and by running a stream of de-ionized water over each plate.

The 10x10 chromatograms were then placed into three different solvents. First, they were dipped into de-ionized water and were placed, right-side up, into D3 solvent (3.6 M lithium formate, 8.5 M urea, pH 3.5). After the solvent had worked its way up the chromatograph, the plates were washed, as described previously, and air-dried. The chromatograms were then dipped into de-ionized water and were placed, on their right side, into D4 solvent (0.8 M lithium chloride, 0.5 M tris HCl, 8.5 M urea, pH 8.0). After the solvent had worked its way up the chromatograph, the plates were once again washed and air-dried. A wick was stapled to the left side of each chromatogram and they were then placed, again on their right side, into D5 solvent (1.5 M sodium phosphate, pH 6). Once chromatography in the D5 solvent was completed, the plates were air-dried (not washed) and were labeled and marked using a diluted ^{32}P solution.

The marked plates were placed with film into autoradiography cassettes. The film was allowed to be exposed at -80°C for 24-48 hours. The film was developed and used as a template to circle adducts on each chromatogram.

Circled adducts and controls were cut out of the chromatograms and placed into liquid scintillation vials. Five (5) μl of 70% ethanol was added to each vial. The vial were then placed into either the Tri-Carb Model 1900 CA or the Tri-Carb Model 2200 CA Liquid Scintillation Analyzer. These instruments used Cerenkov counting to determine the radioactivity of each cut-out. Calculation of relative adduct labeling (RAL) was obtained from the following formula:

$$RAL = \frac{\text{Adduct cpm}}{1.25 \times 10^6 \text{ cpm} / \text{pmol(ATP)} \times 3240 \text{ pmol(dNP)} / \mu\text{gDNA}} \times 10^6$$

Arsenic Concentration Analysis

Arsenic levels in lung and distal skin tissues were determined using ICP-mass spectrometry (ICP) following the method reported by Evans *et al.* [13]. The technician performing the arsenic concentration analysis was blinded to the samples. Each skin sample was given a 6 digit random number generated by Microsoft Excel. The samples were sorted by random number and were relabeled accordingly. The same process was done with the lung samples.

Statistical Analysis

Data were initially imported in Microsoft Excel. This program was used to organize and export data and to do basic statistical (t-test) comparisons. SAS was used to do descriptive statistics on the various groups—this included tests for normality. The Shapiro-Wilkes test statistic (W) was used to test for normality for the arsenic concentrations and for adduct levels. The natural logarithms (ln) of this data were also examined using this test. Both arsenic concentrations and adduct levels fit a lognormal distribution better, and correlations were done

using natural log transformed data (Appendices B-E). SAS was also used to perform ANOVA and Two-Factor Analyses required to evaluate null hypotheses 1 and 2. Box plots, scatter plots and correlations were done using SigmaPlot. The boundaries of the box on each box plot represents the 25th and 75th percentiles. The median is shown as a line inside the box. Whiskers represent the 90th and 10th percentiles and all outliers are displayed as dots. The scatter plots show a regression line and the 95 percent confidence intervals of that line. The scatter plots and correlations generated in SigmaPlot were used to evaluate hypotheses 3 through 5.

Results

Animal Group Comparisons

No significant difference was observed between animals receiving arsenic treatments and those not receiving arsenic treatments. Mean weights of the animals at sacrifice were very similar—a two-sided t-test returned a p-value of 0.72 (see Table 2). Cage weights were monitored. The mean change in cage weights from initial treatment to sacrifice was not significantly different between arsenic-treated animals and non-arsenic-treated animals (see Table 3).

Table 2: Comparison of weight at sacrifice

As Treatment	Mean (g)	Standard Deviation	P-value
No Arsenic	17.18	1.03	0.72
Arsenic	17.09	0.98	

Table 3: Comparison of total weight change

As Treatment	Mean (g)	Standard Deviation	P-value
No Arsenic	1.14	0.27	0.37
Arsenic	1.28	0.28	

Water consumption was also measured and tracked per cage. Total water consumed by arsenic and non-arsenic treated cages was not statistically different (Table 4), nor was water consumed by the different groups per animal per day (Table 5).

Table 4: Comparison of total water consumed

As Treatment	Mean (ml)	Standard Deviation	P-value
No Arsenic	416.80	59.34	0.84
Arsenic	422.08	45.05	

Table 5: Comparison of water consumed per animal per day

As Treatment	Mean (ml)	Standard Deviation	P-value
No Arsenic	17.33	1.44	0.63
Arsenic	16.88	1.80	

DNA Adducts

Figures 2 and 3 are included here as representative autoradiograms for skin and lung samples respectively. Four adducts were readily visible for most skin samples, where as only one adduct was found in the lung samples. Adduct 1 was found in the same place for both skin and lung samples—approximately 1-2 millimeters up and to the right of the point of origin. Adduct 2 was found as a tail from adduct 1. Adducts 3 and 4 were found further up and to the

right with respect to adducts 1 and 2. This pattern follows reported patterns for BaP-adducts [13, 28].

Figure 3: Representative autoradiogram for skin sample

Figure 4: Representative autoradiogram for lung sample

A two factor multivariate analysis was used to compare all treatment groups. As expected, controls were significantly different than treated animals. Without the control animals, this analysis showed no statistical significance between treatment groups (see Appendix H).

Null hypothesis #1 was examined by using analysis of variance (ANOVA) to compare skin RAL and lung RAL among the four different 100 nM BaP treatment groups (A1, A2, A3 and A4). Figure 4 shows the box plot of RAL in skin per treatment group and Figure 5 shows the box plot of RAL in lung per treatment group. No significant differences were observed among adducts in the skin tissues—an F-value of 0.90 was calculated (see Appendix F).

Figure 5: Comparisons of adducts in skin for 100 nM BaP treatment groups

Figure 6: Comparisons of adducts in lung for 100 nM BaP treatment groups

A significant difference was observed in the lung tissues (F-value of 2.19), but this trend was opposite of what was expected. Adducts in the lung tissues of animals receiving no arsenic (group A1) were significantly higher than those receiving arsenic treatments. Based on this observation, null hypothesis #1 was rejected for adducts in the lung, but the trend was counterintuitive.

