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Deleterious effect of mercuric chloride on human epithelial cells

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Deleterious effect of mercuric chloride on human epithelial cells

By

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A Thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Applied Science

in the program of

Environmental Applied Science and Management

Toronto, Ontario, Canada, 2003

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Abstract

Changhai (Kevin) Ji, 2003. *Deleterious effect of a soluble mercuric compound on human epithelial cells.* A thesis presented to Ryerson University in partial fulfillment of the requirements for the degree of Master of Applied Science in the Program of Environmental Applied Science and Management

This thesis studied the cytotoxicity of mercuric chloride on human epithelial cells, Hep2. Results of crystal violet viability assay showed that treatment with HgCl_2 at concentrations $< 5 \times 10^{-6} \text{ M}$, had no effect on cell viability, while concentrations $> 5 \times 10^{-6} \text{ M}$, resulted in a significant decrease in cell viability. Using a comet assay to assess DNA damage showed maximum comet formation and length at concentrations $< 5 \times 10^{-6} \text{ M}$ HgCl_2 , DNA damage also increased as exposure time increased. Results of an acridine orange/ethidium bromide assay indicated that at low HgCl_2 concentrations, cell death occurred by a mix of apoptosis and necrosis while at high concentrations of HgCl_2 , cell death occurred primarily by necrosis. Mercury speciation study indicated that predominate form of mercury in MEM was labile to resin Ionac SR4. This is the first study to demonstrate a deleterious effect of soluble mercuric chloride on human epithelial cells.

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Chapter 1 Introduction

Human activities play a major role in the pollution of environment with toxic and carcinogenic metal compounds. Industrial pollution of the environment with metal compounds is becoming a significant problem. Unlike most organic pollutants, metals are not degraded and could be accumulated in the environment.

Mercury (Hg) is recognised as a serious threat to human health because it causes central nerves system (CNS) and renal damage and forms neurotoxic organic mercury compounds (Aschner, 1992; WHO, 1991). The effects of Hg on the CNS include neurological damage, irritability, paralysis, blindness, insanity, chromosome damage, and birth defects (Manahan, 1994). The most infamous case of mercury poisoning was the Minamata Bay area of Japan in the 1950's when 111 cases of mercury poisoning were reported and 43 deaths occurred (Manahan, 1994). This event was attributed to the consumption of seafood contaminated with mercury waste from a chemical manufacturing plant.

A lot of studies carried out over 50 years have investigated the activity of Hg compounds in experimental test systems evaluating a variety of genetic endpoints (D'itri & D'itri, 1977; Cantoni & Costa, 1983; Cantoni *et al.*, 1984; Omata *et al.*, 1991). Different mercury compounds tended to produce qualitatively comparable genetic effects, which suggested the involvement of a common toxic entity, methylmercury

derivatives and other ionisable organomercury compounds, were more active in short-term tests than either non-ionizable mercury compounds or inorganic mercury salts.

Mercury is genotoxic. In last 25 years, attention was paid to the assessment of possible genetic effects in exposed humans. Genotoxicity of mercury compounds has usually been attributed to the ability of the metal to bind with tubulin sulfhydryl, which impairs spindle function and causes chromosome aberrations. In human populations, structural chromosomal aberrations, micronuclei or sister chromatid exchanges have been induced by occupational exposure to Hg (Ogura *et al.*, 1996; Popescu *et al.*, 1979 & Anwar *et al.*, 1991).

Many biochemical effects of Hg have been reported. Hg interferes with synthesis of proteins and nucleic acids, inhibits several membrane-bound or cytosolic enzymes, diminishes albumin secretion and alters porphyrin metabolism.

1.1 Scope of Study

In this thesis, deleterious effects of low-level mercuric chloride to human epithelial cells are investigated. The methods applied will be (1) comet assay for detection of DNA damage, (2) crystal violet assay for detection of cell viability and (3) acridine orange/ethidium bromide dying assay for the apoptosis study. Based on the literature review, the single cell gel electrophoresis or comet assay is rather a new test with

widespread potential applications in genotoxicity testing and biomonitoring. The commonly used alkaline version of the test detects DNA strand breaks and alkali liable lesions with high sensitivity. This thesis examines the effect of mercuric chloride on cell viability, DNA damage, nature of cell death of human epithelial cells (HEp2), and includes a preliminary study of mercury speciation in cell culture medium.

1.2 Objectives

The objective of the thesis is to study the deleterious effect of mercuric chloride on human epithelial cells, which represent the portal of entry into the human system. The results of this study will contribute to the knowledge of the risk of soluble mercuric compounds to human health. The detection techniques were developed to monitor the cytotoxicity of soluble mercuric chloride to human health.

1.3 Anticipated results

Mercury is a xenobiotic metal that is well known to adversely affect the immune system. The genotoxic effects of mercuric compounds are different at different concentration levels. There will be induced DNA damage at very low levels (even less than 5 μM), and cell death will occur primarily through apoptosis or necrosis. Through this study we expect to adopt sensitive detection techniques to evaluate the cytotoxicity of mercuric compounds on human health.

Chapter 2 Literature Review

In this chapter, the literature is reviewed to learn about mercury, toxicity of mercury and mercuric compounds, and biomonitoring of mercuric pollutants. First of all, the physical and chemical properties of mercury are reviewed. Then, the sources, the impacts of mercuric pollutants, effects of mercuric compounds on the health of human and other organisms are investigated. The cytotoxicity and DNA damage of mercuric compounds on human cells are discussed. Finally, the detection techniques of DNA damage and identification of cell death are reviewed.

2.1 Physical and chemical properties of mercury

Mercury is an element that occurs naturally in the environment, among the most common of the metal pollutants in the environment (Maier *et al.*, 2000). It is a dense silver-white metal that is liquid at room temperature and standard pressure, thus it is the only metal characterized by low electrical resistance, high surface tension and high thermal conductivity with no hardness, crystal structure, cleavage or streak at room temperature (Wren *et al.*, 1991). It is widely used in its pure form in thermometers, barometers, and other common consumer products. The chemical properties are shown in Table 1.

Mercury occurs in three valence states (0, +1 and +2) and may be present in various physical and chemical forms in the natural environment. The main chemical forms of

Table 1. Chemical properties of mercury

Atomic number	80
Atomic mass	200.59 g.mol ⁻¹
Electronegativity according to Pauling	1.9
Density	13.6 g.cm ⁻³ at 20°C
Melting point	- 38.9 °C
Boiling point	356.6 °C
Vanderwaals radius	0.157 nm
Ionic radius	0.11 nm (+2)
Isotopes	12
Electronic shell	[Xe] 4f ¹⁴ 5d ¹⁰ 6s ²
Energy of first ionisation	1004.6 kJ.mol ⁻¹
Energy of second ionisation	1796 kJ.mol ⁻¹
Energy of third ionisation	3294 kJ.mol ⁻¹
Standard potential	+ 0.854 V (Hg ²⁺ / Hg)
Discovered by	The ancients

mercury exist: elemental mercury, inorganic mercury and organic mercury. The nature and reactions of these species determine the solubility, mobility, and toxicity of Hg in the environment (Ullrich, *et al.*, 2001).

The ecological and toxicological effects of Hg are strongly dependent on the chemical forms (species) present (Clarkson, 1998). The most common form of mercury released to the environment is element form Hg^0 and the divalent form Hg^{2+} . Inorganic Hg forms may be transformed to organic forms. Organic mercury, such as phenylmercury, methylmercury, ethylmercury and methoxyethylmercury, is of great concern because of its adverse effects on human health. Methylmercury is one of the most hazardous environmental pollutants (WHO, 1989, 1991). Hg^+ is only stable as a dimer (Hg_2^{2+}) in aqueous solution and readily disproportionates into H^0 and Hg^{2+} (Ullrich *et al.*, 2001).

2.2 Sources of mercury

The sources of Hg can either be naturally weathered from the Earth's crust or from anthropogenic activities (Aschner, 1992). Mercury enters the environment as a result of normal breakdown of minerals in rocks and soil through exposure to wind and water. Release of mercury from natural sources has remained fairly the same over the years.

Still mercury concentrations in the environment are increasing; this is ascribed to human activity (Suzuki *et al.*, 1991).

Mercury occurs naturally in the environment mainly as mercuric sulphide from the degassing of the earth's crust through volcanic gases, the weathering of rock in mountains and by evaporation from the oceans (Erwin & Munn, 1997; Zelikoff & Thomas, 1998). Mercury is found in soil and rocks typically as an ore known as cinnabar, consisting of insoluble mercuric sulphide. Concentrations in soil and rock average 0.5 mg/kg, though actual concentrations vary considerably depending upon location. It was reported that as much as 25,000 and 125,000 tonnes of Hg are released to environment from natural processes annually (WHO, 1989).

Anthropogenic sources are important, and may contribute more than half of the total emissions (Suzuki *et al.*, 1991). For example, gold mining operations use mercury amalgamation process, which releases a large amount of mercury into environment and have caused substantial mercury pollution in the Madira River of the Amazon River basin in South America (Beyer *et al.*, 1996). Another major human source is the burning of fossil fuels especially coal. Other sources include metal smelter industries, cement manufacture and crematorium. An increasingly important source is the incineration of municipal waste.

Desirable properties make mercury an important industrial metal. Organic mercury compounds including methylmercury have been commercially produced since 1930 (Watanabe & Satoh, 1996). Mercury has over 3,000 uses in many fields like industry, agriculture, the military, medicine, and dentistry (WHO, 1989). For example, some common uses of mercury in industrial products are electrical equipment, chloralkali, paint, thermometers, fungicides, and as preservatives in pharmaceuticals and cosmetics (Maier *et al.*, 2000). The amount of mercury released from the industrial products was about 10, 000 tonnes annually (Maier *et al.*, 2000).

2.3 Fate of mercury in the environment

Mercury and mercury compounds occur naturally in the environment. Through natural resources and anthropogenic activities, elemental mercury vapour is released into the air and changes into inorganic mercury by photochemical oxidation (D'itri & D'itri, 1977; Mahaffey, 1999). Finally, mercury and mercury compounds will redeposit on the earth or be discharged into the water bodies with precipitation as a global mercury cycle. Mercury deposition in the lake has increased at the rate about 1.7% per year over the past 140 years (Minnesota Department of Health, 2001).

2.3.1 Mercury in the aquatic environment

Human exposure to mono-methylmercury (MMeHg) through consumption of freshwater and marine fish is the principal public health concern (Porvari, 2003).

Elevated methylmercury concentrations in fish are common in inland lakes and in the oceans even very far from point sources. Natural and anthropogenic sources and processes affect the distribution and fate of different Hg species. Long-range atmospheric transport and deposition of anthropogenically-derived Hg has caused widespread contamination of soils and lakes even in remote areas, including the boreal forest zone. Building of reservoirs for hydroelectric and flood control purposes has also caused elevated methylmercury concentrations in predatory fish in the northern hemisphere (Potter *et al.*, 1975; Cox *et al.*, 1979; Meister *et al.*, 1979; Schetagne & Verdon, 1999).

When mercury is discharged into water bodies, it is first oxidized to the divalent mercuric ion (Hg^{2+}). In the presence of special group of methylating micro-organisms, the mercuric ion is then transformed into highly toxic, poisonous methylmercury (CH_3Hg^+) and dimethylmercury $(\text{CH}_3)_2\text{Hg}$ in both aerobic and anaerobic environments (Alexander, 1999; Boening, 2000). The primary generator of methylmercury in the environment is the sulphate reducing bacteria, although a variety of microorganisms are capable of methylating mercury. More microbes in the nutrient enrichment water body increase the methylation of mercury. There are more than 10^{10} tonnes of sediments carried to water each year. The organic rich sediment becomes a natural sink for Hg^{2+} and the formation of methylmercury is enormous in the water (WHO, 1976).

2.3.2 Mercury in sediments

Mercury contaminants in sediment could be transferred to the water column via a variety of processes, including diffusion and advection from sediments, sediments re-suspension and release, and biotransfer through aquatic organisms that feed at the sediment-water interface (Masion & Lawrence, 1999). Methylation of inorganic mercury in the sediment of lakes, rivers, and other water-body would transport methylmercury to the water. It does not only affect the microorganism but it also a key step in the aquatic food chain leading to eventual human consumption.

2.3.3 Mercury in the food chain

In the aquatic ecosystem, fish fed zooplankton with a high concentration of methylmercury have a significantly higher concentration of mercury in muscle than fish fed with low methylmercury concentration zooplankton (Hall *et al.*, 1997). In the upper trophic level, there's a great increase in methylmercury concentration through the food chain, called bioaccumulation or bioconcentration (Hemond & Fechner-Levy, 1999). Bottom fauna and plankton absorb methylmercury from the sediments and water; they are eaten by small fish that are in turn eaten by large carnivorous fish (Waldron, 1980). Longer food chains generally result in greater bioaccumulation of mercury (Rasmussen *et al.*, 1990). The bioaccumulation effect is generally compounded throughout the lives of the fish, and large fish and those with longer life span will likely have greater methylmercury level. Finally, humans consume the predatory fish, accounting in part for high methylmercury level in humans.

