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14. ABSTRACT

This work supports the U.S. Army Medical Research and Materiel Command's (USAMRMC) Biotechnology High Performance Computing Software Applications Institute (BHSAI) in its efforts to construct predictive models to assess liver, kidney and heart damages of chemical toxicants and drugs. To validate the platform, metabolic changes induced by toxicant exposure is supposed to be experimentally identified from the comprehensive analysis of in vivo transcriptomic data collected from the liver, kidney, heart, plasma, and urine of animals. To translate the results obtained in animal models to human, multiple species of animals, rats and guinea pigs, were used. To dissect the primary from the secondary transcriptomic response of liver, kidney and heart cells, resulted from altered metabolism of other organs/tissues, to toxicant exposure, transcriptomic analysis was performed on primary hepatocytes, renal tubular epithelial cells, cardiac myocytes originated from rats. We examined toxic effect of mercury chloride and thioacetamide.

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INTRODUCTION

This work supports the U.S. Army Medical Research and Materiel Command's (USAMRMC) Biotechnology High Performance Computing Software Applications Institute (BHSAI) in its efforts to construct predictive models to assess liver, kidney and heart damages of chemical toxicants and drugs. To validate the platform, metabolic changes induced by toxicant exposure is supposed to be experimentally identified from the comprehensive analysis of in vivo transcriptomic data collected from the liver, kidney, heart, plasma, and urine of animals. To translate the results obtained in animal models to human, multiple species of animals, rats and guinea pigs, were used. To dissect the primary from the secondary transcriptomic response of liver, kidney and heart cells, resulted from altered metabolism of other organs/tissues, to toxicant exposure, transcriptomic analysis was performed on primary hepatocytes, renal tubular epithelial cells, cardiac myocytes originated from rats. We examined toxic effect of mercury chloride and thioacetamide.

KEYWORDS

Rat; guinea pig; liver; kidney; heart; toxicant exposure; transcriptomics; biomarker; thioacetamide; mercury chloride; RNA-seq differential expression; Hepatocytes, renal proximal tubular epithelial cells, cardio-myocytes.

ACCOMPLISHMENTS

[1] Assessment of toxic effect of mercury chloride on gene expression profiles of liver, kidney and heart cells *in vivo* system (rats and guinea pigs).

The DoD team and we selected mercury chloride for Y-3 study.

Mercury is naturally present in the earth's crust. It is also present in the atmosphere either derived naturally from the degassing of the earth's crust, emissions from volcanoes, evaporation from the world's seas, or from industrial pollution, which has greatly increased our exposure. Some of these industries include the health care sector, in which mercury is used in measuring instruments or as a disinfectant and in dentistry; the mining industry, such as power plants and crematoria; and the charcoal industry. A matter of serious concern is mercury exposure via environmentally contaminated food, mainly seafood, where mercury bio-accumulates in the food chain (Bose-O'Reilly S et al. Curr Probl Pediatr Adolesc Health Care 40: 186-215, 2010). Mercury salts occur in both mercury (I) (or mercurous) and mercury (II) (mercuric) forms. Mercury (II) salts are usually more toxic than their mercury (I) counterparts because their solubility in water is greater; thus, they are more readily absorbed from the gastrointestinal tract (Langford NJ et al. J Human Hypertension 13: 651-656, 1999). Mercury has a high affinity for sulfuryl groups, which inactivate numerous enzymatic reactions, amino acids, and sulfur-containing antioxidants (NAC, ALA, GST) with decreased oxidant defense and increased oxidative stress. Mercury induces mitochondrial dysfunction which occurs primarily at the ubiquinone-cytochrome B region and with NADH dehydrogenase causing displacement of Fe^{++} and Cu^{++} ions in

the a3Cub center of cytochrome C. This results in depolarization and autoxidation of the inner mitochondrial membrane with lipid peroxidation and severe mitochondrial dysfunction. These effects of mercury increase oxidative stress (Houston MC. J Clin Hypertens 13: 621-627, 2001; Syversen T and Kaur P. J Trace Elem Med Biol 26: 215-226, 2012). Mercury irreversibly inhibits selenium-dependent enzymes and may also inactivate S-adenosyl-methionine, which is necessary for catecholamine catabolism by catechol-o-methyl transferase. Due to the body's inability to degrade catecholamines, a person suffering from mercury poisoning may experience profuse sweating, tachycardia (persistently faster than normal heart beat), increased salivation, and hypertension (Reeves MA and Hoffmann PR. Cell Mol Life Sci 66: 2457-2478, 2009; Carvalho CM et al. J Biol Chem 283: 11913-11923, 2008). The organs primarily affected after acute poisoning of mercuric mercury are the intestine and kidneys. In the intestine the corrosive effects will dominate and ingestion of high dose of mercuric chloride produces extensive precipitation of intestinal mucosal proteins, mucosal necrosis, generalized abdominal pain, bloody diarrhea and shock (Bernhoft RA. J Environ Public Health 2012:460508, 2012; Pollard KM and Hultman P. Met Ions Biol Syst 34: 421-440, 1997). Kidneys accumulate highest levels of mercury compared to brain and liver (Husswain S et al. J Environ Sci Health B 32: 395-409, 1997). Renal failure may occur within 24 h due to necrosis of the tubular epithelium. As little as 1 g can prove fatal to an adult human. The most prominent effect of mercuric mercury is tubular necrosis in the kidney and after prolonged exposure glomerulonephritis can also be seen (Pollard KM and Hultman P. Met Ions Biol Syst 34: 421-440, 1997). Mercury toxicity include hypertension, generalized atherosclerosis, coronary heart disease, myocardial infarction, cardiac arrhythmias, heart rate variability, sudden death, cerebrovascular accidents, carotid artery disease, renal dysfunction and total mortality (Houston MC. J Clin Hypertens 13: 621-627, 2001).

[1-1] Assessment of toxic effect of mercury chloride on gene expression profiles of liver, kidney and heart in rats.

[1-1-1] Define a dose and treatment period which causes detectable injury in the liver, kidney and/or heart within a period of 5 days without the development of severe complications secondary to the injury of these organs.

In this project, we use three doses, no drug (vehicle), low dose and high dose, and two exposure period, T1 and T2. Our criteria for each dose and exposure periods were as below:

High dose – T2: Appropriate dose and appropriate administrated time which causes detectable injury in the liver, kidney and/or heart prior to the development of obvious clinical symptom(s)

High dose – T1: Appropriate dose and intermediate administrated time

Low dose – T2: The half dose of appropriate dose and appropriate administrated time

Low dose – T1: The half dose of appropriate dose and intermediate administrated time

Reported toxicity of mercury chloride in rats: In rats, a single dose of mercury chloride at 50 and 3.26 mg/kg ip cause hepatic injury within 24 hours and 4 days after the dose, as shown by elevated plasma ALT, AST ALP and bilirubin levels and histological changes such as disorganization of normal radiating pattern of cell plates, degenerated hepatocytes, dilatation of central vein and prominent sinusoidal congestion (Ansar S and Iqbal M. Human and Experimental Toxicology 35: 1305-1311, 2016; Joshi D et al. Biomedicine & Pharmacotherapy 91: 645-655, 2017). In addition, a single ip injection of mercury chloride at 9 (Wilks MF et al. Toxicologic pathology 22: 282-290, 1994), 7.5 (Yanagisawa H et al. Toxicology Letter 98: 181-188, 1998), 5 (Perottoni et al. Food Chem Toxicol 42: 17-28, 2004; Lund BO et al. Biochem Pharmacol 45: 2014-2024, 1993; Augusti PR et al. Food Chem Toxicol 46: 212-219, 2008), 4 (Hazelhoff MH et al. Int J Mol Sci 13: 10523-10536, 2012) and 3.36 mg/kg (Joshi D et al. Biomedicine & Pharmacotherapy 91: 645-655, 2017) brought about renal injury 3 hours – 4 days after dosing, as shown by elevated plasma levels of urea, uric acid, creatinine and blood urea nitrogen accompanied by histological changes such as degenerated renal tubules with obstructed lumen, shrunken glomerulus, vacuolated cells, disrupted brush border membrane, cellular detachment, disrupted tubular basement membranes and necrosis. On the other hand, when mercury chloride was administered daily basis by gavage, 0.1 mg/kg oral administration daily for a period of 3 days (Reus IS et al. J Biochem Molecular Toxicology 17: 161-168, 2003) caused slight increase in serum ALT, APL and bilirubin levels. 0.4 mg/kg oral administration daily for a period of 7 days (Othman MS et al. Food and Chemical Toxicology 69: 175-181, 2014) exhibited its hepatotoxicity, such as increased serum levels of ALT (2 times), AST (1.6 times) and ALP (1.8 times), and severe hepatic necrosis, disappearance and disarrangement of hepatic lobules structures, extensive granular and vesicular degeneration, vacuolation and inflammatory cell infiltrations in portal region, and renal toxicity, such as elevated plasma levels of urea (5 times), uric acid (3 times), creatinine (8 times) and blood urea nitrogen (3.5 times), and degenerated renal tubules with obstructed lumen and shrunken glomerulus.