Null hypothesis #2 was examined by comparing skin RAL and lung RAL among treatment groups B1, B2, B3 and B4. Respectively, Figures 7 and 8 show the box plots of RAL in skin per treatment group and the RAL in lung per treatment group. ANOVA was used to compare means returning F-values of 0.95 and 1.12 respectively. No significant differences were observed between groups, although the trend was more inline with theoretical expectations—particularly in the lung. Based on this data, null hypothesis #2 was not rejected.

Figure 7: Comparisons of adducts in skin for 200 nM BaP treatment groups

Figure 8: Comparisons of adducts in lung for 200 nM BaP treatment groups

Arsenic data

Water for treatment groups was tested using ICP. The target doses were 2.0 mg/L, 4 mg/L and 8 mg/L. The actual measurements (mean) were 3.7 mg/L, 4.3 mg/L and 10.0 mg/L,

respectively. The arsenic doses administered to the 2 mg/L and the 4 mg/L treatment groups were actually very close together, as shown in Table 6. Blank milli-Q water had non-detectable limits of arsenic—these blanks were taken from the same source from which water was given to groups C, A1 and B1.

Arsenic concentrations measured by ICP in the skin and lung followed an expected upward trend. One notable exception was the high concentration found in the lung samples of the control groups (Table 7). This data was influenced by three particularly high values in group A1. These values triggered the need to retest certain samples (the outcomes of this retest will be discussed in the discussion section of this thesis). The raw data can be further examined in Appendix A.

Table 6: Actual arsenic concentration of water administered to treatment groups

Water Samples from Treatment Groups	As Concentration (mg/L)
2 mg/L (A2, B2)	3.6
2 mg/L (A2, B2)	3.8
4 mg/L (A3, B3)	4.4
4 mg/L (A3, B3)	4.2
8 mg/L (A4, B4)	11.0
8 mg/L (A4, B4)	9.0
Blank	0.0
Blank	0.0

Table 7: Average arsenic concentrations in the skin and lung samples of different treatment groups

As Dose	Average As Conc in Skin (ng/g)	Average As Conc in Lung (ng/g)
Control (C, A1, B1)	18.376	58.756
2 mg/L (A2, B2)	27.029	47.618
4 mg/L (A3, B3)	62.750	101.161
8 mg/L (A4, B4)	130.302	172.454

Arsenic data vs. adducts

Null hypothesis #3 was tested evaluating the correlation between RAL in skin and ICP arsenic concentration for both the 100 nM and 200 nM BaP treatment groups. Figure 9 illustrates this relationship in skin for the 100 nM treatment group ($r^2 = 6.63 \times 10^{-4}$). Figure 10 graphs this relationship in skin for the 200 nM treatment group ($r^2 = 0.028$). Neither of these relationships was deemed to be significant, thus null hypothesis # 3 had to be accepted.

Figure 9: $\ln [\text{As}]$ vs. $\ln (\text{RAL in skin})$ for 100 nM BaP treatment groups

Figure 10: $\ln [\text{As}]$ vs. $\ln (\text{RAL in skin})$ for 200 nM BaP treatment groups

Null hypothesis #4 was evaluated by examining the correlation between RAL in lung and ICP arsenic concentration for both the 100 nM and 200 nM BaP treatment groups. A correlation of $r^2 = 3.09 \times 10^{-3}$ was found in the 100 nM treatment group (Figure 11). The scatter plot for the

200 nM BaP treatment group (Figure 12) was also inconclusive with an r^2 of 5.42×10^{-3} . Null hypotheses #4 was not rejected.

Figure 11: ln [As] vs. ln (RAL in lung) for 100 nM BaP treatment groups

Figure 12: ln [As] vs. ln (RAL in lung) for 200 nM BaP treatment groups

Lung RAL vs. Skin RAL

For both BaP treatment groups, the RAL in lung was compared to the RAL in skin.

Figures 13 and 14 show the scatter plots depicting these relationships. The correlations of these two figures— $r^2 = 6.62 \times 10^{-3}$ and $r^2 = 0.022$, respectively—were inconclusive and based on that fact null hypothesis #5 was not rejected.

Figure 13: ln (RAL in skin) vs. ln (RAL in lung) for 100 nM BaP treatment groups

Figure 14: $\ln(\text{RAL in skin})$ vs. $\ln(\text{RAL in lung})$ for 200 nM BaP treatment groups

Discussion

Animal data comparisons demonstrated that the animals were not adversely affected by their treatments in terms of weight gain or water consumption. No differences were noted with respect to these two variables. No behavioral differences were observed between treatment groups, also indicating that the low doses did not produce an overt toxic response in the mice.

Topical applications of BaP presented a potential source of error, as it can be somewhat difficult to apply via pipette. Great care was taken to ensure BaP was applied to the bare skin. A complete miss only took place once, and the animal was retreated immediately. Care was also taken to reduce evaporation of the BaP/acetone solution. Despite these precautions, both the 100 nM and the 200 nM solutions had to be remade prior to the last treatment. This could have been due to evaporation and the animals, subsequently, could have been administered higher concentrated treatments. Concern for the solution damming against the border of the shaved skin did not seem to be warranted because the 25 μ l treatments were quickly absorbed into the skin. As an extra precaution, extracted DNA was taken from skin tissues removed from the center of the sample.

The most notable discrepancies in the study involved the arsenic treatment regimen and the ICP measurements of tissues. The arsenic treatments were intended to be 0 mg/L, 2 mg/L, 4 mg/L and 8 mg/L. The actual measurements were 0 mg/L, 3.7 mg/L, 4.3 mg/L and 10.0 mg/L, respectively. Due to the fact that the 2mg/L and 4mg/L doses were actually very similar to one another, no differences in adduct levels between these two groups were expected. And, in fact, these two treatments did yield very similar results.