2.4 Effect of mercury

2.4.1 Effect on health

Mercury can easily enter the body if its vapor is inhaled or if it is eaten in organic forms in contaminated fish or other foods. It can also enter the body when food or water contaminated with inorganic mercury is eaten or drunk, although, at a particular dose level, less will enter the body this way. Mercury, in all forms, may also enter the body directly through the skin (ATSDR Public Health Statement, 1990).

Humans have mercury in their bodies, but at a level that is typically not high enough to cause any health effects (Erwin & Munn, 1997). All forms of mercury are toxic to humans, but methylmercury is especially toxic because the human body has a less well-developed defense mechanism against it (Maier *et al.*, 2000). Methylmercury accumulates in liver, kidney, brain or blood may cause acute or chronic health effects. Moreover, methylmercury affects the central nervous system, and in severe cases, irreversibly damages brain (Thomson, 2000). The nervous systems of embryos and fetuses are more sensitive to methylmercury than mature nervous systems.

Metallic mercury is used in a variety of household products, such as barometers, thermometers and fluorescent light bulbs. The mercury in these devices is trapped and usually does not cause any health problems. However, if a thermometer breaks a

significantly high exposure to mercury through breathing can occur for a short period of time while it vaporizes. This can cause harmful effects, such as nerve, brain and kidney damage, lung irritation, eye irritation, skin rashes, vomiting and diarrhoea (D'tri & D'itri, 1977).

Mercury may turn up in food as it can be spread within food chains by smaller organisms that are consumed by humans, for instance through fish (WHO, 1976).

Mercury concentrations in fish usually greatly exceed the concentrations in the water they live in (Wooding, 1972). Cattle breeding products can also contain eminent quantities of mercury. Mercury is not commonly found in plant products, but it can enter human bodies through vegetables and other crops, when sprays that contain mercury are applied in agriculture.

Mercury has a number of effects on humans, that can all be simplified into the following main effects:

- Disruption of the nervous system
- Damage to brain functions
- DNA damage and chromosomal damage
- Allergic reactions, resulting in skin rashes, tiredness and headaches
- Negative reproductive effects, such as sperm damage, birth defects and miscarriages

Damaged brain functions can cause retardation, personality changes, tremors, vision

changes, deafness, muscle incoordination and memory loss. Chromosomal damage is associated with many congenital diseases, including Down's syndrome (WHO, 1989).

2.4.2 Effect on the environment

Most of the mercury released into the environment from human activities, such as fossil fuel combustion, mining, smelting and solid waste combustion, is released into air. Some forms of human activity release mercury directly into soil or water, for instance the application of agricultural fertilizers and industrial wastewater disposal (Waldron, 1980). All mercury that is released in the environment will eventually end up in soils, waters.

Acidic surface waters can contain significant amounts of mercury. When the pH values are between five and seven, the mercury concentrations in the water will increase due to mobilization of mercury in the ground. Once mercury has reached surface waters, soils microorganisms can convert it to methyl mercury, a substance that can be absorbed quickly by most organisms and is known to cause nerve damage (Mitra, 1986).

Fish absorb great amounts of methyl mercury from surface waters every day. As a consequence, methylmercury can accumulate in fish and in the food chains that they are part of (Post *et al.*, 1996). Developmental effects, even mortality, will happen in fish with a high level of methylmercury (Henry & Heinke, 1996). Different levels of

toxicity could be found in different fish at the same concentration.

Exposure to mercury, such as ingestion, breathing or skin contact could cause animal kidneys damage, stomach disruption, and damage to intestines, reproductive failure and DNA alteration (Boening, 2000).

2.5 Toxicity of mercury

Toxic effects of mercury are expressed in different ways according to the chemical forms of mercury, the dose and the route of exposure in various species of animals. There are two major forms of toxic effects of mercury, nephrotoxic effects and neurotoxic effects (Suzuki *et al.*, 1991). Mercury induces lymphocyte proliferation, increased level of immunoglobulin, autoantibody production, and immune-complex deposits (Pelletier *et al.*, 1988, Hultman & Enestrom, 1987).

Elemental mercury is very toxic (Carroll *et al.*, 2000). Organic forms of mercury are more toxic to aquatic organisms compared with inorganic forms. Methylmercury is recognized as one of the most hazardous environmental pollutants (Watanabe & Satoh, 1996). Developmental effects, tremors, deformities, birth defects, reproductive failure and mortality will happen in fish with a high level of methylmercury (Henry & Heinke, 1996).

The divalent ionic form of inorganic mercury primarily damages the kidney, while both organic and metallic mercury are known to penetrate into the central nervous system (CNS) and produces severe toxicity in this organ (Clarkson *et al.*, 1988). Harmful effects appear to be related to both cell density and concentration of mercury in a substrate. The 96 h LC50 values for freshwater fish ranges between 33 and 400 µg/l, while the LC50 is generally higher for marine fish. There is little indication that fish themselves either methylate or demethylate mercury. As is the case with other groups of ecological receptors, organic mercury compounds are most toxic to birds. Sea birds and those feeding in estuaries are usually most contaminated (WHO, 1989).

2.5.1 Mechanisms of mercury toxicity

It is well known that the toxic symptoms of mercury vary with its chemical forms. A large number of studies have been conducted to gain access to molecular mechanisms of toxic actions exerted by mercury compounds (Suzuki *et al.*, 1991). Mercury is an extremely reactive, very soft metal whose ions form coordinate covalent bonds with ligands such as sulfhydryl groups. The reactivity and toxicity of Hg^{2+} was the highest observed for any divalent metal ion (Williams *et al.*, 1982). Methylmercury is even more toxic than inorganic mercury, particularly to nerve cells (Clarkson *et al.*, 1988).

Mercury is bound to the cell walls or membranes of microorganisms. The basis for the nerve cell specific toxicity of methylmercury may reside in its ability to affect

microtubule functions and also, due to its high lipid solubility, it readily enters cells (Clarkson *et al.*, 1988). Inorganic mercury appears to move into the cells after damage to the membrane barrier, while methylmercury can penetrate cells without damage to the cell membrane (Christensen *et al.*, 1993).

2.5.2 Nephrotoxic effects

Mercurials cause two types of renal injury, tubular damage and glomerular injury (Endou & Jung, 1991). The kidney has been well known as one of the target organs of mercurial compounds. HgCl_2 directly inhibits water channels in the kidney proximal tubule (Pratz *et al.*, 1986). Mercurial compound act on the vasopressin-sensitive water channel, result in inhibition of water flow in the proximal tubule and the toad urinary bladder (Hoch *et al.*, 1989).

Another type of renal injury is glomerular injury. Various mercurials may induce autoimmune glomerular lesions, and other pathological conditions such as pink disease and possibly Kawasaki disease, an acute febrile mucocutaneous syndrome. Some studies have showed that HgCl_2 has a suppressive effect that was considered to be the consequences of direct or indirect alteration or inhibition of enzymes such as pyruvate kinase (Dieter *et al.*, 1983). HgCl_2 may act by modifying "self" cell-surface structure such as class I or class II molecules leading to a type of mixed-lymphocyte reaction (Nordlind, 1985; Reardon & Lucas, 1987).

2.5.3 Neurotoxicity

The central nervous system is one of the critical organs for exposure to mercury. In animals, inorganic mercury can bypass the blood brain barrier and enter motor neurons (Pamphlett & Waley, 1996). The chemical forms of mercury most relevant to the neurotoxic effect are elemental mercury vapour or methylmercury. The mechanism of neurotoxicity caused by methylmercury has been one of the major topics in heavy metal toxicology. Repeated outbreaks of intoxication by this metal alkyl that has occurred in various regions in the world clearly show that the nervous system is the most sensitive tissue to methylmercury exposure (Watanabe & Satoh, 1996; WHO, 1991).

Mercury interacts with brain tubulin and disassembles microtubules that maintain neurite structure. Experiment with rats revealed that chronic inhalation of low-level Hg^0 can inhibit polymerization of brain tubulin essential for formation of microtubules (Pendergrass *et al.*, 1997). Methylmercury affect differently the synthesis of brain proteins and the unusual reduction or elevation of certain protein species caused by perturbation of the synthesis rates by methylmercury may be responsible for the impairment of normal nerve function (Omata *et al.*, 1991).

Membrane structure of the cell may be the first target of methylmercury in conjunction with lipid peroxidation (Verity & Sarafian, 1991). In the occipital cortex of cats administered with a toxic or subclinical dose of methylmercury, the histological changes were contrasted to the change in alpha-norepinephrine receptors (Sato &

Nakamura, 1991). The capacity of the receptors in the occipital cortex was reduced even in the cats showing no historical abnormalities with brain Hg concentrations of around 10 $\mu\text{g/g}$ wet weight.

2.6 DNA damage study

DNA can also be a target for mercury toxicity. Mercuric chloride is very potent at producing DNA damage in mammalian cells (Cantoni *et al.*, 1982; Cantoni & Costa, 1983; Cantoni *et al.*, 1983; Cantoni *et al.*, 1984; Christie *et al.*, 1986). HgCl_2 induced DNA single strand breaks in Chinese hamster ovary cells (Costa *et al.*, 1991). There is evidence that low-dose exposure (0.5 mM) to mercury (II) may lead to DNA damage in fibroblasts and Chinese hamster ovary cells (Ariza *et al.*, 1994; Hamilton-Koch *et al.*, 1986). DNA could be fragmented as a result of mercury dependent induction of apoptosis as has been suggested (Shenker *et al.*, 1997; Insug *et al.*, 1997).

2.6.1 Molecular mechanism of HgCl_2 toxicity

Molecular mechanisms of mercury toxicity are not well understood. The DNA damage induced by mercuric chloride was probably due to superoxide radical formation that was confirmed by cytochrome C reduction and its depression in the absence of superoxide dismutase. HgCl_2 induced DNA damage has many similarities to those caused by X-rays, but the single strand breaks induced by HgCl_2 are not easily repaired in contrast to those induced by X-rays (Costa *et al.*, 1991). HgCl_2 has been shown to

induce single strand breaks, not alkali-labile sites. The binding of mercury to DNA was shown to be very tight since it resisted extraction with high salt and chelating agents (Christie *et al.*, 1986; Costa, *et al.*, 1991).

The incomplete repair of DNA strand breaks caused by mercuric mercury may account for the incapability of mercuric chloride to function either as a mutagen or as a carcinogen. Methylmercury was shown to be more potent in causing DNA strand breaks than inorganic mercury especially in nerve cells in which the strand breaks were produced even at concentrations as low as 10^{-6} M (Costa *et al.*, 1991). The higher sensitivity of nerve cells in terms of DNA damage by methylmercury can be explained by efficient uptake of methylmercury by nerve cells as in the case of its cytotoxicity towards mouse glioma cells (Miura & Imura, 1991).

2.6.2 The genotoxicity of HgCl_2

The genotoxic effects of mercury have been reported from a variety of systems. The genotoxicity of HgCl_2 has been evaluated and found to be inconsistent. Following oral administration of chronic and acute sub-toxic doses of HgCl_2 to male rats, the percentage of chromosomal changes increased and mitotic index decreased in proportion to the dosages and the time of treatment to a significant extent (Das *et al.*, 1983). A mutagenicity assay using NIH 3T3 cells transfected with vector containing *lacZ* as a reporter for mutational events, showed a significantly increased mutational

frequency in the *lac Z* gene with HgCl_2 , even at the lowest concentration tested (Schurz *et al.*, 2000). HgCl_2 was found to be genotoxic in the mutatox and SOS tests. However, it was not mutagenic in Ames test with different *Salmonella typhimurium* strains (Codina *et al.*, 1995).

2.6.3 Genotoxicity Assay

Short-term genotoxicity tests are widely used as screening tools to evaluate the mutagenic potential of industrial chemical and pharmaceutical products. The single cell gel electrophoresis (SCGE) test or comet assay is rather a new test with widespread potential applications in genotoxicity testing and biomonitoring (Tice *et al.*, 1991; Collins *et al.*, 1997). The single cell gel electrophoresis assay is a rapid and sensitive method for the detection of DNA damage in individual cells, induced by a variety of genotoxic agents (Singh *et al.*, 1988). The assay is based on the embedding of cells in agarose, their lysis in alkaline buffer and finally subjection to an electric current.

The commonly used alkaline version of the test detects DNA strand breaks and alkali labile lesions with high sensitivity. During the last few years, many known mutagens and genotoxic carcinogens have been tested with the comet assay and the sensitivity of the test to assess directly induced DNA damage has been undoubtedly demonstrated (Bucio *et al.*, 1999). Furthermore, since the DNA migration data are obtained on a cell basis, this assay can measure the intracellular distribution of DNA damage and repair (Singh *et al.*, 1991). Previous studies have suggested that cytotoxicity and the DNA

breakage induced by heavy metals may be associated with active oxygen species inside cells, because active oxygen scavengers prevented metal induced cellular injuries such as DNA breaks (Snyder, 1988; Tsuzuki *et al.*, 1994). The relationship between active oxygen and cytotoxicity of Hg is not clear.