Therefore, to find appropriate doses and exposure period that match with our criteria mentioned above, we tested the effect of daily treatment of mercury chloride at the doses in range between 0.1and 9.0 mg/kg by gavage for a period of 5 days or a single dosing by intraperitoneal injection.

[1-1-1-1] Examine dose- and exposed period-dependency of the toxicity of mercury chloride administered by gavage.

Animals: Eight-week-old male Sprague-Dawley rats (~300g) were purchased from Charles River Laboratories (Wilmington, MA). Rats were fed a normal diet (No. 5001, Purina Mills, St. Louis, MO) and provided water ad libitum in an environmentally controlled room with a 12:12-h light-dark cycle in the Animal Care facility at Vanderbilt University. 7 days before each study, we implanted a silicon catheter in the jugular vein for collecting blood. Animals were transferred from regular housing care to metabolic cage 2 days before each study.

Administration of mercury chloride and collection of blood and urine: Mercury chloride was dissolved in water and administrated by gavage at 9:00 am daily for 5 days as shown in **Appendix #1**. Blood were collected twice daily, just before drug administration and 5 pm during 5 days of experimental period. Cumulated urines were collected at 9 am and 5 pm daily.

Results: We tested 6 different doses of mercury chloride, vehicle, 1 mg/kg, 3 mg/kg, 5 mg/kg, 7 mg/kg and 9 mg/kg on three rats, respectively. As shown in **Appendix #2**, rats, which were treated with 3, 5 and 7 mg/kg, lost weight dose-dependently. Rats treated with 9 mg/kg were worse and thus euthanized in the first days. Urine levels of KIM-1, renal injury marker, were elevated 24 hours after the dose in rats treated with 3, 5 and 7 mg/kg, compared with rats treated with vehicle and 1 mg/kg. On the other hand, we did not see significant elevation of plasma ALT and AST levels in rats administered with any dose of mercury chloride, compared with rats administered vehicle.

[1-1-1-2] Examine dose- and treatment period-dependency of the toxicity of mercury chloride administrated intraperitoneally.

Animals: Eight-week-old male Sprague-Dawley rats (~300g) were purchased from Charles River Laboratories (Wilmington, MA). Rats were fed a normal diet (No. 5001, Purina Mills, St. Louis, MO) and provided water ad libitum in an environmentally controlled room with a 12:12-h light-dark cycle in the Animal Care facility at Vanderbilt University. 7 days before each study, we implanted a silicon catheter in the jugular vein for collecting blood. Animals were transferred from regular housing care to metabolic cage 2 days before each study.

Administration of mercury chloride and collection of blood and urine: Mercury chloride was dissolved in saline and administrated intraperitoneally at 9:00 am in Day 1 as shown in **Appendix #3**. Blood were collected twice daily, at 9 am and 5 pm during 3 days of experimental period. Cumulated urines were collected at 9 am and 5 pm daily.

Results: We tested 8 different doses of mercury chloride, vehicle, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8 mg/kg on three rats, respectively. As shown in **Appendix #4** and **Appendix #5**, rats, which were treated with 0.125, 0.25, 0.5, and 1.0 mg/kg, did not lose their weight, suggesting that these doses did not affect animal's eating activity. The dose of 0.125 mg/kg did not alter plasma AST and urine KIM-1 levels during 3 days test period. The dose of 0.25 mg/kg did not alter plasma AST level during 3 days test period, it caused the elevation of Urine KIM-1 levels from 9 hours after the dose. Rats treated with 0.5 mg/kg had mild elevation of plasma AST levels within 8 hours after the dose and tended to be higher compared with the vehicle treated rats. On the other hand, urine KIM-1 levels after 9th hour of the dose. In rats treated with 1 mg/kg, plasma AST levels were increased by 50% within 8 hours after dose and urine KIM-1 levels increased markedly from 9 hours after the dose. Rats, which were treated with 2, 4 and 8 mg/kg, lost their body weight by 10 – 18% and had marked increase in plasma AST levels within 8 hours and urine KIM-1 levels after 9th hour of the dose.

Conclusion: The oral administration of mercury chloride did not cause the physical injury of the liver, while it caused body weight loss and the physical injury of the kidney. On the other hand, the intraperitoneal administration of lower dose (0.25, 0.5 and 1.0 mg/kg) of mercury chloride brought about a mild physical injury in both of the liver and the kidney, without a loss of body weight and the development of clinical symptom (**Appendix #6**). Therefore, we chose a single intraperitoneal administration as the route for the drug administration, two doses of 0.25 mg/kg as low dose and 0.5 mg/kg as high dose, and two exposure periods of 10 hours as shorter exposure and 34 hours as longer exposure, for the final study.

[1-1-2] Assessment of toxic effect of mercury chloride on gene expression profiles of liver, kidney and heart in rats.

Animals: Eight-week-old male Sprague-Dawley rats (~300g) were purchased from Charles River Laboratories (Wilmington, MA). Rats were fed a normal diet (No. 5001, Purina Mills, St. Louis, MO) and provided water ad libitum in an environmentally controlled room with a 12:12-h light-dark cycle in the Animal Care facility at Vanderbilt University.

Administration of mercury chloride and collection of liver, kidney and heart: Based on the results in our preliminary studies, rats were treated as shown below:

Group	Dose	Exposure time	Number of animals
Group #1	Vehicle (saline, 1 ml/kg)	10 hours	5
Group #2	0.25 mg/kg	10 hours	5
Group #3	0.5 mg/kg	10 hours	5
Group #4	Vehicle	34 hours	5
Group #5	0.25 mg/kg	34 hours	5
Group #6	0.5 mg/kg	34 hours	5

Mercury chloride was dissolved in saline at 0.25 mg/ml and 0.5 mg/ml. Vehicle (Group #1 and Group #4), mercury chloride at 0.25 mg/kg as low dose (Group #2 and Group #5) and 0.5 mg/kg as high dose (Group #3 and Group #6) were administrated intraperitoneally at 7:00 am in Day 1. Groups, which were exposed for 10 hours, were fasted from right after the treatment with the vehicle or drug. At 5:00 pm (10 hours after the treatment), the liver, kidney and heart were harvested from these rats after anesthesia by intraperitoneal injection of sodium pentobarbital (80 mg/kg). Groups, which were exposed to the drug for 34 hours, were allowed to access food freely after the dose. In Day 2, they were fasted from 7:00 am and at 5:00 pm (34 hours after the dose), the liver, kidney and heart were harvested from these rats after anesthesia by intraperitoneal injection of sodium pentobarbital (80 mg/kg).

These final studies were completed and RNA were extracted from the liver and kidney from all of animals. 60 samples of extracted RNA were sent to VANTAGE for RNA sec analysis.