Discrepancies in ICP measurements in tissue were somewhat inexplicable. Three values in control animals were very high and prompted the need to retest a number of tissues. Thirty (30)

tissues were retested, and the results were very different (p-value was 8.05×10^{-5} using t-test—see Table 8). This finding puts all of the ICP measurements into question, and methods were revisited to find sources of error. The most probable confounder is the presence of chlorides in the mouse tissue. During ICP analysis these chlorides may mix with argon to form argon chloride (ArCl). ArCl has the same mass as arsenic and could cause interference. To account for this in the future, known blank tissues should be sent first for calibration purposes. Split samples should also be sent for analysis in order to check for agreement.

Table 8: Comparison ICP measurements in same tissues

Run	Mean (ng/g)	Standard Deviation	P-Value
1st	57.88	73.07	8.05E-05
2nd	154.78	78.21	

The presence of BaP adducts was apparent in all groups but the control group (C) indicating that metabolic activation occurred *in vivo* in response to BaP treatments. As previously stated, the adduct pattern was similar to those reported in previous studies [13, 28].

For the most part, the adduct data supported null hypotheses 1 & 2 suggesting that arsenic treatment does not effect BaP adduct levels in skin and lung tissues. General trends did show higher adduct levels in the 8mg/L arsenic treatment groups than in the 2 mg/L and 4 mg/L treatment groups. This trend was slightly more evident in the 200 nM BaP treated animals. A possible reason for this may have been due to a more uniform uptake and metabolism of BaP in this higher treatment group, however, other studies have had little problem seeing a uniform response in treatments lower than 100 nM [28].

A surprising observation was that the non-arsenic treated animals had higher levels than the 2 mg/L and 4 mg/L arsenic groups. This directly contradicts the findings reported from Evans *et al* [13], which saw a two fold increase in adducts from the 0 mg/L group to the 2 mg/L group in 200 nM BaP treated animals. One of the main differences between these two studies was the time the animals were treated with arsenic prior to treatment to BaP. Evans provided sodium arsenite in drinking water for 9 days prior to his BaP treatments. Here the animals were provided sodium arsenite for 21 days prior to BaP treatments.

Longer arsenic treatments were supposed to improve the experimental results by allowing arsenic levels to reach a uniform and steady state. Turning our attention once again to Figure 1, the two main point of arsenic interaction involve detoxification and DNA repair. These interactions are dependent on the types of arsenic species present. The initial detoxification mechanism of sodium arsenite is methylation to both pentavalent and trivalent forms of methylarsonic acid (MMA) and dimethylarsinic acid (DMA), of which the trivalent forms are more toxic [11, 17]. Of these metabolites, MMA(III) and DMA(III) have been shown to effect adduct formation and repair mechanisms greater than MMA(V) and DMA(V) [29]. Animals undergoing longer treatments may have different amounts of these metabolites, and may in turn respond differently accounting for the differences in these two studies, however physiologically based pharmacokinetic modeling does not support this idea showing steady state for all forms at approximately 96 hours [30].

In addition to the initial detoxification of arsenic via methylation, GSH and related enzymes also play a role in metabolizing arsenic species [11] and in dealing with oxidative stress caused by methylation [18]. *In vitro*, arsenic has been shown to stimulate glutathione levels [18]. This may partially explain the reason for higher observed adduct levels among the non-arsenic treated

animals (groups A1 and B1). Those animals in treatment groups A2, A3, B2 and B3 may have had higher levels of cellular glutathione, and this would have helped them to metabolize and detoxify the BaP treatments resulting in lower RAL.

Because more treatment groups were used in this study, the statistical power per group was lower than in the Evans study. Here each group had 10 animals. Fifteen (15) animals were used by Evans. Not all postlabelling experiments were duplicated in this study, whereas Evans duplicated each experiment. Had these experiments been duplicated (or triplicated), the variability seen in the treatment groups would have been reduced.

The ^{32}P -postlabeling method is difficult to master, and there is good reason to believe that the experiments done towards the end of this study were better than those done at the beginning. As mentioned in the methods section, there was a major change in plate washing technique that occurred after experiment BAM9. After BAM9 the plates were cleaner and the RAL were lower. Although analysis of the data without experiments BAM1-BAM8 has shown no drastic changes to the outcome of the study, this may have warranted the need to redo the samples analyzed at the beginning of the postlabelling process.

Based on the inconsistencies in ICP measurements, it is not surprising that no correlation was observed between adduct levels and arsenic concentration in the skin and lung tissues. Null hypotheses 3&4 were therefore accepted and the relationships here remain in question. This data does support the findings outlined in the discussion of hypotheses 1&2 and the same hindsight is also applicable here.

Null hypothesis #5 was that RAL in Lung would correlate with RAL in Skin. This correlation was not observed to be significant by Evans *et al* [13], and was not observed to be significant here.

Conclusion

The study was decidedly inconclusive with respect to the possible synergistic interactions between arsenic and BaP in the mouse model. None of the null hypotheses were rejected. For both the 100 nM and the 200 nM BaP treatment groups, there were no significant changes of note between the four arsenic treatment groups. RAL was not significantly correlated with ICP concentration for both skin and lung tissues. There was also no significant relationship between skin RAL and lung RAL.

The results of this study demonstrate the complexity of the problem in question. Assuming that the carcinogenic mechanisms of BaP are fairly straightforward, the role of arsenic must be great and varied. The two most probable points of arsenic interaction include competition with BaP for detoxification pathways and interference with DNA repair. Different species of arsenic may have different effects on these two points of interaction. The metabolism and uptake of arsenic, therefore, plays an important role and must be considered in future studies.

Besides determining the speciation of arsenic in tissues, the potential interference of ArCl must be addressed. Another possible improvement to the study design is to use a broader range of arsenic doses so that the treatment groups are more distinct. Future studies should also focus on a single BaP treatment group. Not much was gained here by examining the 100 nM treatment group and this study would have been better served by increasing the numbers of animals in each arsenic treatment group.

The evaluation of mixtures is a very complex process. Experiments using a mouse model may not translate well to the actual human environmental and occupational exposure events. To that end, steps must be taken to make such extrapolations.