There is considerable evidence to suggest that programmed cell death (apoptosis) is a distinct biochemical pathway essential for all multicellular organisms. Under normal conditions, a careful balance between proliferation and cell death is required to maintain homeostasis (Vaux, 1993; King & Cidlowski, 1995). However, a number of diseases including autoimmune diseases and infectious microorganisms can trigger increased levels of apoptosis, thereby leading to organ failure and even death. There are also a number of exogenous agents that can trigger apoptosis in human cells.

Mercury may compromise immune function by initiating apoptosis in T cells and monocytes (Insug *et al.*, 1997; Shenker *et al.*, 1997). DNA could be fragmented as a result of mercury-dependent induction of apoptosis. To compare mercury-induced comets with comets known to result from apoptotic DNA damage, control cells were exposed to anti-fas. Fas, membrane receptor that controls apoptosis in many cells, normally binds to fas ligand to trigger apoptosis (Bucio *et al.*, 1999). However, when fas is cross-linked by anti-fas antibodies, apoptosis is also initiated.

Low concentrations of methylmercury (10^{-10} – 10^{-7} M) cause apoptosis of the astroglial component in a three-dimensional cell culture system of fetal rat telencephalon at different developmental periods (Monnet-Tschudi, 1998). Moreover, methylmercury induced apoptosis has been demonstrated in D384 human astrocytoma cells (Dare' *et al.*, 2001). It is difficult to identify the actual mechanism or mechanisms by which methylmercury triggers apoptosis, however, because multiple effects on a series of subcellular targets have been described in different models. Alteration of calcium homeostasis (Hare *et al.*, 1993), microtubule depolymerization (Miura *et al.*, 1999; Castoldi *et al.*, 2000), lysosomal damage (Dare' *et al.*, 2001) and reactive oxygen species (ROS) generation (Atchison & Hare, 1994; Yee & Choi, 1996; InSug *et al.*, 1997; Shenker *et al.*, 1999) are among the most acknowledged effects, some of which are associated with apoptosis.

Although the direct oxidative properties of mercury and its compounds are largely accepted, the actual effects of ROS generation are still controversial. In this regard, it is noteworthy that in vitro experiments concerning the protective action of different antioxidants and manipulation of the intracellular scavenging attitude have led to contrasting results (Park *et al.*, 1996; Ou *et al.*, 1999; Dare' *et al.*, 2001). No matter how ROS are generated, they have several intracellular targets with different pathogenetic implications. Aside from the effects due to lipid peroxidation and thiol groups reactivity, specific oxidative DNA damage could be of particular relevance, as it has been assessed in human lymphocytes (Ogura *et al.*, 1996; Lee *et al.*, 1997).

2.7 Mercury speciation

The most excessive use of mercury in industry and agriculture has ceased due to the poisonous and insidious nature of mercury and mercury compounds. Methylmercury may still be found at high concentrations (0.5 - 1.0 mg/kg) in fish caught in rural parts of Sweden, Canada and the US. Since most of the mercury found in the atmosphere is in its metallic form, in natural waters as divalent inorganic mercury, and in fish as monomethylmercury, the need for analytical methods able to differentiate between these mercury species is obvious (such determinations are termed "speciation"). Although many methods for the determination of total mercury exist, extraction of useful information concerning mercury species is limited.

The toxicity, biochemical behavior and transportation of mercury in the environment are highly dependent on its physio-chemical form. It has been shown that organic mercury compounds, which may be up to one thousand times more toxic than inorganic mercury, may also be formed through methylation of inorganic mercury by organisms and bacteria. Organic mercury found in fish and waters is almost always methyl mercury. As with all instrumentation for speciation, it is imperative that the techniques offer good separation and sensitivity in order to quantify the individual species with both accuracy and precision.

Speciation is the determination of the individual physico-chemical forms of an element, which together make up its total concentration in a sample. Knowledge of speciation is important because toxicity, bioavailability, bioaccumulation and transport of a particular element depend critically on the chemical form.

Most experimental studies on the toxicokinetics as well as toxicodynamics of mercuric compounds have used water-soluble salts and administered the compounds in solution. The occupational or dietary exposure of humans to mercuric compounds is often a mixed exposure to different species of mercury and with concomitant exposure to potential ligands. Bioavailability is the combination of bioaccessibility and absorption. If the mercuric compounds are bound strongly to ligands in the intestinal tract, they might in some cases not be accessible for the absorptive process, whereas other ligands may even facilitate the absorption. If the mercuric species is insoluble, absorption may be low.

2.8 *Detection techniques for DNA damage and cell death*

Due to the increasing input of mercuric contaminants into the environment, it has become necessary to develop sensitive and reliable methods to assess the impact of mercury and its compounds on organisms at the early exposure stage. Recent work in biomonitoring has concentrated on biomarkers of exposure, such as induction of cytochrome and the metallothionein, as specific indicators of respectively contaminants

(Livingstone, 1993). Considerable success has been achieved correlating these measurements with pollution levels in vertebrates, particularly in fish. In terms of biomonitoring, sessile organisms are ideal candidates for this purpose and some progress has been achieved using these biochemical measurements (Mitchelmore *et al.*, 1998).

The consequences of exposure and metabolism of contaminants may be assessed by investigating biomarkers of contaminant damage, for example, DNA adducts, DNA strand breaks (SBs) and apoptosis. The ^{32}P -postlabelling assay has been employed to detect DNA adducts in field and laboratory studies. However, increase in DNA adduct levels have been demonstrated with laboratory exposure to organic xenobiotics, field studies have had conflicting results, with both increase and no differences being seen between clean and polluted sites (Marsh *et al.*, 1993).

Strand breaks are a potential biomarker because these are common modifications that may be produced by a wide range of agents and mechanisms. Breaks may be directly produced by chemicals such as hydrogen peroxide and other reactive oxygen species. The latter may results from endogenous metabolism or may be produced in excess from redox cycling or other free radical interactions associated with organic xenobiotics, metabiolites, and transition metals. Alternatively, SBs may results from alkali-labile sites or during excision repair of DNA adducts (Fairbairn *at al.*, 1995).

Apoptosis and necrosis are two distinct forms of cell death (Renvoize *et al.*, 1998; Balla *et al.*, 2001; Martin, 2001). In the early 1970s the discovery of new patterns of cell death led to emergence of the concept of apoptosis (Kerr *et al.*, 1972). Apoptosis is considered as an example of a programmed cell death (PCD) and it is a distinct mode of cell death that is responsible for deletion of cells in normal tissues. Morphologically, apoptosis involves rapid condensation and budding of the cell, with the formation of membrane-enclosed apoptotic bodies containing well-preserved organelles. There are various assay methods for evaluating the apoptosis on cultured cells. The crystal violet staining assay is a simple and reproducible assay of cytotoxicity which stains only viable cells (Chiba *et al.*, 1998). Acridine orange / ethidium bromide staining assay is proven a valid technique to assess apoptosis and necrosis. Acridine orange is excluded from live cells but stains nuclear fragments in apoptotic cells.

The main morphologic criteria for apoptosis usually are cellular shrinkage, condensation and margination of the nuclear chromatin, DNA fragmentation, cytoplasmic vacuolisation, and cell lysis. A characteristic biochemical feature of the process is double-strand cleavage of nuclear DNA at the linker regions between nucleosomes leading to the production of oligonucleosomal fragments. Recently, it has been suggested that mercury may induce immune dysfunction by triggering apoptosis in immune cells (Ben-Ozer *et al.*, 2000).

Necrosis process can start only and exclusively when the cell dies and an irreversible process, a 'no return' way in the cell life (Popper, 1988). Necrosis is ordinary cell death with the characteristics of a passive process while apoptosis is special form of cell death with the characteristics of an active process (Denecker *et al.*, 2001 and Rana *et al.*, 2001). Necrosis is associated traditionally with inflammation (Kelly *et al.*, 2001), whereas apoptosis is a genetically controlled, energy-dependent method of cellular deletion without inflammation. Necrosis occurs in response to more severe forms of the same types of injury, but apoptosis occurs in response to any mild injury (Uezono *et al.*, 2001). Apoptosis occurs via a coordinated, predictable and pre-determined pathway, while necrosis results from the additive effect of a number of independent biochemical events that are activated by severe depletion of cell energy stores. Necrosis is difficult to prevent, whereas the apoptotic pathway can potentially be modulated to maintain cell viability (Kanduc *et al.*, 2002).

Techniques commonly used to detect SBs in organisms are alkaline elution, alkaline unwinding and agarose gel electrophoresis. Recently a new technique, the single cell gel electrophoresis (Comet Assay), is being investigated (Pandurangi *et al.*, 1995; Steinert, 1998). This technique exhibits many advantages over the previous assays (Wilson *et al.*, 1998). It is sensitive, required small samples of any eukaryotic cell population, and allows the quantitation of single cells so that heterogeneity and subcellular population responses can be examined (Fairbarin *at al.*, 1995). Although there are potential limitations in the use of this technique for the screening of chemicals

for genotoxicity, it may be exploited as an initial indicator of general DNA damage in organisms of genotoxicity and the specific nature of the DNA lesions.

Chapter 3 Materials and Methods

3.1 Cell culture

The human epithelial cell line HEp2 (human laryngeal cell line) was obtained from American Type culture Collection, Rockville, MD. All the cells used in this study were between passages 14-40. Cells were routinely maintained in minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% FCS (fetal calf serum, Cansera International Inc.) with 0.1% gentamycin (400 mg/ml) in 25-cm² flasks in a 5% CO₂ incubator at 37 °C. The medium was replaced every 2-3 days and the cells were harvested and diluted seven-fold every 7 days.

3.2 Crystal Violet Assay

Crystal violet assay is based on the growth rate reduction reflected by the colorimetric determination of the stained cells. It could be used to test the viability or apoptosis of cultured cells. Surviving cells after treatment were fixed and stained with crystal violet. After washing off the dead cells the absorbance at 570 nm of the remaining cells attached to the plastic plate can be measured using a spectrophotometer and the plate can also be photographed for a visual record of the assay.

3.2.1 Chemical preparation

Cells will be treated with various concentrations of mercuric chloride (HgCl_2) in 96 well cell culture clusters for 24, 48 and 72 h. Mercuric chloride concentrations were 0, 0.5, 1, 2, 5, 10, 20, 50 μM . MgCl_2 was used as controls (test the impacts of total ion strength on cell viability). Each treatment has 3 replications.

3.2.2 Cell preparation

HEp2 cells were spited one day before the experiment. When cells confluence reaches 90%, cells will be transfer to 96 sterile well plates. When cell grew until 80% confluence in MEM media with FCS and antibiotics (gentomycin), Added treatment medium to plates for 24, 48 or 72 hours.

3.2.3 Crystal Violet Microtitre Plate Stain

After 24 hours treatment, treatment solution and dead cells were poured off plate wells after vigorous shaking. Remained living cells were fixed using a 2% solution of formalin in Phosphate Buffered Saline (PBS) (65 μl /well) and incubated for 1 minute at room temperature.

Formalin solution was poured off into waste beaker. 65 μl of tissue culture stain was added to each well and incubated at room temperature for a minimum of 20 minutes (or 2 hours to overnight). All wells were washed with de-ionized H_2O twice and air dried. Plates were stored indefinitely or photographed for a visual record of the assay.

To enable graphing of the results, 100 μ l 10% acetic acid was added to each well, tapped gently to disperse the dye, and read at 570 nm in ELISA plate reader.

3.3 Comet assay

3.3.1 Reagent Preparation

Phosphate Buffered Saline (PBS) (Ca^{++} and Mg^{++} free, 0.001M) was diluted from 10 X PBS (0.01M) (Trevigen) stock solutions and stored at room temperature. Lysis solution (40 mL) was chilled at 4°C, or on ice, for at least 20 minutes before use. (Lysis solution contained 2.5 M sodium chloride, 100 mM EDTA pH 10, 10 mM tris base, 1% sodium lauryl sarcosinate, and 1% triton X-100).

The comet LMAgarose was ready to use once molten. The cap was loosening to allow for expansion when the bottle was heated in a 90 -100°C water bath for 5 minutes, or until the agarose was molten. The bottle was placed in a 37°C water bath for at least 20 minutes to cool. The LMAgarose would remain molten at 37°C for sample preparation indefinitely. (Comet LMAgarose contains 1% low melting point agarose in 1X PBS.)

Alkaline solution (50 mL, pH >13) was made with NaOH pellets (0.6g0, EDTA (250 mL 200 mM), and deionized water (49.75 mL). After all theses components were stirred until fully dissolved. The solution would warm during preparation and to cool to room temperature before use.

TBE (1x) electrophoresis buffer was used as electrophoresis solution. Concentrated TBE (10x) electrophoresis buffer was prepared with Tris (0.89 M), Boric acid (0.89 M) and EDTA (0.02 M) in aqueous solution. TBE (1x) electrophoresis buffer was diluted from TBE (10x) electrophoresis buffer with deionized water. This buffer solution was autoclaved and stored at room temperature.