[1-2] Assessment of toxic effect of mercury chloride on gene expression profiles of liver, kidney and heart in guinea pigs.

[1-2-1] Define a dose and treatment period which causes detectable injury in the liver, kidney and/or heart within a period of 5 days without the development of severe complications secondary to the injury of these organs.

In this project, we use three doses, no drug (vehicle), low dose and high dose, and two exposure period, T1 and T2. Our criteria for each dose and exposure periods were as below:

High dose – T2: Appropriate dose and appropriate administrated time which causes detectable injury in the liver, kidney and/or heart prior to the development of obvious clinical symptom(s)

High dose – T1: Appropriate dose and intermediate administrated time

Low dose – T2: The half dose of appropriate dose and appropriate administrated time

Low dose – T1: The half dose of appropriate dose and intermediate administrated time

There are few reports about toxicity of mercury chloride on guinea pigs. It has been reported that guinea pig is more sensitive to methyl mercury that the rat and mouse (Iverson F et al. Toxicol Appl Pharmacol 24: 545-554, 1973; Swensson A. Acta Med Scand 143: 365, 1952). Bret L et al. (Blet L et al. Enzyme Protein 47: 27-36, 1993) reported that a single intraperitoneal injection of 1.35 mg/kg of mercuric chloride caused dramatic increases in kidney injury markers in urine within 24 hours after the dosing, indicating that guinea pig is more sensitive to renal toxicity of mercury chloride compared with rat, since such severe renal injury is caused within 24 hours by a single intraperitoneal injection of >4 mg/kg of mercuric chloride in rats as mention above. Therefore, to find appropriate doses and exposure period that match with our criteria mentioned above, we tested the effect of daily treatment of mercury chloride at the doses in range between 0.5 and 6.0 mg/kg by gavage for a period of 5 days or a single dosing by intraperitoneal injection at the doses in the range between 0.25 and 2.0 m/kg.

[1-2-1-1] Examine dose- and treatment period-dependency of mercury chloride toxicity with gavage administration.

Animals: Five-week-old male Hartley guinea pigs (~320g) were purchased from Charles River Laboratories (Wilmington, MA). Guinea pigs were fed a normal diet (No. 5025, Purina Mills, St. Louis, MO) and provided water ad libitum in an environmentally controlled room with a 12:12-h light-dark cycle in the Animal Care facility at Vanderbilt University. 7 days before each study, we implanted a silicon catheter in the jugular vein for collecting blood.

Administration of mercury chloride and collection of blood and urine:

Mercury chloride was dissolved in water and administrated by gavage at 9:00 am daily for 5 days as shown in **Appendix #7**. Blood were collected twice daily, just before drug administration and 5 pm during 5 days of experimental period.

Results: We tested eight different doses of mercury chloride, vehicle, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/kg, respectively. As shown in **Appendix #8**, we did not see significant elevation of plasma ALT and AST levels in animals treated with mercury chloride compared with animals treated with vehicle.

[1-2-1-2] Examine appropriate dose- and treatment period-dependency of mercury chloride toxicity with intraperitoneal administration.

Animals: Five-week-old male Hartley guinea pigs (~320g) were purchased from Charles River Laboratories (Wilmington, MA). Guinea pigs were fed a normal diet (No. 5025, Purina Mills, St. Louis, MO) and provided water ad libitum in an environmentally controlled room with a 12:12-h light-dark cycle in the Animal Care facility at Vanderbilt University. 7 days before each study, we implanted a silicon catheter in the jugular vein for collecting blood.

Administration of mercury chloride and collection of blood and urine:
Mercury chloride was dissolved in water and administrated intraperitoneally at 9:00 am daily in Day 1 as shown in **Appendix #9**. Blood were collected twice daily, just before drug administration and 5 pm during 5 days of experimental period.

Results: We tested six different doses of mercury chloride, vehicle, 0.25, 0.5, 1.0, 1.5 and 2.0 mg/kg. As shown in **Appendix #10**, the body weight and plasma levels of creatinine, ASI and ALT were not changed by the treatment with mercury chloride at the dose of 0.25, 0.5 or 1.0 mg/kg, and were not different significantly compared with that by the treatment with the vehicle. Animals treated with 1.0 mg/kg had marked elevation of plasma AST and ALT levels within 8 hours after the dosing and after which these retuned to the basal levels progressively, while they did not exhibit any significant changes in body weight and plasma creatinine levels. Animals treated with 1.5 mg/kg had marked elevation of plasma levels of creatinine, ALT and AST levels 8 hours after the dosing without change in body weight. With further increase in the dose to 2.0 mg/kg, animals lost body weight with marked elevation of plasma creatinine, ALT and AST.

Conclusion: The oral administration of mercury chloride did not cause the physical injury of the liver, while it caused body weight loss and the physical injury of the kidney. On the other hand, the intraperitoneal administration of mercury chloride at 1.0 mg/kg brought about a mild physical injury in the liver, but not the kidney, without a loss of body weight. The intraperitoneal administration of mercury chloride at 1.0 mg/kg brought about a mild physical injury in both the liver and kidney, without a loss of body weight. Therefore, we chose a single intraperitoneal administration as the route for the drug administration, two doses of 1.0 mg/kg as low dose and 1.5 mg/kg as high dose, and two exposure periods of 9 hours as shorter exposure and 33 hours as longer exposure, for the final study.

[1-2-2] Assessment of transcriptomic response of liver, kidney and heart to mercury chloride on in guinea pigs.

Using the doses and exposure times that were determined by the preliminary study shown above, we performed the final study to assess the transcriptomic response of the liver and kidney to mercury chloride exposure in guinea pigs.

Animals: Five-week-old male Hartley guinea pigs (~320g) were purchased from Charles River Laboratories (Wilmington, MA). Guinea pigs were fed a normal diet (No. 5025, Purina Mills, St. Louis, MO) and provided water ad libitum in an environmentally controlled room with a 12:12-h light-dark cycle in the Animal Care facility at Vanderbilt University.

Administration of mercury chloride and collection of liver, kidney and heart: Based on the results in our preliminary studies which had done in previous quarter, we chose intraperitoneal administration, 1.0 mg/kg as low dose and 1.5 mg/kg as high dose, and 9 hours as shorter exposure and 33 hours as longer exposure, for the final study. Therefore, we have 6 groups and each group had 5 guinea pigs.

Group	Dose	Exposure time	Number of animals
Group #1	Vehicle	9 hours	5
Group #2	1.0 mg/kg	9 hours	5
Group #3	1.5 mg/kg	9 hours	5
Group #4	Vehicle	33 hours	5
Group #5	0.25 mg/kg	33 hours	5
Group #6	0.5 mg/kg	33 hours	5

Mercury chloride was dissolved in saline at 1.0 mg/ml and 1.5 mg/ml. Vehicle (saline, 1 ml/kg), mercury chloride at 1.0 mg/kg as low dose and 1.5 mg/kg as high dose administrated intraperitoneally at 7:00 am in Day 1. Group#1, Group#2 and Group#3, which were exposed for 9 hours, were fasted from right after the treatment with the vehicle or drug. At 4:00 pm (9 hours after the treatment), the liver, kidney and heart were harvested from these animals after anesthesia by intraperitoneal injection of sodium pentobarbital (80 mg/kg). Group#4, Group#5 and Group#6, which were exposed to the drug for 33 hours, were allowed to access food freely after the dose. In Day 2, animals of these groups were fasted from 7:00 am and at 4:00 pm (33 hours after the dose), the liver, kidney and heart were harvested from these rats after anesthesia by intraperitoneal injection of sodium pentobarbital (80 mg/kg).

The final studies were completed and extracted RNA were sent to VANTAGE for RNA seq analysis.

[2] Mercury chloride toxicity liver, kidney and heart primary cultured cells.

To measure the primary transcriptomic response of liver, kidney and heart cells to toxicant exposure, transcriptomic analysis was performed on cultured primary hepatocytes, renal tubular epithelial cells, cardiac myocytes originated from rats.