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Appendix A: Summary Data

Summary Data

Animal	As Dose (mg/L)	BaP Dose (nM)	Total Adducts-Skin (RAL)	As-Skin (ng/g)	Total Adducts- Lung (RAL)	As-Lung (ng/g)
A1-1	.	100	332.8	18.2	3.8	202.1
A1-2	.	100	204.9	0.0	7.4	7.9
A1-3	.	100	78.0	59.8	1.4	76.9
A1-4	.	100	119.0	22.8	6.1	47.9
A1-5	.	100	177.7	14.9	1.0	240.3
A1-6	.	100	721.3	26.4	0.4	50.6
A1-7	.	100	105.5	0.0	2.3	282.1
A1-8	.	100	187.7	66.7	2.0	36.2
A1-9	.	100	292.3	51.2	3.6	0.0
A1-10	.	100	180.0	0.0	6.7	0.0
A2-1	2	100	313.5	47.8	2.4	49.8
A2-2	2	100	211.9	14.9	0.6	0.0
A2-3	2	100	237.3	0.0	0.8	122.0
A2-4	2	100	115.8	0.0	0.7	113.1
A2-5	2	100	32.9	15.5	0.2	0.0
A2-6	2	100	406.5	22.4	0.3	53.8
A2-7	2	100	67.4	26.0	2.9	82.8
A2-8	2	100	318.6	0.0	0.5	49.0
A2-9	2	100	185.0	39.1	0.3	70.7
A2-10	2	100	245.5	0.0	6.9	4.8
A3-1	4	100	205.0	25.2	5.0	67.4
A3-2	4	100	48.3	27.5	1.8	59.1
A3-3	4	100	109.5	55.8	2.6	24.8
A3-4	4	100	108.7	130.6	0.2	108.5
A3-5	4	100	147.8	84.2	1.5	179.2
A3-6	4	100	148.1	13.6	2.7	183.9
A3-7	4	100	394.8	36.6	0.6	153.5
A3-8	4	100	7.1	41.0	0.6	20.1
A3-9	4	100	234.4	49.8	0.3	192.6
A3-10	4	100	221.6	30.4	3.2	173.1
A4-1	8	100	144.0	163.0	3.4	479.7
A4-2	8	100	143.9	126.8	1.3	180.6
A4-3	8	100	256.2	366.7	0.8	155.4
A4-4	8	100	143.8	314.1	0.9	126.4
A4-5	8	100	110.6	45.8	1.2	108.1
A4-6	8	100	166.7	48.2	3.6	41.7
A4-7	8	100	91.0	66.0	1.7	173.9
A4-8	8	100	218.1	54.5	2.4	80.0
A4-9	8	100	186.0	185.1	0.8	200.0
A4-10	8	100	195.2	73.6	1.7	302.3

B1-1	.	200	141.1	0.0	4.2	53.9
B1-2	.	200	176.4	8.0	1.0	25.3
B1-3	.	200	215.3	3.9	3.3	0.0
B1-4	.	200	182.4	45.3	2.7	65.6
B1-5	.	200	158.8	6.8	6.7	51.8
B1-6	.	200	99.4	0.0	2.8	50.0
B1-7	.	200	105.4	26.1	0.4	0.0
B1-8	.	200	108.2	26.2	1.6	0.0
B1-9	.	200	205.8	28.8	2.2	0.0
B1-10	.	200	131.5	0.0	1.5	0.0
B2-1	2	200	86.9	36.8	1.0	0.0
B2-2	2	200	109.1	37.5	2.5	15.3
B2-3	2	200	42.0	44.4	9.5	0.0
B2-4	2	200	78.0	34.7	0.5	108.9
B2-5	2	200	109.0	0.0	0.8	29.4
B2-6	2	200	468.4	28.7	0.2	0.0
B2-7	2	200	185.2	79.6	15.8	67.5
B2-8	2	200	169.9	19.4	1.3	35.5
B2-9	2	200	129.5	45.1	1.3	150.0
B2-10	2	200	377.8	48.5	1.8	0.0
B3-1	4	200	227.0	59.3	1.6	0.0
B3-2	4	200	75.7	48.3	1.2	141.8
B3-3	4	200	86.2	39.1	5.1	23.8
B3-4	4	200	131.5	246.5	3.5	58.8
B3-5	4	200	79.1	50.2	1.4	0.0
B3-6	4	200	311.6	49.9	0.4	0.0
B3-7	4	200	175.2	0.0	2.3	0.0
B3-8	4	200	100.2	66.3	3.0	17.2
B3-9	4	200	247.0	0.0	0.1	80.0
B3-10	4	200	98.8	14.4	1.2	53.1
B4-1	8	200	118.5	148.9	10.2	209.0
B4-2	8	200	157.0	106.4	8.1	104.9
B4-3	8	200	125.9	254.2	1.2	25.4
B4-4	8	200	377.2	120.8	1.5	67.0
B4-5	8	200	74.4	129.1	0.5	561.4
B4-6	8	200	377.4	140.8	12.6	250.0
B4-7	8	200	257.9	65.5	5.0	437.0
B4-8	8	200	241.1	84.8	4.5	160.6
B4-9	8	200	105.5	96.8	3.7	67.2
B4-10	8	200	366.5	66.5	0.2	62.3
C-1	.	.	0.2	0.0	0.2	48.1
C-2	.	.	-0.7	0.0	0.3	126.3
C-3	.	.	-0.3	15.4	-4.3	37.5
C-4	.	.	1.8	20.5	0.3	7.5

Animal Group	As Dose (mg/L)	BaP Dose (nM)	Total Adducts-Skin (RAL)	As-Skin (ng/g)	Total Adducts-Lung (RAL)	As-Lung (ng/g)
A1	.	100	239.9	26.0	3.5	94.4
A2	2	100	213.4	16.6	1.6	54.6
A3	4	100	162.5	49.5	1.9	116.2
A4	8	100	165.6	144.4	1.8	184.8
B1	.	200	152.4	14.5	2.6	24.7
B2	2	200	175.6	37.5	3.5	40.7
B3	4	200	153.2	57.4	2.0	37.5
B4	8	200	220.1	121.4	4.7	194.5
C	.	.	0.3	9.0	-0.9	54.9