SYBR® Green staining solution was prepared from the SYBR Green concentrate provided (10,000X concentrate in DMSO). SYBR Green stain (1 μ L) was added to 10 mL pH 7.5 TE Buffer (TE buffer: 10 mM Tris-Cl pH 7.5, 1 mM EDTA). The diluted stock was stable for several weeks when stored at 4°C in the dark.

Anti-fade solution contained 500 mg p-Phenylenediamine dihydrochloride and 4.5 mL 0.001 M PBS. NaOH (400 μ L, 10 N) was added dropwisely with stirring until pH of solution reached 7.5-8.0. 0.001 M PBS was added to increase the volume to 5 mL, and then added 45 mL of glycerol for a final volume of 50 mL. Vortex mixture thoroughly and applied 10 μ L per sample, covering samples with cover slip. Nail polish may be used to seal cover slip. Anti-fade solution was stored at -20°C for up to one month.

3.3.2 Sample Preparation and Storage

Treated cells were gently scraped off from each well with a rubber policeman. Cells and medium were transferred to centrifuge tubes; cells were counted and then pelleted.

Cells were washed with ice cold 0.001M PBS (Ca^{++} and Mg^{++} free) and were resuspended at 1×10^5 cells/ml in ice cold 0.001M PBS (Ca^{++} and Mg^{++} free).

Lysis solution was prepared and chilled at 4°C or on ice for at least 20 minutes before use. LMAgarose was melted in a beaker with boiling water for 5 minutes, with the cap loosened. LMAgarose bottle was stored in a 37°C water bath for at least 20 minutes to cool. The temperature of the agarose was critical or the cells may undergo heat shock. Heat blocks are not recommended for regulating the temperature of the agarose.

Treated cells were combined at $1 \times 10^5/\text{mL}$ with molten LMAgarose (at 37°C) at a ratio of 1: 10 (v/v) and were immediately pipetted 75 μL agarose/cells mixture onto comet slide. A side of pipette tip was used to spread agarose/cells over sample area to ensure complete coverage of the sample area. Aliquots of the molten agarose were placed into pre-warmed micro-centrifuge tubes, and the tubes were stored at 37°C . Cells were added to one tube, mixed well by gently pipetting once or twice, then 75 μL aliquots were transferred onto each sample area as required.

Comet slide with cells was placed flat at 4°C in the dark for 10 minutes. A 0.5 mm clear ring would appear at the edge of comet slide area. Gelling time was increased to 30 minutes to improve adherence of samples in high humidity environments.

Comet slide was immersed in prechilled Lysis Solution and left on ice for 30 minutes to 60 minutes. Excess buffer was taped off from slide and immersed in freshly prepared Alkaline Solution. Comet slide was left in alkaline solution for 20 to 60 minutes at room temperature, in the dark.

Comet slide was removed from the alkaline solution and gently taped off excess buffer from slide, and immersed in 1X TBE buffer for 5 minutes twice washed the slide.

Comet slides were transferred from 1X TBE buffer to a horizontal electrophoresis apparatus. Slides were placed flat onto a gel tray and align equidistant from the electrodes. TBE buffer (1x) was poured until buffer level just covered all slides. Power supply was set to 1 volt per cm (measured electrode to electrode) and applied voltage for 10 minutes.

Slides were removed from electrophoresis apparatus after 10 minutes. Excess TBE was taped off very gently, and then dipped the slides into 70% ethanol for 5 minutes. Slides were air-dried. Drying brought all the cells in a single plane to facilitate observation. At this stage, samples might be stored at room temperature, with desiccant.

Diluted SYBR® Green (50 μ L) was added onto each circle of dried agarose on the slides. Each slide was checked under epifluorescence microscopy. (SYBR Green's

maximum excitation and emission are respectively 494nm/521nm. Fluorescence filter was adequate).

3.3.3 Data Analysis

When excited (425 - 500 nm) the DNA-bound SYBR® Green emits green light. In healthy cells the fluorescence is confined to the nucleoid: undamaged DNA is supercoiled and thus does not migrate very far of the nucleoid under the influence of an electric current. In cells that have accrued damage to the DNA, the alkali treatment unwinds the DNA, releasing fragments that migrate from the cell when subjected to an electric field. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage. When using TBE as the electrophoresis buffer, the length of the comet tail may be correlated with DNA damage. The characteristics of the comet tail including length, width, and DNA content may also be useful in assessing qualitative differences in the type of DNA damage.

3.3.3.1 Qualitative Analysis

The comet tail can be scored according to DNA content (intensity). The control (untreated cells) should be used to determine the characteristics of a healthy cell. Scoring can then be made according to nominal, medium or high intensity tail DNA content. At least 75 cells should be scored per sample. Comet tail lengths were measured by displaying the stored images on a diskette that was calibrated for distance.

Comet tail lengths did not include the size of the nucleus. Comparison between groups was performed with Student's *t*-test. A *p*-value < 0.01 was considered statistically significant. Each mercury treatment was replicated nine times with at least 150 cells being examined for each concentration. The positive controls were the cells exposed to Verotoxin (VT1); the negative controls were those cells allowed to proliferate for 24, 48, and 72 h in the absence of mercuric chloride or VT1.

3.3.3.2 Quantitative analysis and statistical analysis

Cells were exposed to various concentrations of mercury for 24, 48, and 72 h. Cells forming comets was then assessed as described above. In an individual experimental setup, for each concentration of mercury at least 200 cells were analyzed at each time point. Statistical comparisons were performed using one- or two-way analysis of variance (ANOVA) and Student's *t*-test.

3.4 Apoptosis analysis

3.4.1 Reagent preparation

Acridine orange is excluded from live cells but stains nuclear fragments in apoptotic cells. Acridine orange / ethidium bromide dye mixture: 10 mg of acridine orange and 10 mg of ethidium bromide each were dissolved in 5 mL 0.001 M PBS solution.

3.4.2 Cell preparation and treatment

HEp2 cells were harvested and transferred to 6-well plates one day before the treatment. The cells were grown to 80% confluence in MEM media with FCS and antibiotics (gentomycin), and washed twice with 2 mL 0.001 M PBS each well, then incubated with 2.5 μ M mercuric chloride for 24 hours at 37 °C in 5% CO₂. MEM without mercuric chloride served as negative control. Verotoxin (VT1, 50ng/mL) was used as control for induction of apoptosis, as positive control.

3.4.3 Apoptosis protocol

After treatment, all culture media in each wells was transferred into 15 mL falcon tubes, And each well was rinsed with 1 mL PBS and dumped into the same Falcon tube. Trypsin (1 mL) was added to each well and incubated 2-3 minutes at 37⁰C in 5%CO₂. FCS (200 μ L) was added to each well and collected into the same Falcon tubes. Each well was rinsed at least once with 1 mL PBS to ensure that all cells have been harvested. Finally all wells were checked under the inverted microscope to ensure all cell have been collected. All samples were stored on ice or in the fridge.

The Falcon tubes contained cells were centrifuged for 10 minutes at 4°C and 1200 RPM. Supernatant in the tubes was removed. Cells in the tubes were resuspended in 0.5 mL 0.001 M PBS and were transferred to 1.5 mL eppendorf tubes. The samples in the

eppendorf tubes were centrifuged at the same condition above, removed supernatant and resuspended the cells in 100 μ L 0.001 M PBS.

After 10 μ L of acridine orange/ethidium bromide staining mixture was added to the 100 μ L suspension, the samples in the eppendorf tubes were centrifuged for 10 minutes at the same condition, and removed supernatant, resuspended in 100 μ L 0.001 M PBS. The samples were centrifuged again and resuspended in 100 μ L 0.001 M PBS after removed the supernatant.

Mixed well, 10 micro-liters of the stained samples was added to a slide and identified under the inverted fluorescence microscope. 200 cells were counted each slide, scored each cell as early apoptotic, late apoptotic, necrotic or viable cell by morphological difference, and converted to percentage. Under fluorescence microscope, the chromatin started to condense and marginate in early apoptotic cells but still in green color. In late apoptotic cells the chromatin showed clearly condensation and margination with orange color. Viable cells showed a normal chromatin and green color whereas necrotic cells appeared red color without chromatin condensation or margination.

3.5 *Mercury speciation*

3.5.1 Hg^{2+} MEM preparation

Hg^{2+} may be binded by amino acid components or other organic components of the MEM.

A thiol-based ion exchange resin Ionac SR 4 (Bostick and Klasson, 1998) (Table 2 & 3) was packed in the buret with 610 mm bed depth. Ionac SR-4 is a macroporous, weakly acidic polystyrene/divinylbenzene cation resin with thiol functional groups. HgCl_2 contained MEM solution was filtered through the Ionac SR4. 10 micro-liter of the sample would be measured. Hg contents in the solutions before and after passing through the column were measured with CVAFS Mercury Detector 2500 (Tekran).

3.5.2 Instrument

CVAFS Mercury Detector 2500 (Tekran) is very sensitive mercury detector to vapor mercury. The sample was vaporized at 500-600 °C to release vapor mercury, gold trapper at normal temperature trapped the vapor mercury, and then the gold trapper was heated to 500-600 °C to release the trapped mercury and carried to the detector by Argon gas. The mercury detection was showed in Figure 1.

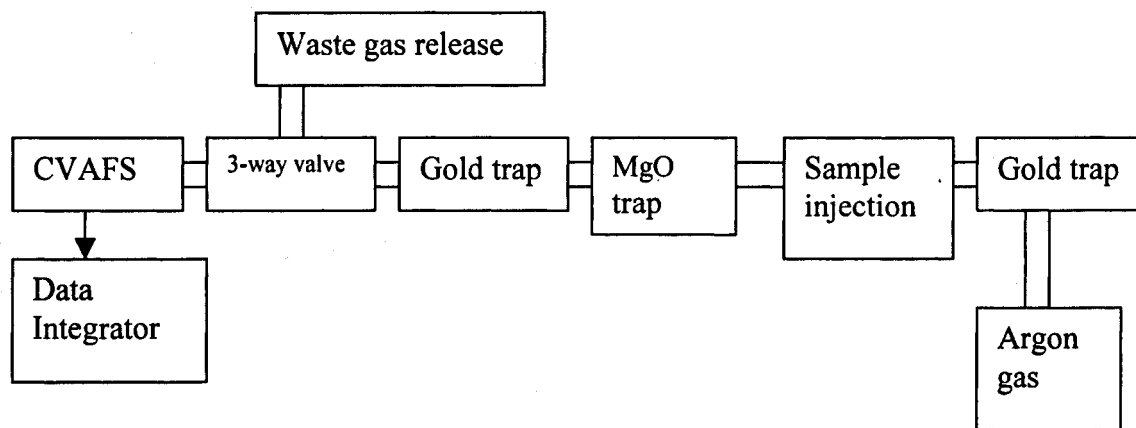


Figure 1. CVAFS Mercury Detection Apparatus

3.5.3 Standard curve

5, 10 and 15 micro-liter of standard mercury source (Model 2505 – Mercury Vapor Calibration Unit) was injected to Cold Vapor Atomic Fluorescence Spectrometry (CVAFS) and measured the areas. The measured areas and the standard weight were used to make a standard curve. The curve was used to calculate the weight of Hg^{2+} MEM samples injected.

3.5.4 Sample measurement

Ionac SR 4 resin was filled into burette with height of 620 mm. Burette was immersed in 4N HCl overnight to clean potential attached mercury, and rinsed with deionized water. After the burette dried SR 4 resin was packed into burette. HgCl_2 MEM (15 mL, 2.5 μM) medium was filtered through the resin column. Filtered sample (10 μL) was injected to sample injection part of the CVAFS apparatus. The sample injection part was heated to 500-600 °C for 6 minutes. Mercury would be vaporized in the sample part and was carried to MgO trap part. The temperature of the MgO trap was 800 °C, and all forms of mercury supposed to become elemental mercury through the reaction with MgO. Elemental mercury was trapped by gold trap when carried by Argon gas to the gold trap. The gold trap was heated to 30 seconds and released all mercury to CVAFS and measured the contents of mercury. Peak areas of samples recorded by Data integrator were used to calculate the contents of Hg in the samples of each

measurement. Each sample was measured 3 times to get the average weight of mercury in each sample.