Cells used:

Rat (Sprague-Dawley) primary hepatocytes (Triangle Research Labs)
Rat renal tubular epithelial cells (Sciencell Research Laboratories)
Rat cardiac myocytes (Sciencell Research Laboratories)

Cell culture: Hepatocytes were plated on collagen 1-coated 96 well plates. Renal tubular epithelial cells and cardiac myocytes were plated on a poly-L-lysine-coated 96 well plates.

[2-1] Preliminary studies to determine the dose and time dependency of mercury chloride toxicity in cultured primary hepatocytes, renal tubular epithelial cells and cardiomyocytes generated from rats.

Our criteria for the condition of high dose at the longer exposure time was to see approximately 20-30% of cell viability.

It has been reported that cell viability of primary cultured mice hepatocytes decreased in concentration rage between 5 and 50 μM and in time ranges between 3 and 12 hours (Lee J et al. Cell Biochemistry & Function 32: 520-529, 2014). Studies using hepatic cell line (WRL-68 cells) reported that exposure of cells to 0.5 or 5 μM $HgCl_2$ for 3 hours increased the percentage of damaged nucleus and the average length of DNA migration (Bucio L et al. Mutation Research 423: 65-72, 1999). In addition, decreased mitochondrial activity and ATP levels, cell round up and incased number of cytoplasmic vacuoles were reported in Kidney OK cell line exposed to 15 μM for 6-9 hours. Based on above information, we tested the doses in the range between 2.5 and 80 μM, and the exposure time periods of 6, 12 and 24 hours.

Experimental protocol: After 24 hours culture, either vehicle (saline) or mercury chloride was added to make the final concentration of 0, 2.5, 5, 10, 20, 40 and 80, μM. The intracellular ATP contents were measured at 6, 12, and 24 hours after the exposure to mercury chloride **(Appendix #11)**. Cell ATP levels were measured using CellTier-Glo 2.0 Assay kit (Promega Co., Madison WI).

Results: Dose dependent cyto-toxicity were observed with all of cell types (**Appendix #12, #13 and #14**). On the other hand, the cyto-toxicity was brought about 6 hours after the exposure of cells to $MgCl_2$ and poor time dependency was observed. The renal cells were more sensitive compared with hepatocytes and cardio-myocytes.

Conclusion: Our criteria for the condition of high dose at the longer exposure time is to see approximately 20-30% of cell viability. Based on these preliminary results, to measure the transcriptomic response of cultured primary hepatocytes and renal tubular epithelial cells to mercury chloride, we decided to use two doses of mercury chloride, 10 μM as low dose and 20 μM as high dose for hepatocytes, and 5 μM as low dose and 10 μM as high dose

for renal tubular epithelial cells. In addition, we chose two exposure period, 12 hours as a shorter period and 24 hours as a longer period for both type of cells.

[2-2] Assessment of transcriptomic response of liver, kidney and heart cells of rats to mercury chloride.

We performed the final study to assess the transcriptomic respnse of cultured rat hepatocytes, renal tubular epithelial cells and cardiac myocytes to mercury chloride.

Experimental protocol: After 24 hours culture, hepatocytes and cardiomyocytes were exposed to either vehicle or mercury chloride at the final concentration of 10 and 20 μM for 12 and 24 hours. Penal Proximal tube epithelial cells were exposed to either vehicle or mercury chloride at the final concentration of 5 and 10 μM for 12 and 24 hours. The RNAs were extracted using trizol method.

Doses and Hours of Treatment for the final study

		Hours of Treatment	
		12 hours	24 hours
Hepatocytes	Vehicle	0 μM	0 μM
	Low Dose	10 μM	10 μM
	High Dose	20 μM	20 μM
Renal Proximal Tube Epithelial Cells	Vehicle	0 μM	0 μM
	Low Dose	5 μM	5 μM
	High Dose	10 μM	10 μM
Cardio-Myocytes	Vehicle	0 μM	0 μM
	Low Dose	10 μM	10 μM
	High Dose	20 μM	20 μM

After exposure of cells to low and high dose for T1and T2, we will harvest cells for extract RNA and extracted RNA were sent to VANTAGE.

[3] Modification of protocols.

[3-1] Replace endosulfan study on guinea pigs and mercury chloride human cell study by thioacetamide-S-oxide and mercury chloride studies on guinea pig cells.

We found that endosulfan does not cause hepatic and renal injury in guinea pigs in the range of the doses and treatment time which do not bring about a neuronal toxicity. In addition, the exposure of rats to the highest dose in range of the dose which did not cause neuronal poisoning for 4 weeks caused a change in very limited number of RNA. The DoD

team requested us to replace the guinea pig-endosulfan in vivo study and mercury chloride human cell study by the thioacetamide-S-oxide and mercury chloride studies on guinea pig hepatocytes and renal proximal tube epithelial cells.

We found that hepatocyte and renal cells of guinea pig are not commercially available. Since we isolate hepatocytes from rat liver routinely, we are ready to isolate guinea pig hepatocytes. On the other hand, we need to examine optimal condition for isolation and culture of renal proximal tubes from guinea pig kidney.

At the first step, using rats because of the cost, we tried the isolation of renal glomerular and proximal tubes from rat kidney. We successfully could isolate renal glomerular and proximal tubes from renal cortex. We will try the next steps, removing of glomerular and isolation of epithelial cells from the proximal tubes. We successfully established the method (**Appendix #15**).

IMPACT

Major findings and accomplishments of this research to date:

- We completed in vivo study to assess the transcriptomic response of the liver and kidney to mercury chloride exposure in both rats and guinea pigs. We also completed the extraction of RNA from the liver and kidney from these animals.
- We completed in vitro study to assess the transcriptomic response of cultured primary rat hepatocytes and renal proximal tubular epithelial cells to mercury chloride. We also completed the extraction of RNA from these cells.
- We established the method for the isolation of proximal tubular epithelial cells from guinea pig's kidney.

CHANGES/PROBLEMS

Nothing to report.

PRODUCTS

Nothing to report.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

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Kelli Boyd, Collaborator
Richard Printz, Collaborator
Chiyo Shota, Collaborator
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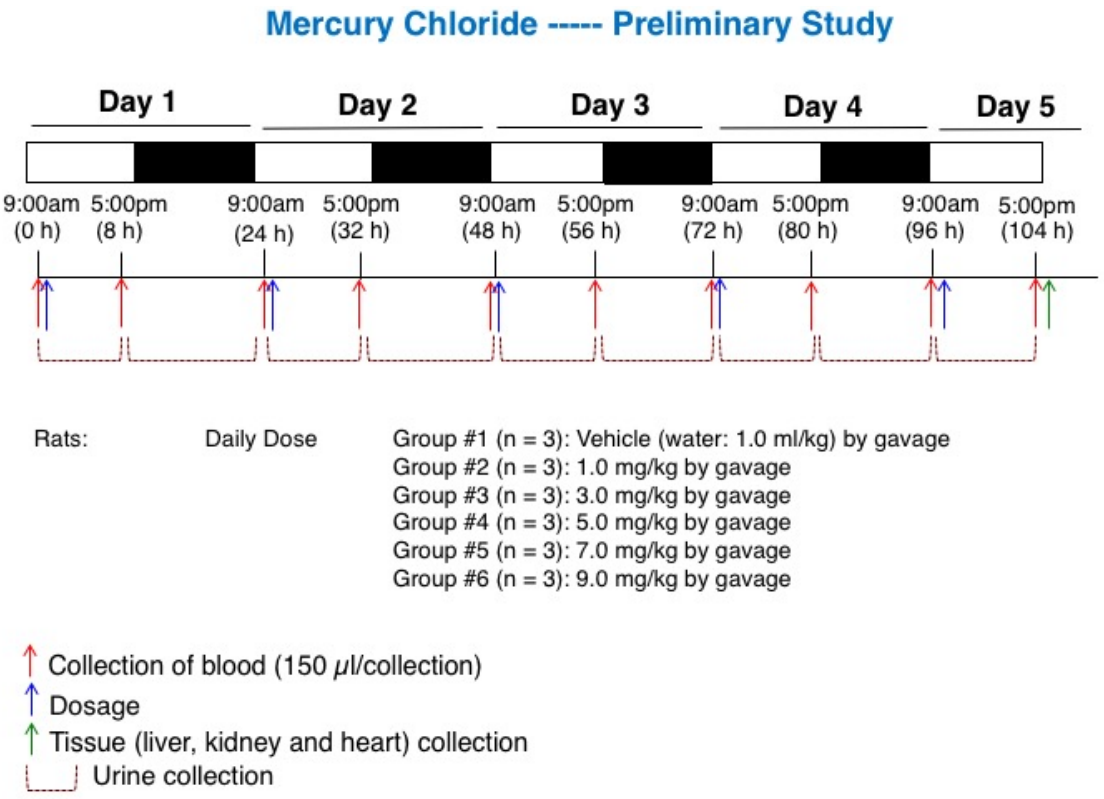
SPECIAL REPORTING REQUIREMENTS