Appendix B: Descriptive statistics for adducts in skin for all treatment groups

Descriptive statistics for adducts in skin for all treatment groups

Group	C	A1	A2	A3	A4	B1	B2	B3	B4
Skin Adducts (RAL)	0.2	332.8	313.5	205.0	144.0	141.1	86.9	227.0	118.5
	-0.7	204.9	211.9	48.3	143.9	176.4	109.1	75.7	157.0
	-0.3	78.0	237.3	109.5	256.2	215.3	42.0	86.2	125.9
	1.8	119.0	115.8	108.7	143.8	182.4	78.0	131.5	377.2
		177.7	32.9	147.8	110.6	158.8	109.0	79.1	74.4
		721.3	406.5	148.1	166.7	99.4	468.4	311.6	377.4
		105.5	67.4	394.8	91.0	105.4	185.2	175.2	257.9
		187.7	318.6	7.1	218.1	108.2	169.9	100.2	241.1
		292.3	185.0	234.4	186.0	205.8	129.5	247.0	105.5
		180.0	245.5	221.6	195.2	131.5	377.8	98.8	366.5
N	4	10	10	10	10	10	10	10	10
Mean	0.250	239.920	213.438	162.531	165.550	152.424	175.584	153.236	220.127
Median	-0.050	183.850	224.583	147.950	155.350	149.950	119.300	115.861	199.008
Std Deviation	1.097	186.613	117.476	109.418	49.734	41.979	138.631	83.032	120.313
Variance	1.2	34824.5	13800.7	11972.3	2473.4	1762.2	19218.6	6894.3	14475.2
Kurtosis	1.873	5.567	-0.647	1.329	-0.174	-1.408	1.297	-0.484	-1.728
Skewness	1.364	2.210	-0.047	0.809	0.354	0.171	1.495	0.901	0.337
Shapiro-Wilkes (W)	0.899	0.749	0.974	0.948	0.974	0.933	0.803	0.863	0.866
In (Skin Adducts (RAL))	-1.6	5.8	5.7	5.3	5.0	4.9	4.5	5.4	4.8
		5.3	5.4	3.9	5.0	5.2	4.7	4.3	5.1
		4.4	5.5	4.7	5.5	5.4	3.7	4.5	4.8
	0.6	4.8	4.8	4.7	5.0	5.2	4.4	4.9	5.9
		5.2	3.5	5.0	4.7	5.1	4.7	4.4	4.3
		6.6	6.0	5.0	5.1	4.6	6.1	5.7	5.9
		4.7	4.2	6.0	4.5	4.7	5.2	5.2	5.6
		5.2	5.8	2.0	5.4	4.7	5.1	4.6	5.5
		5.7	5.2	5.5	5.2	5.3	4.9	5.5	4.7
		5.2	5.5	5.4	5.3	4.9	5.9	4.6	5.9
N	2	10	10	10	10	10	10	10	10
Mean	-0.511	5.279	5.152	4.738	5.067	4.991	4.925	4.908	5.244
Median	-0.511	5.214	5.413	4.997	5.043	5.009	4.778	4.743	5.271
Std Deviation	1.554	0.636	0.784	1.127	0.311	0.282	0.723	0.518	0.594
Variance	2.414	0.404	0.614	1.270	0.097	0.080	0.523	0.268	0.353
Kurtosis	#DIV/0!	0.879	0.990	4.183	-0.169	-1.512	0.000	-1.458	-1.518
Skewness	#DIV/0!	0.684	-1.245	-1.867	-0.311	-0.117	0.339	0.454	-0.140
Shapiro-Wilkes (W)	1.000	0.956	0.880	0.816	0.974	0.930	0.960	0.902	0.908

Appendix C: Descriptive statistics for adducts in lung for all treatment groups

Descriptive statistics for adducts in lung for all treatment groups

Group	C	A1	A2	A3	A4	B1	B2	B3	B4
Lung Adducts (RAL)	0.2	3.8	2.4	5.0	3.4	4.2	1.0	1.6	10.2
	0.3	7.4	0.6	1.8	1.3	1.0	2.5	1.2	8.1
	-4.3	1.4	0.8	2.6	0.8	3.3	9.5	5.1	1.2
	0.3	6.1	0.7	0.2	0.9	2.7	0.5	3.5	1.5
		1.0	0.2	1.5	1.2	6.7	0.8	1.4	0.5
		0.4	0.3	2.7	3.6	2.8	0.2	0.4	12.6
		2.3	2.9	0.6	1.7	0.4	15.8	2.3	5.0
		2.0	0.5	0.6	2.4	1.6	1.3	3.0	4.5
		3.6	0.3	0.3	0.8	2.2	1.3	0.1	3.7
		6.7	6.9	3.2	1.7	1.5	1.8	1.2	0.2
N	4	10	10	10	10	10	10	10	10
Mean	-0.852	3.462	1.559	1.854	1.780	2.643	3.475	1.994	4.740
Median	0.260	2.939	0.643	1.676	1.511	2.446	1.314	1.511	4.098
Std Deviation	2.273	2.489	2.097	1.526	1.025	1.810	5.116	1.514	4.284
Variance	5.2	6.2	4.4	2.3	1.1	3.3	26.2	2.3	18.4
Kurtosis	3.985	-1.323	5.063	0.280	-0.404	2.015	3.630	0.647	-0.567
Skewness	-1.996	0.487	2.202	0.839	0.950	1.241	2.061	0.949	0.762
Shapiro-Wilkes (W)	0.651	0.911	0.687	0.912	0.860	0.917	0.655	0.930	0.905
In (Lung Adducts (RAL))	-1.6	1.3	0.9	1.6	1.2	1.4	0.0	0.5	2.3
	-1.1	2.0	-0.5	0.6	0.3	0.0	0.9	0.2	2.1
		0.3	-0.2	0.9	-0.3	1.2	2.3	1.6	0.2
	-1.1	1.8	-0.4	-1.4	-0.1	1.0	-0.8	1.2	0.4
		0.0	-1.8	0.4	0.2	1.9	-0.2	0.3	-0.8
		-0.9	-1.2	1.0	1.3	1.0	-1.7	-0.8	2.5
		0.8	1.1	-0.5	0.5	-0.9	2.8	0.8	1.6
		0.7	-0.7	-0.5	0.9	0.5	0.3	1.1	1.5
		1.3	-1.2	-1.2	-0.2	0.8	0.3	-1.9	1.3
		1.9	1.9	1.2	0.6	0.4	0.6	0.2	-1.5
N	3	10	10	10	10	10	10	10	10
Mean	-1.288	0.931	-0.207	0.207	0.435	0.736	0.439	0.329	0.965
Median	-1.124	1.056	-0.443	0.512	0.403	0.891	0.273	0.410	1.405
Std Deviation	0.298	0.919	1.161	1.055	0.556	0.782	1.320	1.043	1.356
Variance	0.089	0.845	1.348	1.113	0.309	0.611	1.742	1.087	1.839
Kurtosis	#DIV/0!	-0.073	-0.359	-1.237	-1.165	1.067	0.263	1.386	-0.483
Skewness	-1.726	-0.688	0.634	-0.430	0.335	-0.749	0.394	-1.125	-0.737
Shapiro-Wilkes (W)	0.774	0.939	0.942	0.924	0.934	0.965	0.956	0.997	0.925