Table 2. Typical physical and chemical properties of Ionac SR4

Typical physical and chemical properties		International Units	
Ionic form as shipped			H+
Bead size	> 90%	mm	0.3 - 1.25
Effective size		mm	0.47 +- 0.06
Shipping weight		g/l	736
Density		g/ml	1.02
Water retention		%	35 - 40
Total capacity, min.		eq/l	2
Stability	temperature range	°C	1 - 25
	pH range		0 - 14
Storability	of product	min. years	2
	temperature range	°C	1 - 25

(Sybron Chemical Inc., 2003)

Table 3. Recommended Operating Parameters of Ionac SR4

Recommended Parameters		Operating	International Units	
Operating Temperature		max. °C	35	
Operating pH-range			5 - 10	
Bed Depths		min. mm	610	
Pressure Drop			see chart	
Max. adm. Pressure drop		kPa	200	
	exhaustion	m/h	5 - 24	
Surface Flow Rate				
	backwash	m/h	see chart	
Bulk Flow Rate	exhaustion	BV/h	8 - 32	
Bed Expansion		%	50 - 65	
Freeboard	% of bed depth	%	90 - 100	
Regenerant	type		Thiourea	HCl
	level	g/l	3 - 5	
	concentration	%	5	2 - 4
	regeneration	m/h	1 - 10	
Surface Flow Rate				
	rinsing, slow / fast	m/h	1 - 10 / 5 - 24	
Bulk Flow Rate	regeneration	BV/h	2 - 8	
	rinsing, slow / fast	BV/h	2 - 8 / 8 - 40	
Rinsing Requirement	Water slow / fast	BV	1 - 2 / 3 - 8	

(Sybron Chemical Inc., 2003)

Chapter 4 Results and Discussion

4.1 Cell viability

To study the effects of mercuric chloride on HEp2 cells, we first examined the effects of mercuric chloride on HEp2 cell viability. Cells were initially cultured at 2×10^5 cells/ml. To examine viability, HEp2 cells were exposed to various concentrations of mercury for 24, 48, and 72 h incubation periods, and then tested for viability using a crystal violet assay. MgCl_2 was used as control group since the control medium contained the same ionic strength as mercury treatment group. As shown in Figure 2, Figure 3 and Figure 4, at all time points, concentrations of mercury at $5 \mu\text{M}$ and below had essentially no effect on cell viability. At $1 \mu\text{M}$ mercury, there was a slight trend to decreased viability as exposure time increased, but it was not statistically significant. However, there was a statistically significant decrease in cell viability ($p < 0.01$) between controls and cells exposed to mercury at mercury concentrations above $10 \mu\text{M}$. Mercury concentrations of $10 \mu\text{M}$ and above led to a dramatic decrease in cell number. HgCl_2 concentrations $< 1 \mu\text{M}$ could not be distinguished from controls, whereas $> 10 \mu\text{M}$ HgCl_2 concentration led to a substantial reduction in the number of viable cells relative to controls for cells exposed for 24, 48, or 72 h.

In both 48 h and 72 h treatment groups (Figure 3 and Figure 4), the cell number decreased significantly compared with 24 h group (Figure 2) when exposed to same mercury concentration. Serum starvation may contribute to cell death or at least cell

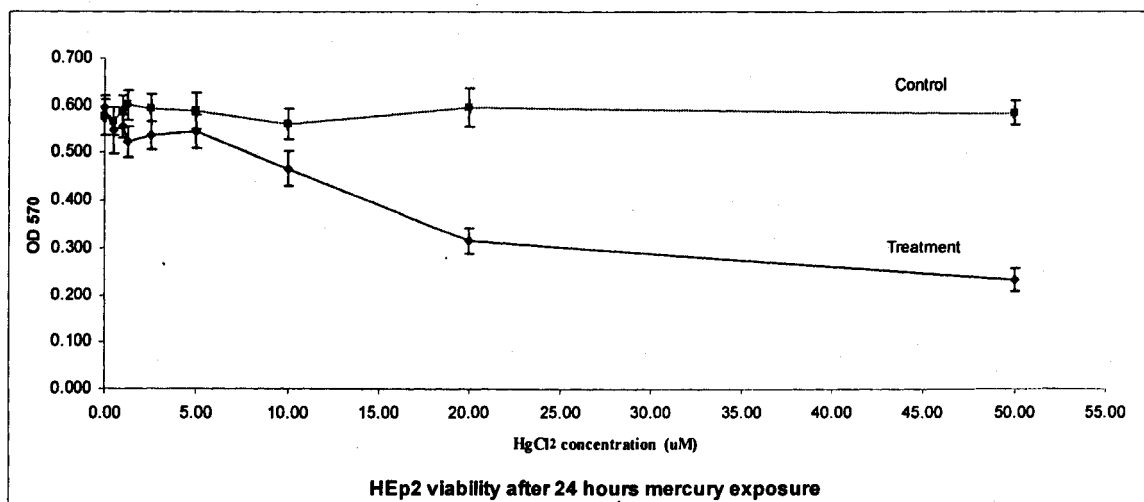


Figure 2. Cell viability of HEP2 after mercuric chloride exposure. The HEP2 cells were treated with concentration of HgCl₂ from 0.5 μ M to 50 μ M for 24 hours (◆). Viability was determined via the crystal violet assay and measured with OD 570. Identical concentrations of MgCl₂ were used as controls (■). Each point is the average of three repetitions.

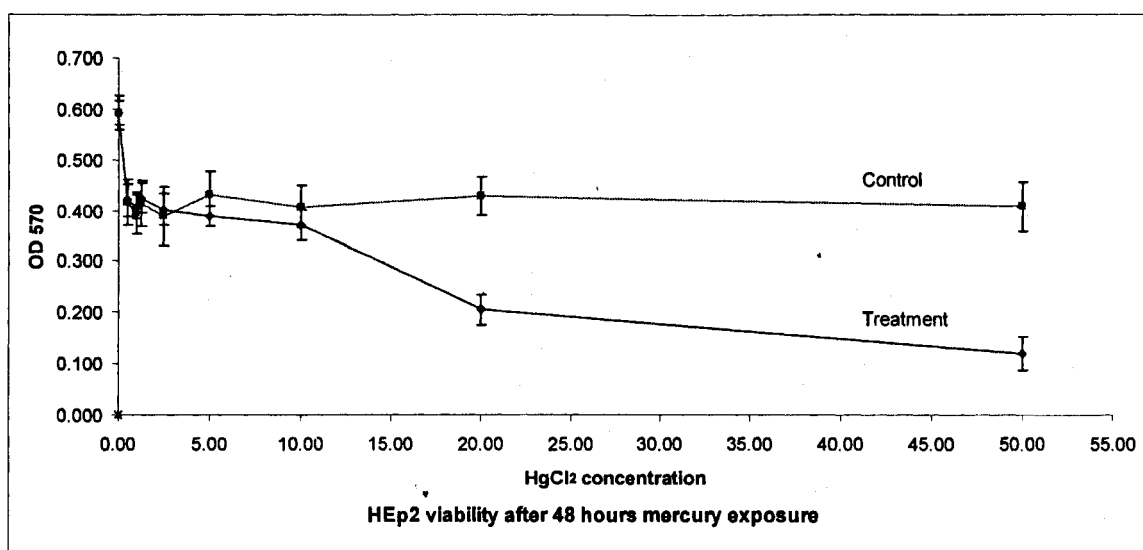


Figure 3. Cell viability of HEP2 after mercuric chloride exposure. The HEP2 cells were treated with concentration of HgCl₂ from 0.5 μ M to 50 μ M for 48 hours (\blacklozenge). Viability was determined via the crystal violet assay and measured with OD 570. MgCl₂ was used as controls (\blacksquare). Each point is the average of three repetitions.

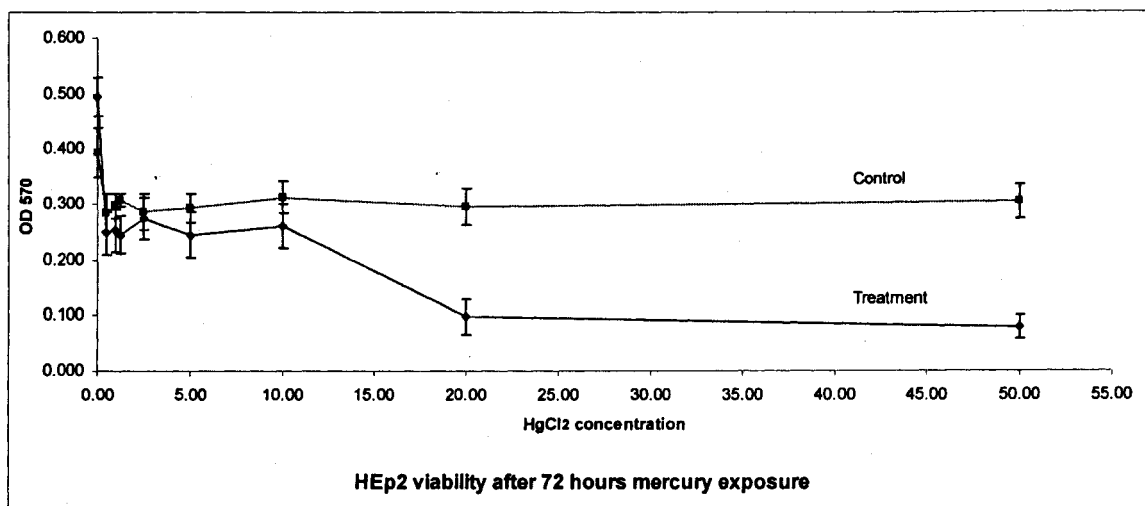


Figure 4. Cell viability of HEP2 after mercuric chloride exposure. The HEP2 cells were treated with concentration of HgCl₂ from 0.5 µM to 50 µM for 72 hours (◆). Viability was determined via the crystal violet assay and measured with OD 570. MgCl₂ was used as controls (■). Each point is the average of three repetitions.

sensitivity to mercury at these later time periods. However the difference between the control and treatment groups in each time period is not due to serum starvation since both groups received the same media.

Monocytic cells such as U-937 exposed to low concentrations of ionic mercury undergo cellular DNA damage. Ben-Ozer *et al.* (2000) found that the LD50 for ionic mercury was approximately 5 μM , below 1 μM cell viability and proliferation was essentially unaffected by mercury (Ben-Ozer *et al.*, 2000). Our results also showed no change in viability of HEP2 cells at mercury concentrations less than 5 μM .

Similar to our results, concentrations of mercury 10 μM and above were toxic to U-937 cells after 24 h, concentrations of mercury <5 μM were not cytotoxic or cytostatic (Ben-Ozer *et al.*, 2000). But this is not to say that mercury does not have a deleterious effect at these concentrations. It appears that the predominant mechanism(s) whereby mercury affects HEP2 cells may be dose-dependent. As it has been suggested, at high and toxic doses mercury may destroy mitochondria, leading to rapid cell death on human T-cells (Shenker *et al.*, 1999). At lower doses, the mechanism may be quite different (Ben-Ozer *et al.*, 2000).

Mercury-induced cytotoxicity was also investigated in both T and B lymphoma cells (Kim *et al.*, 2003). Exposure to mercury for 24 h reduced viability in both cell lines. The viability of T lymphoma cells exposed to 20 μM of mercury for 24 h was reduced

to 79% of control, and viability in B lymphoma cells exposed to 10 μM of mercury for 24 h was reduced to 73% of control. Mercury treatment decreased DNA synthesis in both T and B lymphoma cells in a dose dependent manner. Mercury at 5 μM significantly decreased DNA synthesis in T lymphoma cell and at 50 μM completely blocked DNA proliferation. The B lymphoma cells showed a similar trend after mercury exposure. Mercury at 10 μM started to decrease DNA synthesis of B lymphoma cells and at 50 μM completely blocked DNA proliferation.

4.2 Comet assay

In order to assess mercury-dependent cellular DNA damage in mononuclear cells, we employed the comet assay to detect DNA damage in individual HEp2 cells. The comet assay is a sensitive single cell assay, which detects many DNA changes, including the formation of strand breaks and unwinding (McKelvey-Martin *et al.*, 1998; Kindzelskii & Petty, 1999). DNA damage is apparent by the formation of a comet tail, presumably due to fragmented and/or unwound DNA (McKelvey-Martin *et al.*, 1998).