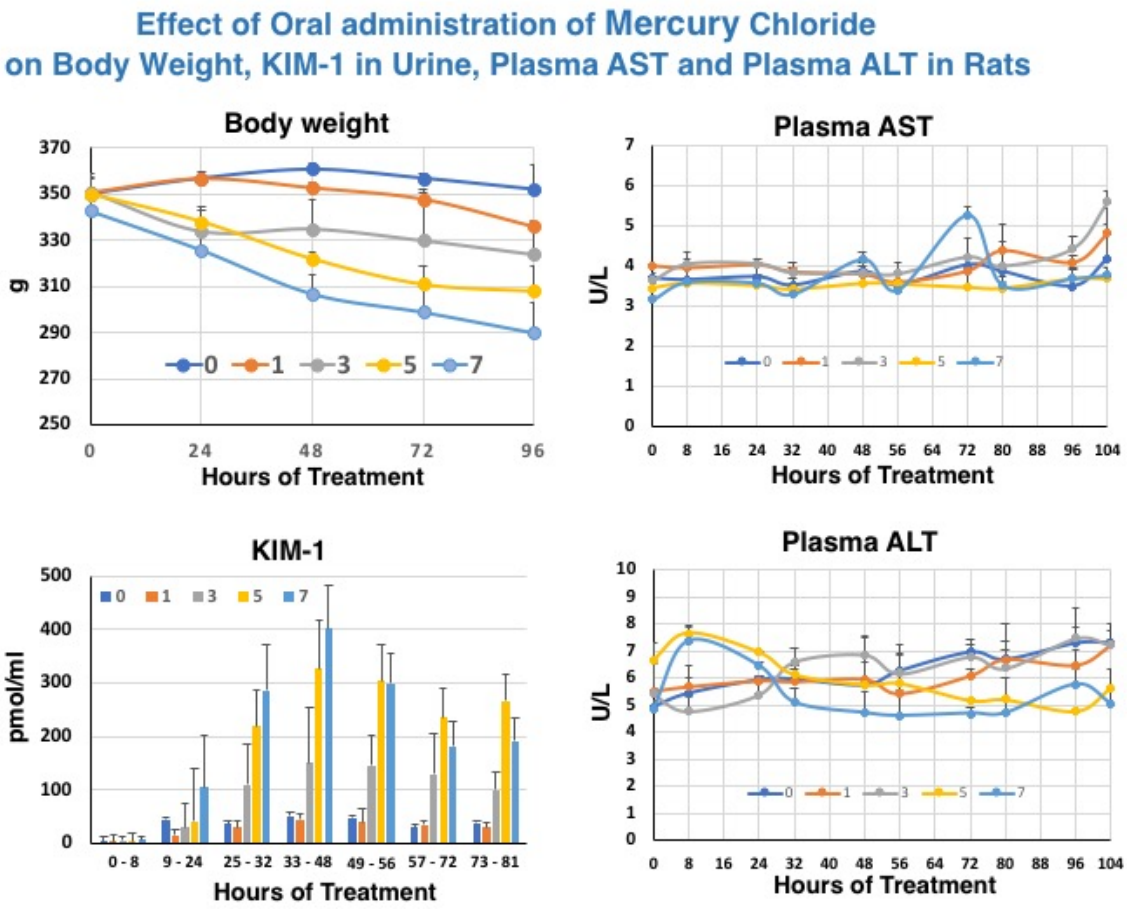
Nothing to report.

Appendix

Appendix #1

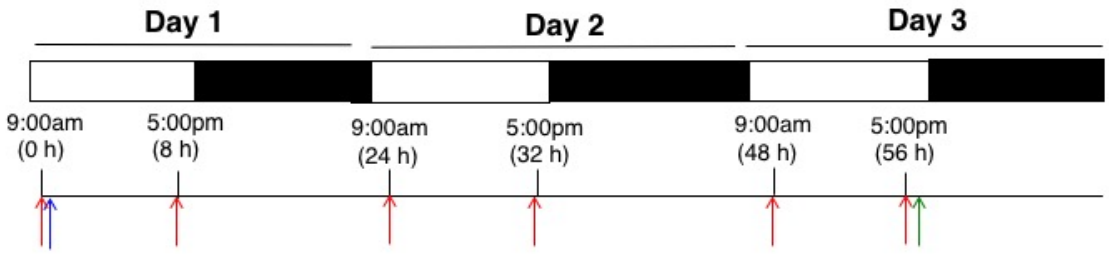


Appendix #2


Effect of Oral administration of Mercury Chloride
on Body Weight, KIM-1 in Urine, Plasma AST and Plasma ALT in Rats
Body weight
g
Hours of Treatment
0 1 3 5 7
Plasma AST
U/L
Hours of Treatment
KIM-1
pmol/ml
0 - 8
9 - 24
25 - 32
33 - 48
49 - 56
57 - 72
73 - 81
Hours of Treatment
Plasma ALT
U/L
Hours of Treatment