Appendix D: Descriptive statistics for arsenic concentration in skin (all treatment groups)

Descriptive statistics for arsenic concentration in skin (all treatment groups)

As Dose	0 mg			2 mg		4 mg		8 mg	
As Conc in Skin (ng/g)	0.0	18.2	0.0	47.8	36.8	25.2	59.3	163.0	148.9
	0.0	0.0	8.0	14.9	37.5	27.5	48.3	126.8	106.4
	15.4	59.8	3.9	0.0	44.4	55.8	39.1	366.7	254.2
	20.5	22.8	45.3	0.0	34.7	130.6	246.5	314.1	120.8
		14.9	6.8	15.5	0.0	84.2	50.2	45.8	129.1
		26.4	0.0	22.4	28.7	13.6	49.9	48.2	140.8
		0.0	26.1	26.0	79.6	36.6	0.0	66.0	65.5
		66.7	26.2	0.0	19.4	41.0	66.3	54.5	84.8
		51.2	28.8	39.1	45.1	49.8	0.0	185.1	96.8
		0.0	0.0	0.0	48.5	30.4	14.4	73.6	66.5
N	24			20		20		20	
Mean	18.376			27.029		53.430		132.867	
Median	15.155			27.383		44.667		113.621	
Std Deviation	20.155			21.423		54.237		88.422	
Variance	406.2			459.0		2941.6		7818.4	
Kurtosis	0.358			0.240		8.548		1.802	
Skewness	1.077			0.460		2.637		1.494	
Shapiro- Wilkes (W)	0.846			0.923		0.720		0.835	
ln (As Conc in Skin (ng/g))		2.9		3.9	3.6	3.2	4.1	5.1	5.0
			2.1	2.7	3.6	3.3	3.9	4.8	4.7
	2.7	4.1	1.4		3.8	4.0	3.7	5.9	5.5
	3.0	3.1	3.8		3.5	4.9	5.5	5.7	4.8
		2.7	1.9	2.7		4.4	3.9	3.8	4.9
		3.3		3.1	3.4	2.6	3.9	3.9	4.9
			3.3	3.3	4.4	3.6		4.2	4.2
		4.2	3.3		3.0	3.7	4.2	4.0	4.4
		3.9	3.4	3.7	3.8	3.9		5.2	4.6
					3.9	3.4	2.7	4.3	4.2
N	16			15		18		20	
Mean	3.066			3.487		3.830		4.710	
Median	3.194			3.606		3.893		4.731	
Std Deviation	0.788			0.465		0.696		0.601	
Variance	0.621			0.216		0.485		0.361	
Kurtosis	0.090			-0.256		1.197		-0.549	
Skewness	-0.592			-0.183		0.456		0.398	
Shapiro- Wilkes (W)	0.951			0.957		0.954		0.961	

Appendix E: Descriptive statistics for arsenic concentration in lung (all treatment groups)

Descriptive statistics for arsenic concentration in lung (all treatment groups)

As Dose	0 mg			2 mg		4 mg		8 mg	
As Conc in Lung (ng/g)	48.1	202.1	53.9	49.8	0.0	67.4	0.0	479.7	209.0
	126.3	7.9	25.3	0.0	15.3	59.1	141.8	180.6	104.9
	37.5	76.9	0.0	122.0	0.0	24.8	23.8	155.4	25.4
	7.5	47.9	65.6	113.1	108.9	108.5	58.8	126.4	67.0
		240.3	51.8	0.0	29.4	179.2	0.0	108.1	561.4
		50.6	50.0	53.8	0.0	183.9	0.0	41.7	250.0
		282.1	0.0	82.8	67.5	153.5	0.0	173.9	437.0
		36.2	0.0	49.0	35.5	20.1	17.2	80.0	160.6
		0.0	0.0	70.7	150.0	192.6	80.0	200.0	67.2
		0.0	0.0	4.8	0.0	173.1	53.1	302.3	62.3
N	24			20		20		20	
Mean	58.756			47.618		76.845		189.640	
Median	42.723			42.227		58.957		157.978	
Std Deviation	78.074			47.696		69.927		150.025	
Variance	6095.5			2274.9		4889.8		22507.6	
Kurtosis	2.816			-0.604		-1.310		1.063	
Skewness	1.856			0.695		0.508		1.322	
Shapiro- Wilkes (W)	0.731			0.883		0.872		0.854	
ln (As Conc in Lung (ng/g))	3.9	5.3	4.0	3.9		4.2		6.2	5.3
	4.8	2.1	3.2		2.7	4.1	5.0	5.2	4.7
	3.6	4.3		4.8		3.2	3.2	5.0	3.2
	2.0	3.9	4.2	4.7	4.7	4.7	4.1	4.8	4.2
		5.5	3.9		3.4	5.2		4.7	6.3
		3.9	3.9	4.0		5.2		3.7	5.5
		5.6		4.4	4.2	5.0		5.2	6.1
		3.6		3.9	3.6	3.0	2.8	4.4	5.1
				4.3	5.0	5.3	4.4	5.3	4.2
				1.6		5.2	4.0	5.7	4.1
N	17			14		16		20	
Mean	3.991			3.939		4.277		4.950	
Median	3.925			4.099		4.296		5.062	
Std Deviation	1.002			0.923		0.851		0.817	
Variance	1.004			0.851		0.725		0.668	
Kurtosis	0.416			2.390		-1.205		-0.294	
Skewness	-0.321			-1.427		-0.446		-0.194	
Shapiro- Wilkes (W)	0.921			0.885		0.893		0.981	