The basic setup was replicated 5 times, so that for each time point and concentration, a total of at least 1000 cells were analyzed. Controls always exhibited comet tails <1mm in length (not including cell nucleus). For mercury-treated cells, comet tails >1 mm were considered positive for mercury-induced comet formation. Cells were exposed to 0, 0.5, 1, 1.25, 2.5, 5, 10, 20, and 50 μM of mercuric chloride for 24, 48, and 72 h. Results showed that DNA damage increased as the exposure time increased. Figure 5

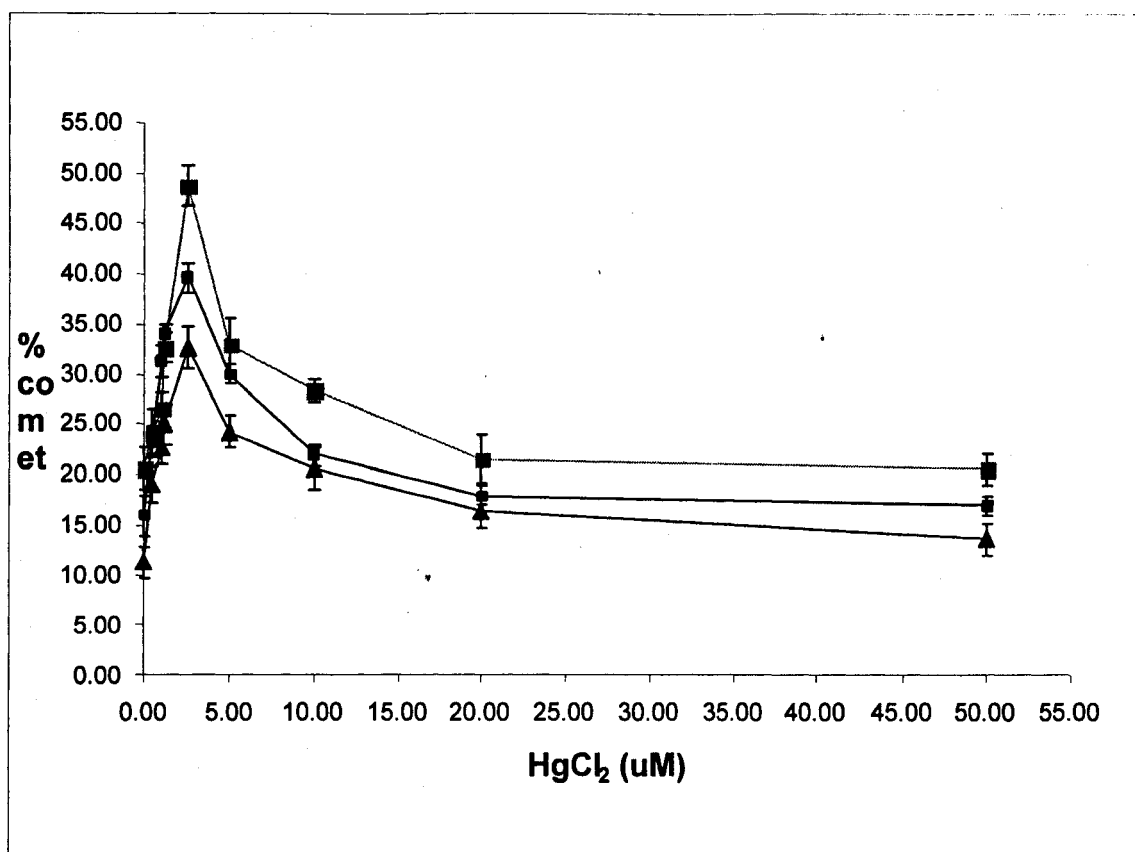


Figure 5. DNA damage induced by mercuric chloride. HEP2 Cells were treated with various concentrations of mercuric chloride for 24, 48 and 72 hours incubation periods and analyzed for percent comet formation. Incubation times were 24 h(▲), 48 h (◆) and 72 h (■).

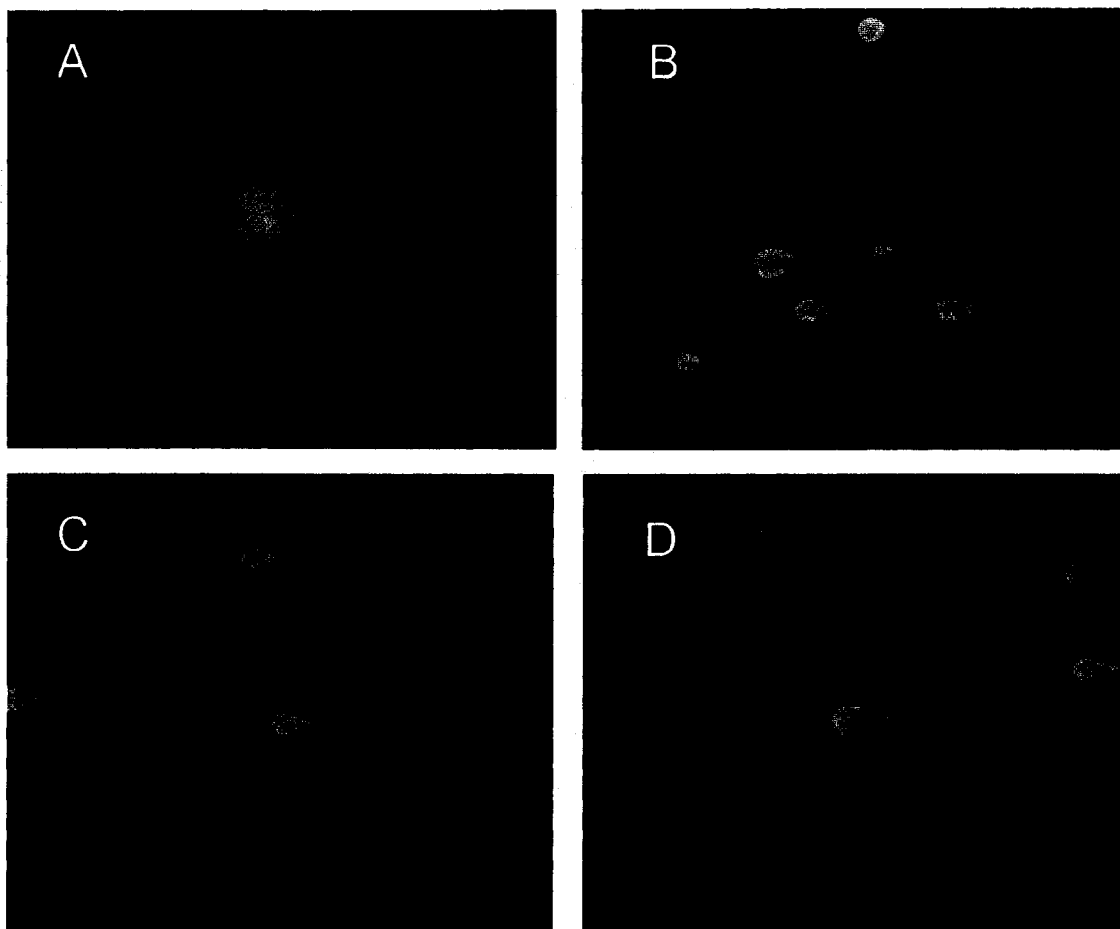


Figure 6. Micrographs of the comet tail lengths after 24 hours mercury exposure. (A) Control; (B) 24 h incubation with 0.5 μ M mercury; (C) 24 h incubation with 1.0 μ M mercury; (D) 24 h incubation with 2.5 μ M mercury. F

showed comet assays of cells treated with 2.5 μM of mercury for incubation periods of 24, 48, and 72 h, respectively.

As shown in Figure 5, from 0 to 2.5 μM of mercury, the percentage of cells forming comets increased as the concentration of mercury increased. At 2.5 μM HgCl_2 , a significant increase ($p < 0.01$) in the fraction of cells forming comet tails was observed. Interestingly, higher concentrations of mercury produced less comet formation, and there were much less cHEp2 cells formed comet when mercury concentration above 10 μM .

DNA damage is visualized when a 'comet tail' (electrophoresed DNA) is observed under fluorescence microscopy (Figure 5). Comet tails are not completely uniform, but rather can have a granular and fragmented structure. Measuring the fraction of cells that undergo comet formation can assess the effect of mercury on DNA damage.

We next quantified the comet tail length for the cells treated with 2.5 μM mercury. Comet tail length increased in a statistically significant manner ($p < 0.01$) with duration of mercury exposure. For those cells forming comets, the mean tail length at 24 h was $5.22 \pm 0.18 \mu\text{m}$; at 48 h, $7.48 \pm 0.16 \mu\text{m}$; and at 72 h, $9.1 \pm 0.13 \mu\text{m}$ (Figure 6). For comparative purposes, a separate experiment was conducted where cells were treated with various concentrations of mercury for 24 h. The results showed that comet lengths were highest with concentration of mercury 2.5 μM (Figure 8).

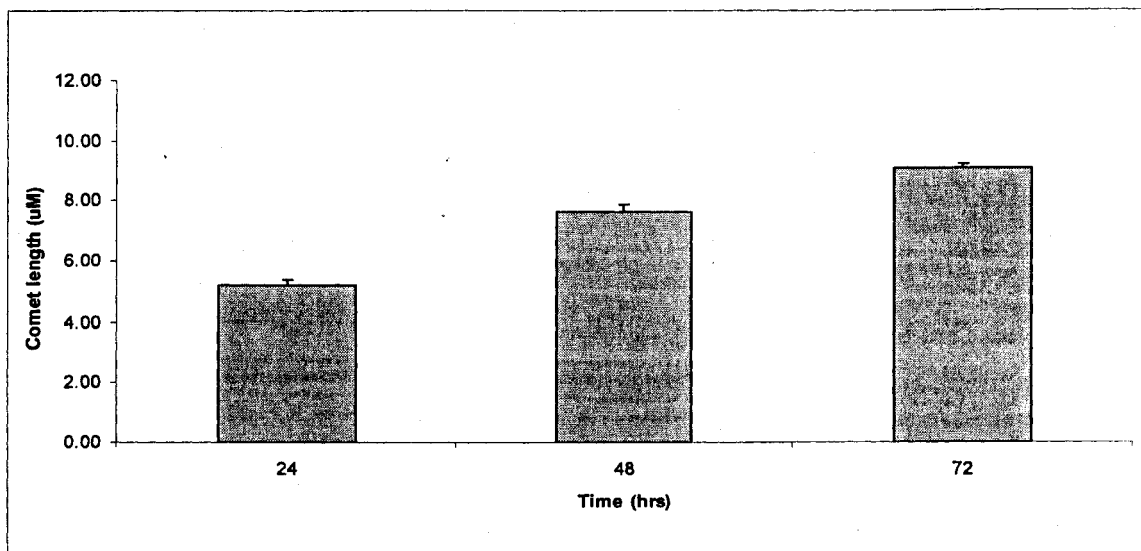


Figure 7. DNA damage induced by mercuric chloride. DNA damage as a function of time. HEp2 cells were treated with 2.5 μM HgCl_2 for 24, 48 and 72 h. DNA damage were analyzed for the content to which DNA damage occurred by measuring comet tail lengths.

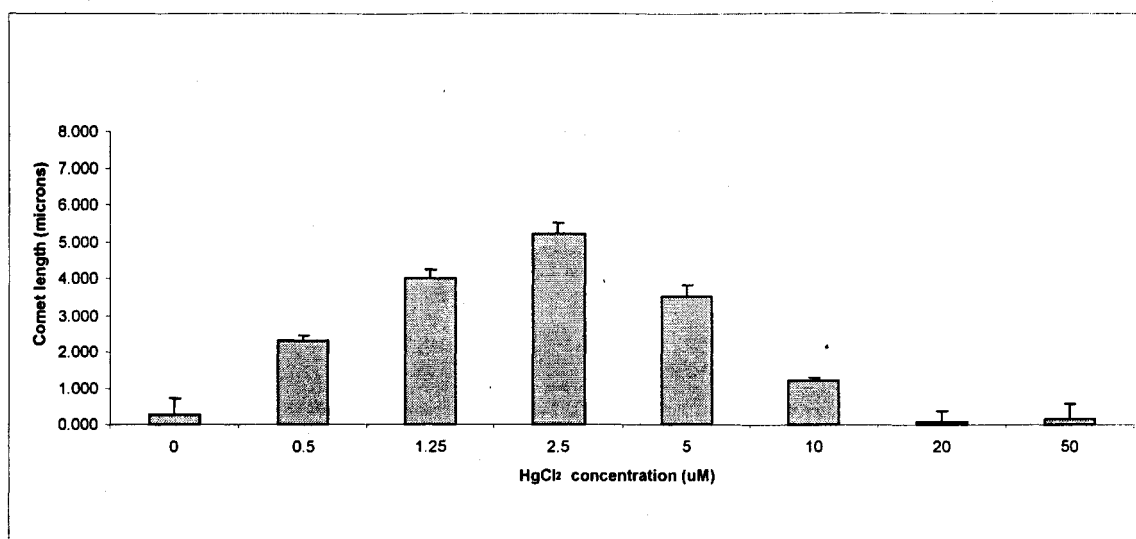


Figure 8. DNA damage induced by mercuric chloride. DNA damage as a function of concentrations. Cells were treated for 24 h with concentrations from 0-50 μ M. DNA damage were analyzed for the content to which DNA damage occurred by measuring comet tail lengths.

In general, the longer the cells were exposed to mercury, the longer the comet tails were. Increased comet tail length is associated with greater DNA fragmentation (McKelvey-Martin *et al.*, 1998; Kindzelskii & Petty, 1999). Therefore, these data suggested that upon increased exposure time to mercury, the cellular DNA became more heavily damaged, but also the fragments were larger than those found in apoptotic cells.

DNA damage was induced when cells exposed 24 hours to low dose ($0.5 \mu\text{M}$) Hg^{2+} in fibroblasts and Chinese hamster ovary cells (Ariza *et al.*, 1994; Hamilton-Koch, 1986).