Appendix #3

Mercury Chloride ----- Preliminary Study



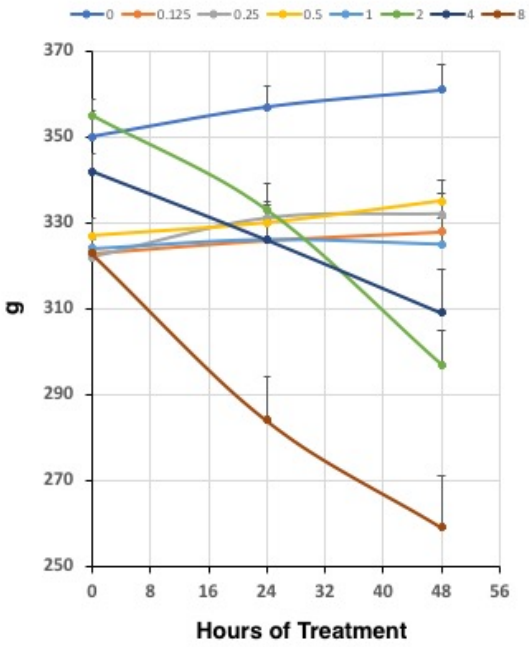
Rats: Daily Dose

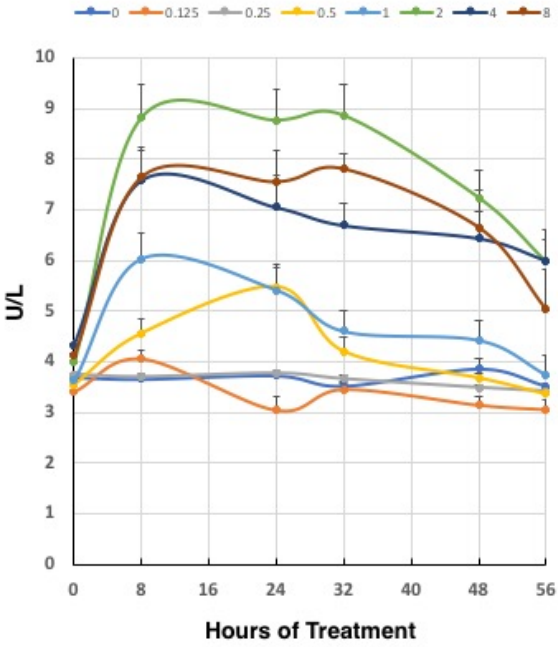
Group #1 (n = 3): Vehicle (saline: 1.0 ml/kg) intraperitoneally
Group #2 (n = 3): 0.125 mg/kg intraperitoneally
Group #3 (n = 3): 0.25 mg/kg intraperitoneally
Group #4 (n = 3): 0.5 mg/kg intraperitoneally
Group #5 (n = 3): 1.0 mg/kg intraperitoneally
Group #6 (n = 3): 2.0 mg/kg intraperitoneally
Group #7 (n = 3): 4.0 mg/kg intraperitoneally
Group #8 (n = 3): 8.0 mg/kg intraperitoneally

↑ Collection of blood (150 μl/collection)
↑ Dosage
↑ Tissue (liver, kidney and heart) collection

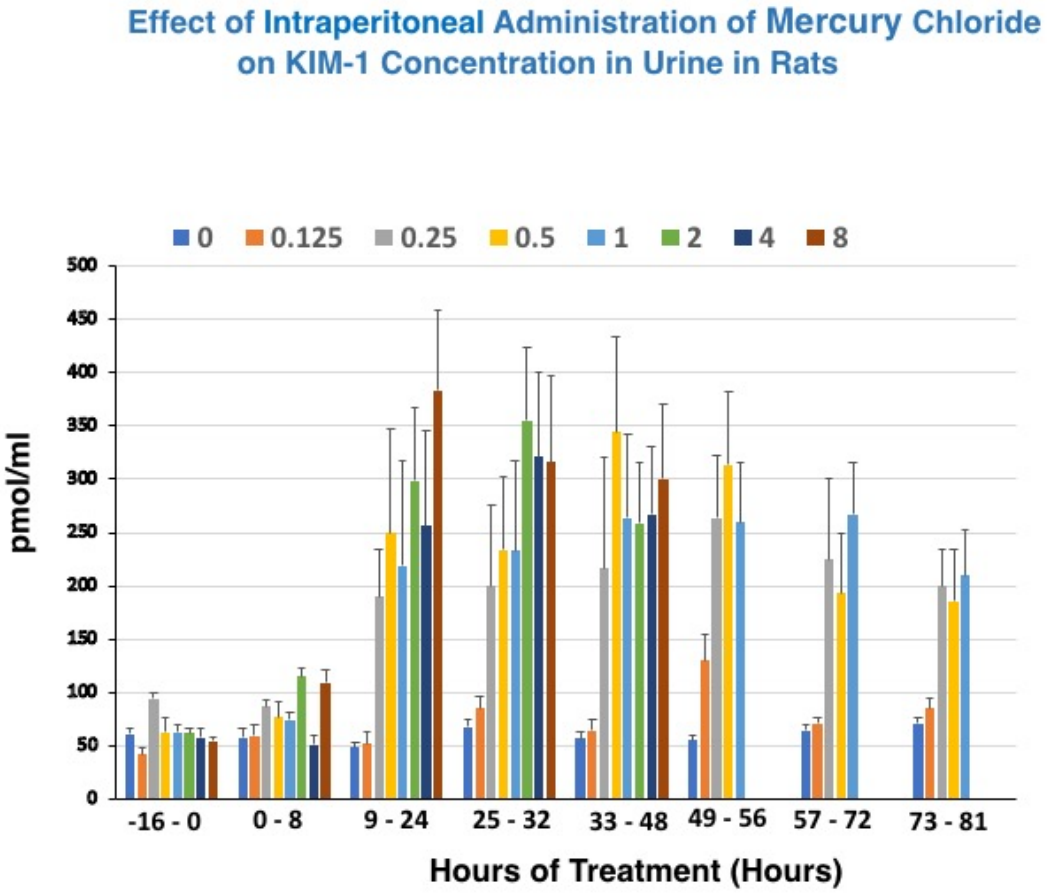
Appendix #4

Effect of Intraperitoneal Administration of Mercury Chloride on Body Weight and Plasma AST in Rats





Appendix #5


Effect of Intraperitoneal Administration of Mercury Chloride on KIM-1 Concentration in Urine in Rats
0
0.125
0.25
0.5
1
2
4
8
pmol/ml
500
450
400
350
300
250
200
150
100
50
0
-16 - 0
0 - 8
9 - 24
25 - 32
33 - 48
49 - 56
57 - 72
73 - 81
Hours of Treatment (Hours)

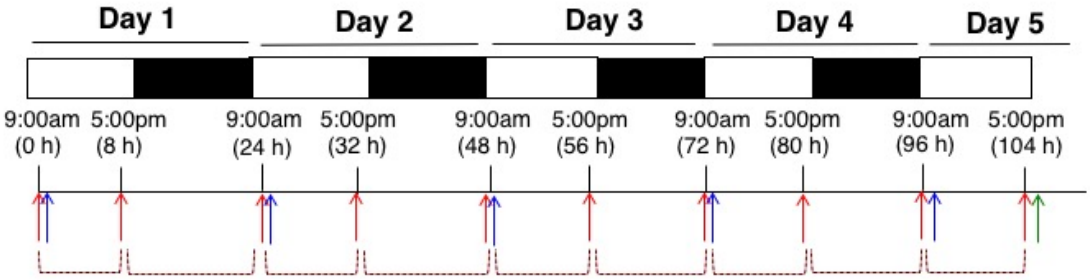
Appendix #6

Doses and Hours of Treatment for the final study

	DAY 1				DAY 2			
	5 h fasted		10 h fasted		5 h fasted		10 h fasted	
Dose (mg/mg)	5 hours		10 hours		29 hours		34 hours	
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
0.125	-	-	-	-	-	-	-	-
0.25	-	-	-	-	-	++	-	++
0.5	+	-	+	-	+	++	+	++
1.0	++	-	++	-	++	+	++	++

Appendix #7

Mercury Chloride ----- Preliminary Study



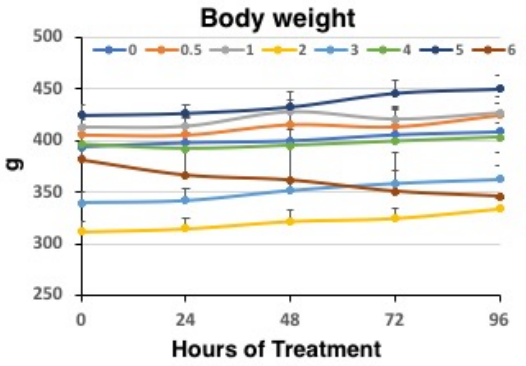
Guinea pigs: Daily Dose

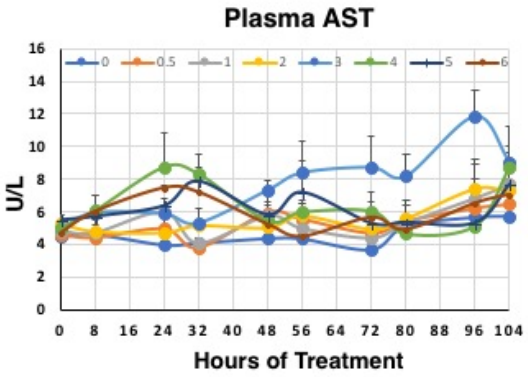
Group #1 (n = 4): Vehicle (water: 1.0 ml/kg) by gavage
Group #2 (n = 3): 0.5 mg/kg by gavage
Group #3 (n = 3): 1.0 mg/kg by gavage
Group #4 (n = 3): 2.0 mg/kg by gavage
Group #5 (n = 3): 3.0 mg/kg by gavage
Group #6 (n = 3): 4.0 mg/kg by gavage
Group #7 (n = 3): 5.0 mg/kg by gavage
Group #8 (n = 3): 6.0 mg/kg by gavage