Appendix F: SAS Printout of ANOVA for adducts in 100 nM treatment groups (A1, A2, A3 & A4)

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treatment	4	A1 A2 A3 A4

Number of observations	40
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The ANOVA Procedure

Dependent Variable: Adducts_Skin Adducts_Skin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	42787.4797	14262.4932	0.90	0.4485
Error	36	567638.3517	15767.7320		
Corrected Total	39	610425.8314			

R-Square	Coeff Var	Root MSE	Adducts_Skin Mean
0.070094	64.27612	125.5696	195.3597

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	3	42787.47973	14262.49324	0.90	0.4485

The ANOVA Procedure

Level of Treatment	N	-----Adducts_Skin----- Mean	Std Dev
A1	10	239.920000	186.613265
A2	10	213.437500	117.476252
A3	10	162.531319	109.418037
A4	10	165.550000	49.733696

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treatment	4	A1 A2 A3 A4
Number of observations		40

The ANOVA Procedure

Dependent Variable: Adducts_Lung

Adducts_Lung

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	22.9480536	7.6493512	2.19	0.1060
Error	36	125.7198038	3.4922168		
Corrected Total	39	148.6678573			

R-Square	Coeff Var	Root MSE	Adducts_Lung Mean
0.154358	86.36034	1.868747	2.163895

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	3	22.94805356	7.64935119	2.19	0.1060

The ANOVA Procedure

Level of Treatment	N	-----Adducts_Lung----- Mean	Std Dev
A1	10	3.46230126	2.48900802
A2	10	1.55926748	2.09654397
A3	10	1.85380897	1.52577721
A4	10	1.78020402	1.02479923

Appendix G: SAS Printout of ANOVA for adducts in 200 nM treatment groups (B1, B2, B3 & B4)

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treatment	4	B1 B2 B3 B4

Number of observations	40
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The ANOVA Procedure

Dependent Variable: Adducts_Skin Adducts_Skin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	30196.6399	10065.5466	0.95	0.4264
Error	36	381153.1283	10587.5869		
Corrected Total	39	411349.7682			

R-Square	Coeff Var	Root MSE	Adducts_Skin Mean
0.073409	58.68271	102.8960	175.3430

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	3	30196.63991	10065.54664	0.95	0.4264

The ANOVA Procedure

Level of Treatment	N	-----Adducts_Skin----- Mean	Std Dev
B1	10	152.424167	41.978977
B2	10	175.584167	138.631229
B3	10	153.236296	83.032136
B4	10	220.127222	120.312758

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treatment	4	B1 B2 B3 B4

Number of observations 40

The ANOVA Procedure

Dependent Variable: Adducts_Lung

Adducts_Lung

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	42.1136514	14.0378838	1.12	0.3534
Error	36	450.8391750	12.5233104		
Corrected Total	39	492.9528265			

R-Square	Coeff Var	Root MSE	Adducts_Lung Mean
0.085431	110.1531	3.538829	3.212647

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	3	42.11365143	14.03788381	1.12	0.3534

The ANOVA Procedure

Level of Treatment	N	-----Adducts_Lung----- Mean	Std Dev
B1	10	2.64262299	1.80966208
B2	10	3.47484079	5.11561182
B3	10	1.99355154	1.51409124
B4	10	4.73957151	4.28443792

Appendix H: SAS Printout of Two by Two Factor Analysis for adducts in all treatment groups (with and without controls)

The GLM Procedure

Class Level Information

Class	Level s	Val ues
As_Dose	4	0 2 4 8
BaP_Dose	3	0 100 200

Number of observations 84

The GLM Procedure

Dependent Variable: Adducts_Skin Adducts_Skin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	211521.343	26440.168	2.09	0.0472
Error	75	948795.090	12650.601		
Corrected Total	83	1160316.433			

R-Square	Coeff Var	Root MSE	Adducts_Skin Mean
0.182296	63.71180	112.4749	176.5370

Source	DF	Type I SS	Mean Square	F Value	Pr > F
As_Dose	3	22802.4687	7600.8229	0.60	0.6165
BaP_Dose	2	135964.9416	67982.4708	5.37	0.0066
As_Dose*BaP_Dose	3	52753.9326	17584.6442	1.39	0.2524

The GLM Procedure

Dependent Variable: Adducts_Skin Adducts_Skin

Source	DF	Type III SS	Mean Square	F Value	Pr > F
As_Dose	3	20230.1871	6743.3957	0.53	0.6610
BaP_Dose	2	135964.9416	67982.4708	5.37	0.0066
As_Dose*BaP_Dose	3	52753.9326	17584.6442	1.39	0.2524

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The GLM Procedure

Class Level Information

Class	Levels	Values
As_Dose	4	0 2 4 8
BaP_Dose	3	0 100 200

Number of observations 84

The GLM Procedure

Dependent Variable: Adducts_Lung Adducts_Lung

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	134.8139554	16.8517444	2.13	0.0426
Error	75	592.0566573	7.8940888		
Corrected Total	83	726.8706127			