There are many potential origins of comet tail non-uniformity; at least one important factor is diversity in the size of DNA fragments. All other things being equal, DNA fragments of smaller size would be expected to migrate farther than larger fragments during electrophoresis. As shown by Figure 7, the longer time the cells exposed to $2.5 \mu\text{M}$ mercury prior to SCGE analysis, the longer the comet tail, suggesting smaller DNA fragments, and greater DNA damage. It has been recently shown that in a similar manner $0.5\text{--}5 \mu\text{M}$ Hg^{2+} leads to single strand breaks in a human fetal hepatic cell line (Bucio *et al.*, 1999).

At $2.5 \mu\text{M}$, the percentage of comets reached peak. At lower concentrations, mercury causes less DNA damage. However, at much higher mercury concentrations, the percentage of comet formation is also reduced. This likely can be attributed to the fact that high mercury concentrations kill cells by a mechanism independent of DNA damage (Cantoni *et al.*, 1984; Schoeny, 1994; Ogura *et al.*, 1996). Genotoxic effects of

mercury are perhaps dose-dependent. At extremely low doses, cells may be unaffected by mercury, while at extremely high doses, cells are likely rapidly killed. We suspect there may be a 'window' between the very low and very high mercury concentrations where DNA is damaged, but cells are not killed. In the case of HEP2 cells, this appears to be between 1 and 2.5 μM . This would support the view that under the proper circumstances, mercury could be mutagenic to epithelial cells. There were similar results in U-937 cells when exposed to low and high dose mercury (Ben-Ozer *et al.*, 2000) as HEP2 cells in this study.

4.3 Apoptosis and necrosis

The acridine orange / ethidium bromide assay was used to undertake the effect of HgCl_2 on HEP2 cells exposed to mercury. Cells incubated in the absence of mercury served as a negative control. These treated with Verotoxin (VT) served as positive control. Consistent with the results by the crystal violet assay, viability decreased in a dependent manner with increasing HgCl_2 concentrations. The results indicated approximately 10% early apoptotic cells and another 10% late apoptotic at all concentrations of mercuric chloride tested.

There was a dependent increase in necrosis with increasing concentrations of mercury, indicating at least at the highest concentrations of mercury, HEP2 cells were dying primarily via necrosis. At the lower concentrations between 2.5 and 5.0 μM mercuric

chloride, there were equal levels of cell death via apoptosis and necrosis. However, at the lowest levels of mercuric chloride (0.5 – 1.25 μ M) HEp2 cells were dying primarily via apoptosis (Figures 9, 10, and 11).

DNA can be fragmented as a result of mercury-dependent induction of apoptosis, as it has been reported in human T-cells (InSug *et al.*, 1997; Shenker *et al.*, 1997). Mercury may also compromise immune function by initiating apoptosis in T cells and monocytes (InSug *et al.*, 1997; Shenker *et al.*, 1997).

Evidence of apoptosis included membrane blebbing, nuclear condensation, and margination. Fluorescence micrographs of HEp2 cells showed chromatin condensation and margination in early and late apoptotic cells (Figure 12). However, there were no obvious chromatin condensation and margination in viable and necrotic cells while viable cells appeared green, necrotic cells were red color when observed by fluorescence microscopy (Figure 12). As reported by Abul-Milh *et al.* (2001) necrotic cells were larger and lighter with plasma membrane lesions and mitochondrial abnormalities.

Some researchers have suggested that in human monocyte-like cells treated with low-concentrations (< 5 μ M) of mercury, fragmentation is not due to apoptosis (Bucio *et al.*, 1999; Ben-Ozer, 2000). The membrane receptor has been shown to bind to fas ligand that triggers apoptosis. Fas controls apoptosis in many cells (Bucio *et al.*, 1999; Ben-

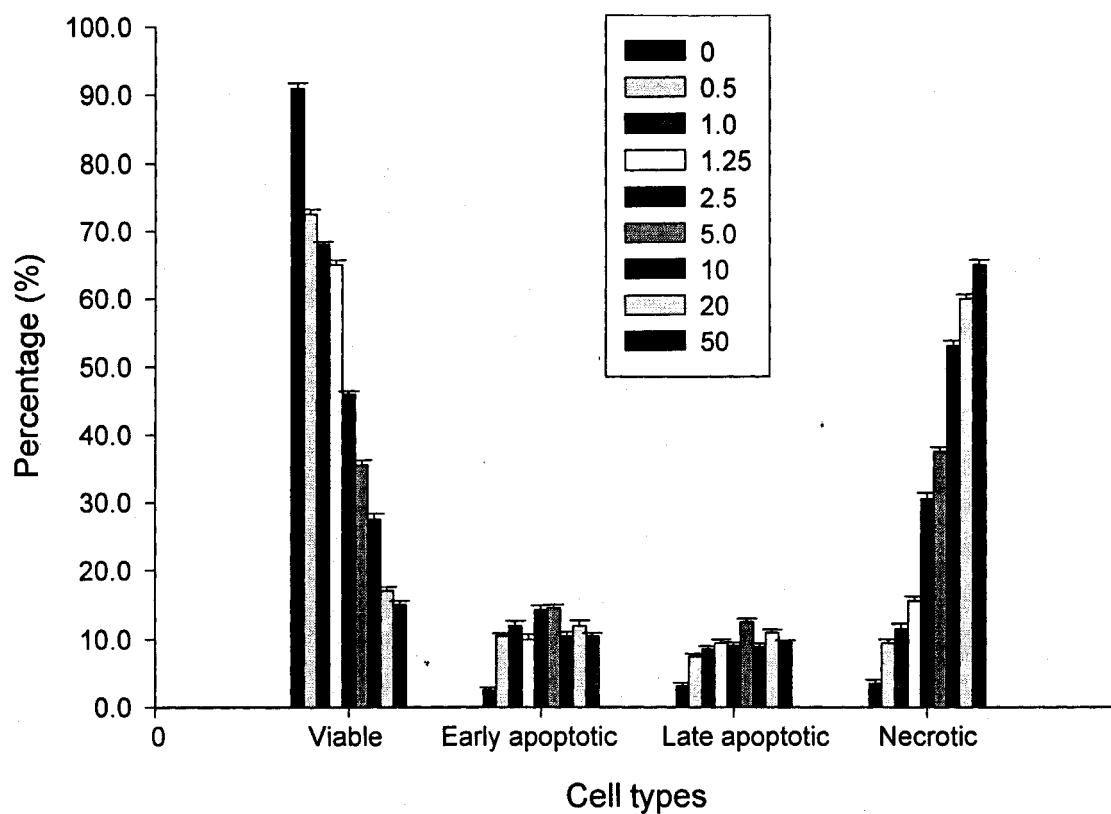


Figure 9. Apoptosis induced by mercuric chloride. Cells were treated with various concentrations (0.5-50 μM) of mercuric chloride for 24 hours. Cells were dyed with acridine orange / ethidium bromide dye mixture and checked for apoptosis under fluorescence microscope. Data were representative of three repetitions.

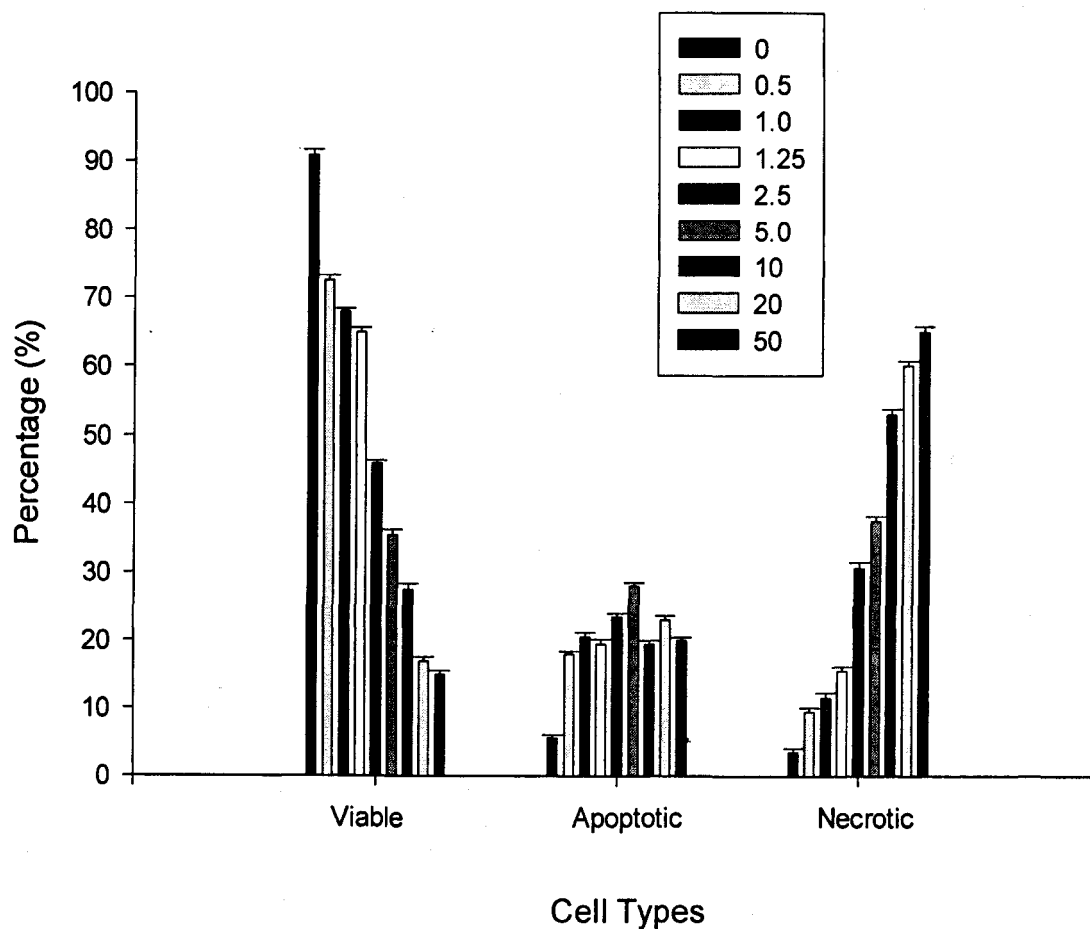


Figure 10. Apoptosis induced by mercuric chloride. Cells were treated with various concentrations (0.5-50 μ M) of mercuric chloride for 24 hours. Cells were dyed with acridine orange / ethidium bromide dye mixture and checked for apoptosis under fluorescence microscope. Data were representative of three repetitions.

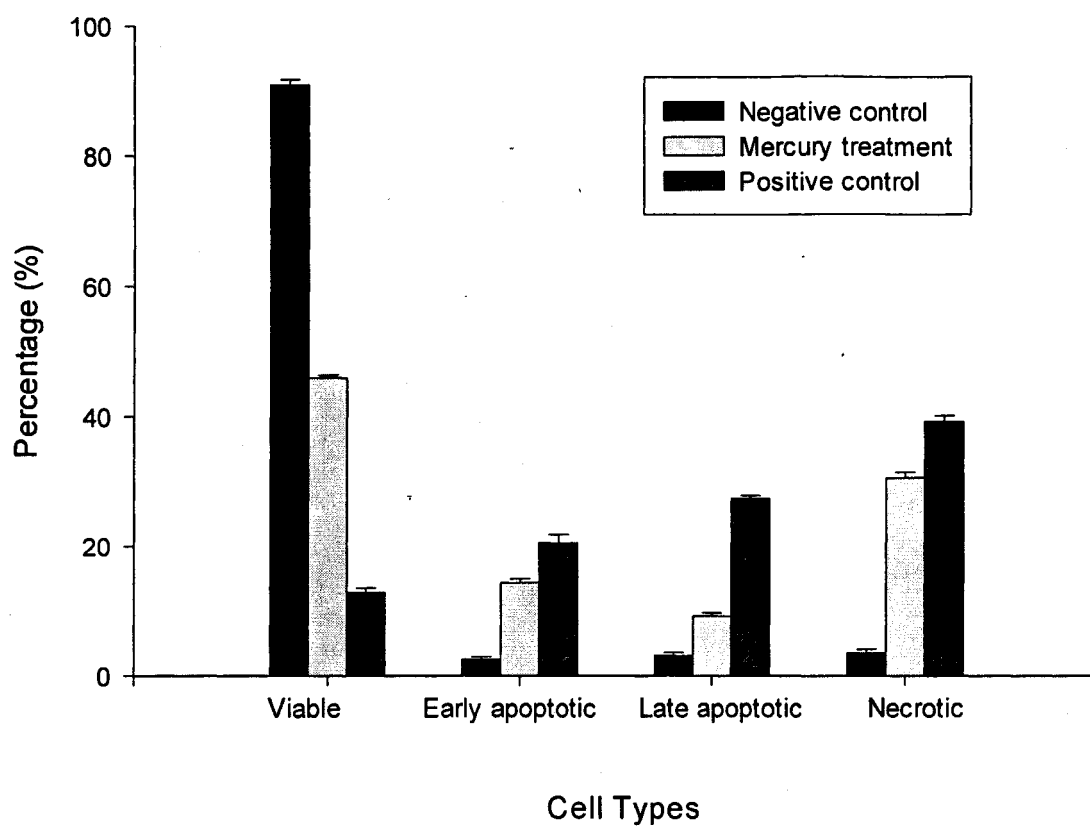


Figure 11. Mercury-induced apoptosis and necrosis. Cells were treated with 2.5 μ M HgCl_2 for 24 hours, 50 ng/ml VT as positive control, culture medium without mercury and VT as negative control. Data are representative of three repetitions.