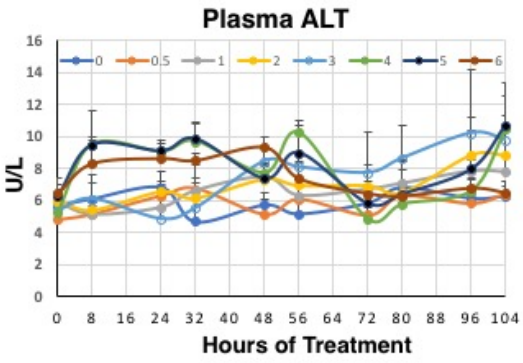
↑ Collection of blood (150 μl/collection)
↑ Dosage
↑ Tissue (liver, kidney and heart) collection
⌐¬ Urine collection

Appendix #8

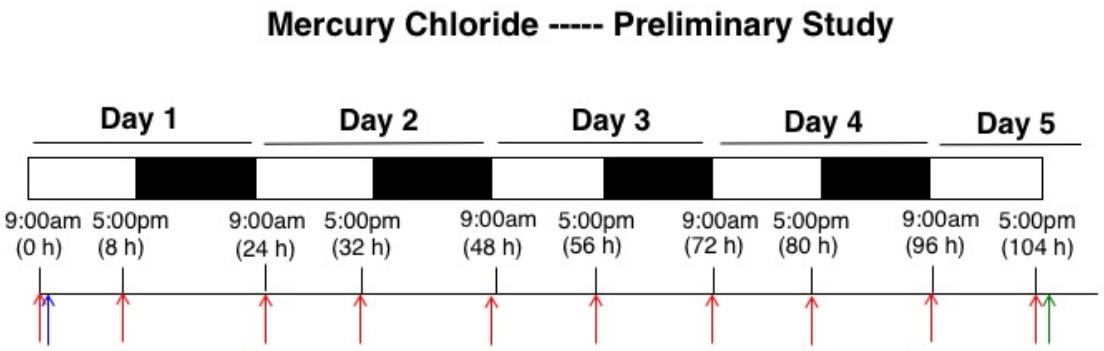
Effect of Oral administration of Mercury Chloride on Body Weight, KIM-1 in Urine, Plasma AST and Plasma ALT in Guinea Pigs







Appendix #9

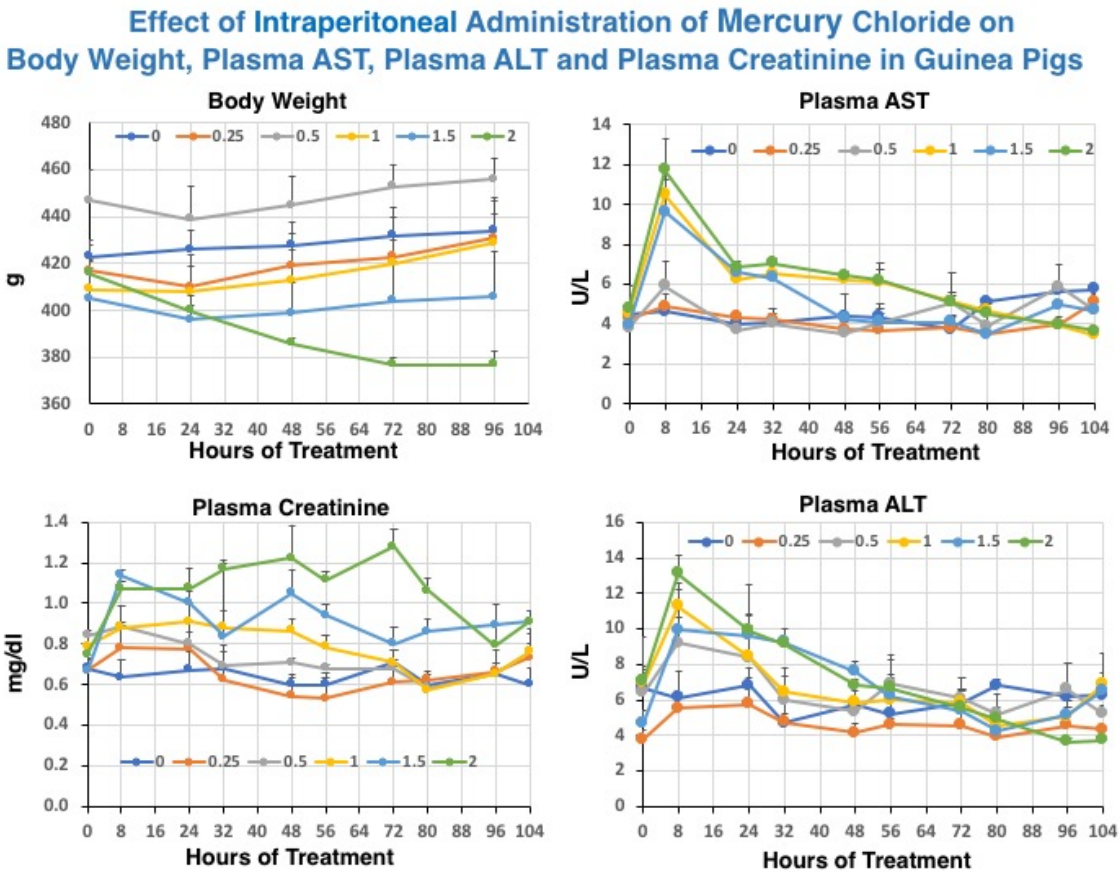


Guinea pigs: Daily Dose

Group #1 (n = 4): Vehicle (saline: 1.0 ml/kg) intraperitoneally
Group #2 (n = 3): 0.25 mg/kg intraperitoneally
Group #2 (n = 3): 0.5 mg/kg intraperitoneally
Group #3 (n = 3): 1.0 mg/kg intraperitoneally
Group #4 (n = 3): 1.5 mg/kg intraperitoneally
Group #5 (n = 3): 2.0 mg/kg intraperitoneally

↑ Collection of blood (150 μl/collection)
↑ Dosage
↑ Tissue (liver, kidney and heart) collection

Appendix #10


Effect of Intraperitoneal Administration of Mercury Chloride on
Body Weight, Plasma AST, Plasma ALT and Plasma Creatinine in Guinea Pigs
Body Weight
g
Hours of Treatment
Plasma AST
U/L
Hours of Treatment
Plasma Creatinine
mg/dl
Hours of Treatment
Plasma ALT
U/L
Hours of Treatment
0
0.25
0.5
1
1.5
2