R-Square	Coeff Var	Root MSE	Adducts_Lung Mean
0.185472	111.5082	2.809642	2.519673

Source	DF	Type I SS	Mean Square	F Value	Pr > F
As_Dose	3	18.39683813	6.13227938	0.78	0.5106
BaP_Dose	2	72.82131468	36.41065734	4.61	0.0129
As_Dose*BaP_Dose	3	43.59580261	14.53193420	1.84	0.1470

The GLM Procedure

Dependent Variable: Adducts_Lung Adducts_Lung

Source	DF	Type III SS	Mean Square	F Value	Pr > F
As_Dose	3	21.46590238	7.15530079	0.91	0.4421
BaP_Dose	2	72.82131468	36.41065734	4.61	0.0129
As_Dose*BaP_Dose	3	43.59580261	14.53193420	1.84	0.1470

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The GLM Procedure

Class Level Information

Class	Levels	Values
As_Dose	4	0 2 4 8
BaP_Dose	2	100 200

Number of observations 80

The GLM Procedure

Dependent Variable: Adducts_Skin Adducts_Skin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	80997.519	11571.074	0.88	0.5282
Error	72	948791.480	13177.659		
Corrected Total	79	1029788.999			

R-Square	Coeff Var	Root MSE	Adducts_Skin Mean
0.078654	61.93319	114.7940	185.3513

Source	DF	Type I SS	Mean Square	F Value	Pr > F
As_Dose	3	20230.18709	6743.39570	0.51	0.6755
BaP_Dose	1	8013.39912	8013.39912	0.61	0.4381
As_Dose*BaP_Dose	3	52753.93256	17584.64419	1.33	0.2699

The GLM Procedure

Dependent Variable: Adducts_Skin Adducts_Skin

Source	DF	Type III SS	Mean Square	F Value	Pr > F
As_Dose	3	20230.18709	6743.39570	0.51	0.6755
BaP_Dose	1	8013.39912	8013.39912	0.61	0.4381
As_Dose*BaP_Dose	3	52753.93256	17584.64419	1.33	0.2699

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The GLM Procedure

Class Level Information

Class	Levels	Values
As_Dose	4	0 2 4 8
BaP_Dose	2	100 200

Number of observations 80

The GLM Procedure

Dependent Variable: Adducts_Lung Adducts_Lung

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	87.0592897	12.4370414	1.55	0.1635
Error	72	576.5589788	8.0077636		
Corrected Total	79	663.6182686			

R-Square	Coeff Var	Root MSE	Adducts_Lung Mean
0.131189	105.2647	2.829799	2.688271

Source	DF	Type I SS	Mean Square	F Value	Pr > F
As_Dose	3	21.46590238	7.15530079	0.89	0.4488
BaP_Dose	1	21.99758476	21.99758476	2.75	0.1018
As_Dose*BaP_Dose	3	43.59580261	14.53193420	1.81	0.1521

The GLM Procedure

Dependent Variable: Adducts_Lung Adducts_Lung

Source	DF	Type III SS	Mean Square	F Value	Pr > F
As_Dose	3	21.46590238	7.15530079	0.89	0.4488
BaP_Dose	1	21.99758476	21.99758476	2.75	0.1018
As_Dose*BaP_Dose	3	43.59580261	14.53193420	1.81	0.1521

Appendix I: Cage weights and water consumption summary tables

Comparisons of cage weights among treated animals

Cage	Group	29-Jul	4-Aug	11-Aug	18-Aug	22-Aug
1	C	16.075	16.3	17.4	17.8	17.675
2	A1	15.9	15.8	16.4	16.12	16.82
3	A1	15.26	15.38	15.98	16.46	16.3
4	B1	16.46	16.44	17.14	17.62	17.46
5	B1	16.6	16.7	17.58	17.94	17.74
6	A2	15.62	15.94	16.52	16.84	16.5
7	A2	16.44	16.56	17.16	18.02	17.58
8	B2	15.14	15.78	16.2	16.64	16.86
9	B2	16.06	16.68	16.72	17.76	17.2
10	A3	15.66	16.52	16.9	17.54	17.4
11	A3	14.94	15.54	15.88	16.52	16.46
12	B3	16.46	16.92	17.72	18.06	17.84
13	B3	15.98	16.24	17.06	17.8	17.34
14	A4	15.96	15.76	16.78	17.3	16.96
15	A4	16.38	16.68	17.44	17.48	17.38
16	B4	15.62	16.28	16.64	17.34	16.7
17	B4	15.52	16.28	16.96	17.46	16.88

Water consumed per cage per week

Cage	Group	29-Jul	4-Aug	Water Consumed per Week	11-Aug	Water Consumed per Week	18-Aug	Water Consumed per Week	22-Aug	Water Consumed per Week	Water Consumed per Animal per Day	Total Water Consumed
1	C	250	180	70	137	113	163	87	190	60	16.5	330
2	A1	250	136	114	119	131	119	131	154	96	18.88	472
3	A1	250	156	94	138	112	143	107	178	72	15.4	385
4	B1	250	153	97	120	130	122	128	172	78	17.32	433
5	B1	250	150	100	100	150	119	131	167	83	18.56	464
6	A2	250	154	96	126	124	119	131	170	80	17.24	431
7	A2	250	131	119	92	158	107	143	146	104	20.96	524
8	B2	250	153	97	128	122	135	115	172	78	16.48	412
9	B2	250	124	126	111	139	114	136	165	85	19.44	486
10	A3	250	156	94	142	108	140	110	175	75	15.48	387
11	A3	250	162	88	136	114	148	102	178	72	15.04	376
12	B3	250	153	97	131	119	140	110	167	83	16.36	409
13	B3	250	133	117	116	134	127	123	168	82	18.24	456
14	A4	250	166	84	140	110	140	110	170	80	15.36	384
15	A4	250	152	98	134	116	140	110	175	75	15.96	399
16	B4	250	156	94	134	116	140	110	176	74	15.76	394
17	B4	250	148	102	124	126	148	102	173	77	16.28	407