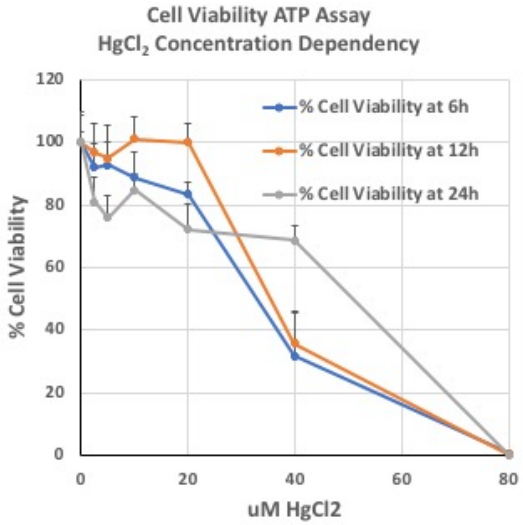
Appendix #11

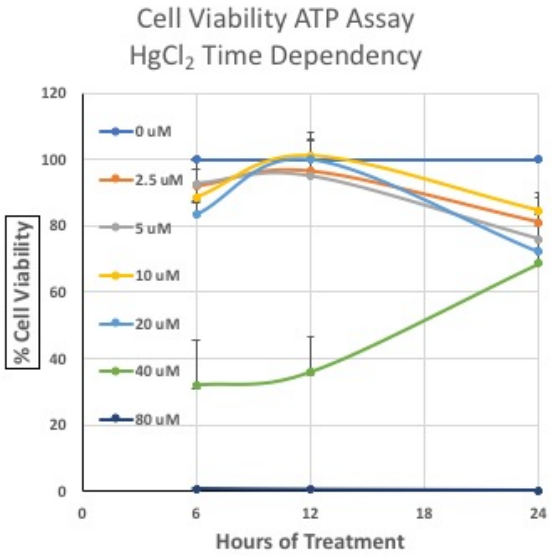
Effect of Mercury Chloride on Functional Viability (ATP levels) of Rat hepatocytes, Renal Cells and cardiomyocytes

Cells used:	**S-D rats Hepatocytes** **S-D rats renal proximal tube epithelial cells** **S-D rats Cardio-myocytes**
$HgCl_2$ Doses (μM):	**0 (n = 5)** **2.5 (n = 5)** **5.0 (n = 5)** **10.0 (n = 5)** **20.0 (n = 5)** **40.0 (n = 5)** **80.0 (n = 5)**
Hours of Treatment:	**6** **12** **24**

Appendix #12

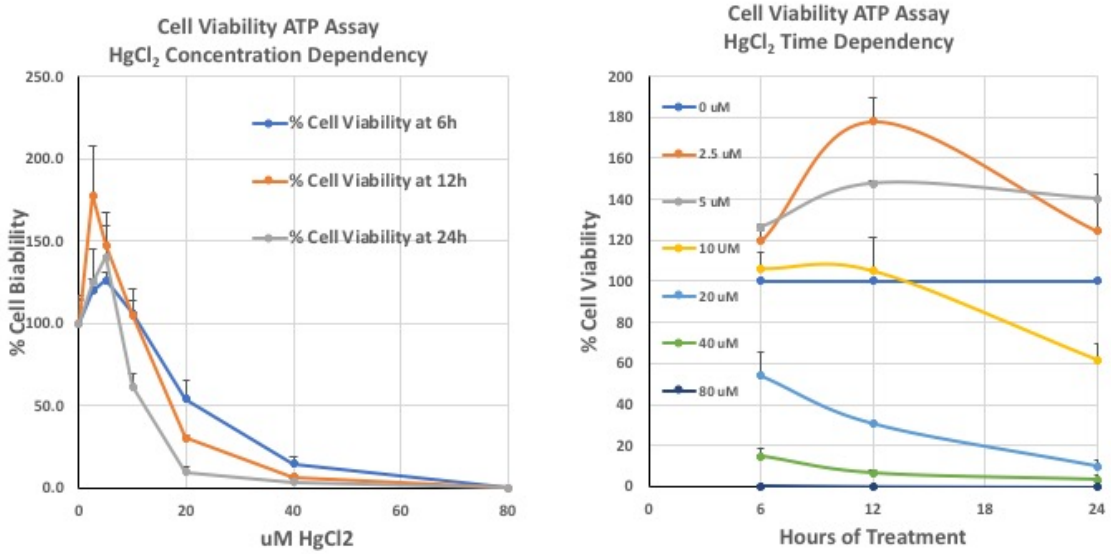
Effect of Mercury Chloride on Functional Viability (ATP levels) of Rat Hepatocytes





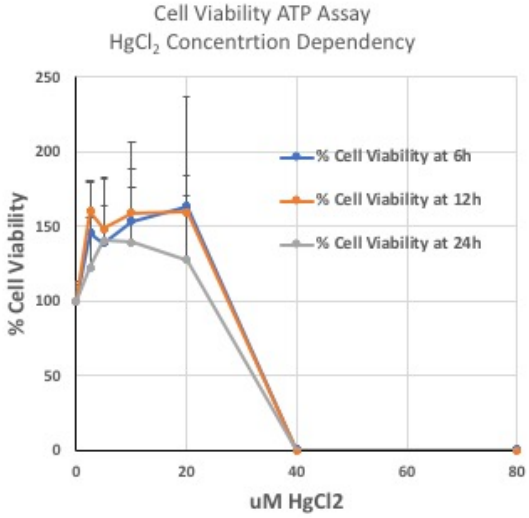
Appendix #13

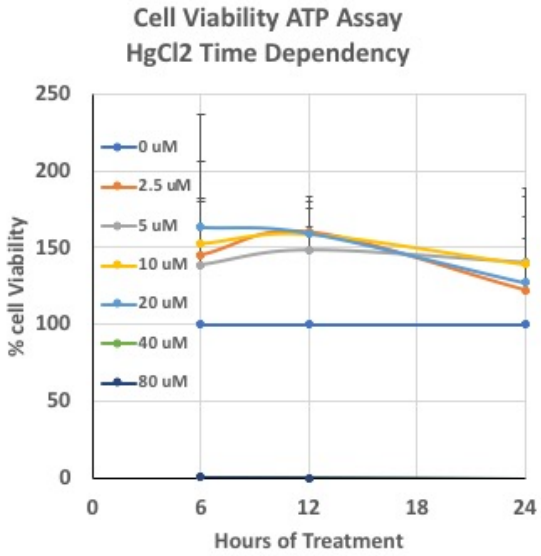
Effect of Mercury Chloride on Functional Viability (ATP levels) of Rat Renal Cells



Appendix #14

Effect of Mercury Chloride on Functional Viability (ATP levels) of Rat Cardiomyocytes





Appendix #15

Animal:	Guinea pig		
Reagents:	DME/F12 (1:1)		
		DMEM	4.5g D-glucose/l
			L-glutamine
			Sodium pyruvate
			Sodium bicarbonate
		F12	HEPES pH7.4
			20 mM sodium bicarbonate
		Penicillin 0.92 x 10^5 IU/l	
	Iron oxide powder <5 µm 95% (Aldrich Lot# MKDC 8301		
		0.5% wv of iron oxide solusion (0.75 g/15 ml DEM/F12	
	Epithelial Cell Medium EpiCM-A, SclenCell Cat# 4131)		
		Adde	5 ml Penicillin/Strep
			10 ml FBS
			Epithelial cell grouth supplement
	Soybean trypsin inhibitor (Type 1-S: soybean, Sigma)		
		0.1% stock solusion)	
	Collagenase H stoch solution (10 mg/ml)		

Procedure	
	Perfuse both kidneys with DME/F12 which in on ice for washing out blood.
	Infise iron particle solution at 1 ml/sec for 15 seconds.
	Remove and transfer the kidneys in ice-cold DME/F12, and decapsulate the kidneys.
	Slice the cortex from the kidney and make 1 mm^2 cubes.
	Put the cubes on 380 µm metal sieve and smash the cube by glass bar.
	Suspend stuffs, which are through the 380 µm metal sieve, in 50 ml ice-cold DME/F12.
	Remove the glomedulars from the suspension using magnet sterlar.
	Filter the suspension through 85µm nylon mesh.
	The proximal tubes are catched by the filter.
	Wash proximal tubes off the nylon mesh into culture dish with 10 ml warmed (37C) DME/F12.
	Add 25 µl soybean trypsin inhibitor and 50 µl collagenase stoch solution.
	Incubate for 2 minutes at 37C.
	Centrifuge it for 5 min at 500 rpm.
	Decant supernatant and add 10 ml of warmed (37 C) "Epithelial Cell Medium
	Centrifuge it for 5 min at 500 rpm.
	Decant supernatant and resuspend thbes in 10 ml of warmed (37 C) "Epithelial Cell Medium
	Plate 5 ml tube suspension into each of two 60 mm plates.