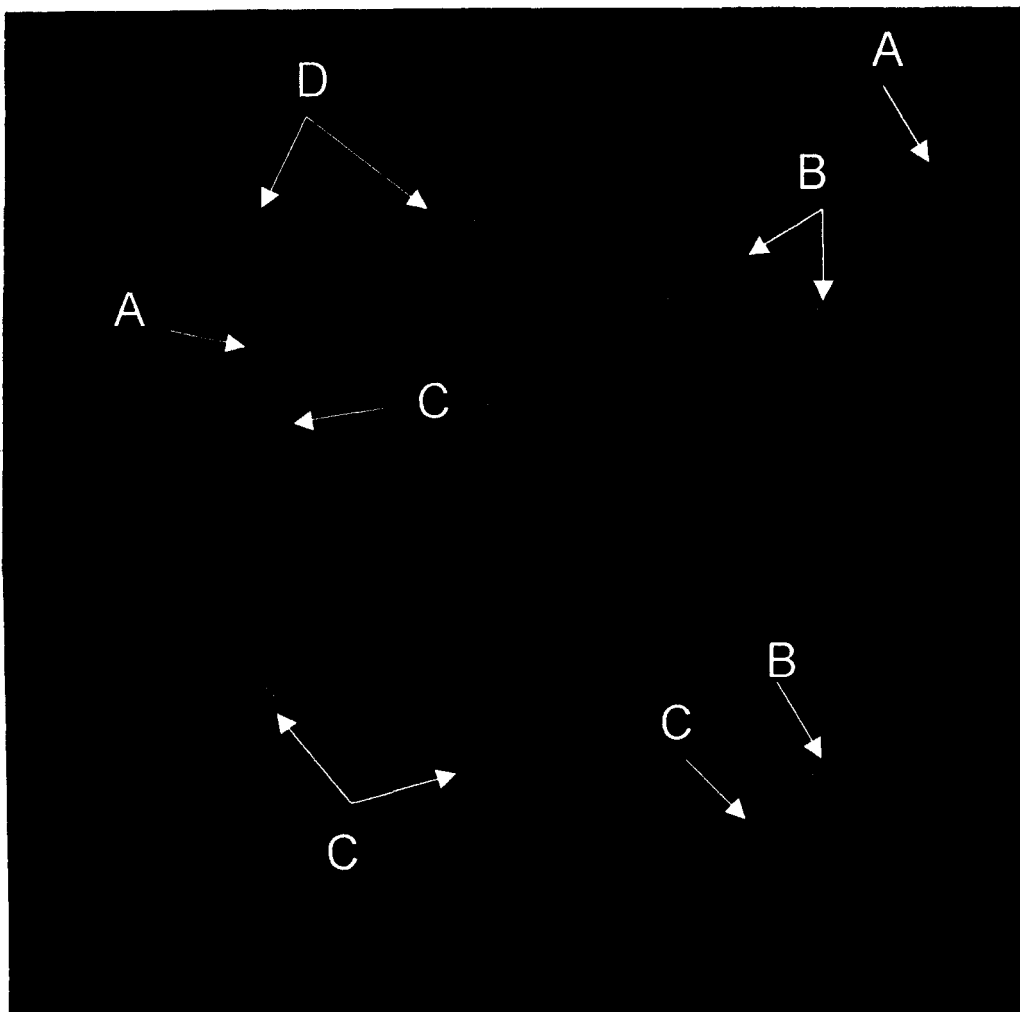


Figure 11. Fluorescence micrographs of HEP2 cells stained with acridine orange / ethidium bromide mixture. (A). Viable cells; (B) early apoptotic cells; (C) Late apoptotic cells; (D) Necrotic cells.

Ozer, 2000). After fas was cross-linked by anti-fas antibodies, apoptosis was also initiated when human fetal hepatic cells that were exposed to anti-fas (Bucio *et al.*, 1999). U-937 cells exposed to anti-fas antibodies displayed comet tails. Since the anti-fas induced comet tail lengths were approximately four times longer than those of the mercury-exposed cells, it appears that anti-fas treated (apoptotic) cells have DNA fragments that are quantitatively different (four times longer) than those found in mercury-treated cells.

Other reports have shown that lymphocytes and monocytes exposed to 0.6–5 μM methylmercuric chloride (MeHgCl) underwent apoptosis (InSug *et al.*, 1997; Shenker *et al.*, 1997). The mechanism of organic mercury damage may differ from that of ionic mercury used in this study. This result supports the view that cellular DNA damage as a result of ionic mercury can occur by a mechanism independent of apoptosis. As Shenker suggests, mercury may indeed target mitochondria and lead to oxidative stress (Shenker *et al.*, 1997).

As opposed to U-937 cells that were previously studied (Ben-Ozer *et al.*, 2000), HEp2 cells of this study suggested that low-level mercury seemed to induce DNA damage and trigger apoptosis. Shenker *et al.* (1998) has also reported that mercury induced apoptosis in human monocytes and T cell. However, some reports mentioned that mercury-induced stress might induce astrocytes to release oxygen free radicals without necessarily leading to cell death (Brawer *et al.*, 1998). Others have shown that

peripheral blood lymphocytes exposed to mercuric compounds underwent genotoxic effects due to an elevated level of 8-hydroxydeoxyguanosine brought by the generation of reactive oxygen species (Ogura *et al.*, 1996).

It is well known that mercury increases the levels of oxygen reactive intermediates in tissues and cells by the depletion of cellular antioxidants (Stancey & Kappus, 1982; Christie & Costa, 1984; Woods, 1988). In this study the HEP2 cells underwent DNA damage when exposed to mercury, perhaps occurring from an increase in reactive oxygen metabolites (ROM), directly attacking DNA (Ogura *et al.*, 1996). At 2.5 μM , the percentage of comets peaks (Figure 6 and Figure 8). At lower concentrations, mercury causes less DNA damage. At higher mercury concentrations ($>10\mu\text{M}$), the percentage of comet formation is very low too. This may suggest that high mercury concentrations kill cells by an independent mechanism without DNA damage typically of apoptosis (Cantoni *et al.*, 1984; Schoeny, 1994; Ogura *et al.*, 1996). Genotoxic effects of mercury are perhaps dose-dependent. When HEP2 cells exposed to high concentrations of mercury, cells were likely rapidly killed while at low concentration cells died primarily by apoptosis. This would support the view that even at low concentrations mercury could be mutagenic.

Involvement of reactive oxygen species (ROS) in mercury-induced cytotoxicity has been reported previously. Those studies revealed that ROS generation caused cell death in several cell types (Buttke & Sandstrom, 1995, Sato *et al.*, 1995). Also, low levels of

mercury increased ROS generation in human lymphocytes, hepatocytes, and brain cells (Shenker *et al.*, 1998, Lund *et al.*, 1993, InSug *et al.*, 1997, Stacey & Kappus, 1982, Hussain *et al.*, 1997). Mercury decreased mitochondrial transmembrane potential and increased ROS generation, and consequent depletion of glutathione (GSH) and lipid peroxidation, the latter is the major cause of mercury-induced cytotoxicity. Mercury compromised the cytosolic thiol redox system and induced an oxidative burst. Lee *et al.*, (2001) reported that mercury-induced cytotoxicity was not associated with generation of ROS and subsequent lipid peroxidation. Further studies demonstrated that treatment of mercury induced ROS production in both EL4 and A20 cells. The antioxidants and silymarin effectively inhibited mercury-induced ROS production. Those results suggested that mercury-induced cytotoxicity is mediated by modulation of ROS and results in increasing membrane permeability.

Chronic exposure to low concentrations of mercury results in disruption of the immune functions (Dieter *et al.*, 1983; Nakatsuru *et al.*, 1985; and McCabe & Lawrence, 1994). Such immunotoxic effects of mercury may lead to immuno-regulatory defects, which may result in persistent infection, cancer or autoimmune disease. The previous reports of mercury as immuno-toxicant and immuno-modulant are contradictory depending on experimental situation, dose and form of mercury, and strain of animal.

Mercury induced apoptosis in human monocytes and T cell, evaluated by the fluorescent probe H33258 (Shenker *et al.*, 1998; InSug *et al.*, 1997). Their results

showed that mercury increased H33258 fluorescence intensity with nuclear condensation and the antioxidants NAC and silymarin decreased such effect in both EL4 and A20 cells. However, treatment of mercury also increased PI fluorescent intensity, which indicated necrosis in both EL4 and A20 cells. This is consistent with the results in this study, that mercury induced both apoptosis and necrosis when HEP2 cells were exposed to mercury at intermediate levels of concentrations, but primarily induced necrosis at the highest mercury concentrations.

The manner in which a cell dies can have a great impact on the resulting response of the surrounding tissue. Death by necrosis has been shown to act as a natural adjuvant inducing oxidative stress and the production of pro-inflammatory cytokines (Anderson *et al.*, 2002). This is due to the indiscriminant release of the cytoplasmic contents from the dying cell. The result is an area of inflammation and immune-mediated cell damage in uninvolved cells surrounding the initial insult. In contrast, death by apoptosis is a controlled event usually with minimal loss of membrane integrity until the later stages termed secondary necrosis. Instead, the cytoplasmic contents are systemically degraded from within. This type of cell death often involves phagocytosis by resident tissue macrophages and the release of anti-inflammatory cytokines (Fadok *et al.*, 1998). Recent studies have however indicated that apoptosis is also accompanied by release of proinflammatory cytokines.

Mercury-induced cytotoxicity does not require de novo protein synthesis (Kim *et al.*, 2002; Kim & Sharma, 2003). The mode of mercury-induced cytotoxicity appears to be a mix of apoptosis and necrosis; both apoptotic and necrotic effects are mediated by ROS in murine lymphoma cell lines. The results in this study also indicated that apoptosis and necrosis were induced by mercuric chloride (Figure 9 & 10).

It has been established that an increase in Ca^{2+} can lead to cytotoxicity through several downstream reactions, such as ROS generation (Gasso *et al.*, 2001). Mercury has been reported to increase intracellular Ca^{2+} concentration (Toimela & Tahti, 2001). Gasso *et al.* (2001) mentioned that some Ca^{2+} channel blockers protected mercury-induced neurotoxicity. It has been known that ROS and lipid peroxidation could increase intracellular Ca^{2+} , although the exact origin of Ca^{2+} was still in controversial (Suzuki *et al.*, 1997). Mercury-mediated oxidative stress may lead to cytotoxicity in part through disrupted Ca^{2+} homeostasis.

Oxidative stress evokes apoptosis by mitogen-activated protein kinase (MAPK)-mediated caspase activation (Kamata & Hirata, 1999). MAPKs have important functions as mediators of cellular response to extracellular signals. Mercury inhibits ROS activation during T cell receptor-mediated signal transduction (Mattingly *et al.*, 2001). Mercury increased TNF α gene expression by regulating p38 MAPKs in macrophages (Kim *et al.*, 2002). The mechanistic studies to MAPKs and downstream

caspase pathway should be elucidated for further understanding of mercury-induced apoptosis.

In summary, results of the present study indicate that mercury induced cytotoxicity in the human epithelial cells line HEP2 is a mix of apoptosis and necrosis. Exposure to low-level mercury induced DNA damage, and morphological changes consistent with apoptosis with minimal necrosis. But exposure to mercury at concentration $> 10 \mu\text{M}$ triggers primarily necrosis, and the increase in necrosis correlated with the increase in content of HgCl_2 . At $2.5 \mu\text{M}$ mercury comet formation percentage peaked, suggesting that DNA damage was maximal.

4.4 Mercury speciation

Many metal ions form complexes with amino acids and small peptides and studies of this complexation have been carried out in order to obtain a better understanding of toxicology of mercury in the medium with organic components (Majid *et al.*, 2002). The location of different metal ions in the hydrophobic cavity of a protein depends on the relative intrinsic bond strength between the metal ions and the various possible metal binding sites.

The mercury contents of $10 \mu\text{L}$ $1.0 \mu\text{M}$ HgCl_2 MEM sample is 2005.90 pg (Table 4). The result of the CVAFS Mercury Detector 2500 was 2034.51 pg calculated according

the standard curve (Figure 13). The relative error was 1.4%. This suggested that this mercury measurement is accurate. When the HgCl_2 MEM solution was passed through the Ionac SR4 column, the resin would absorb mercuric components in the sample that labile to Ionac SR4. Mercuric complexes that non-labile to resin Ionac SR4 in the MEM could pass through the Ionac SR4 resin.

Speciation affects the bioavailability and toxicity of elements and so is important in toxicology and nutrition. The ecological and toxicological effects of Hg are strongly dependent on the chemical form (species) present (Clarkson, 1998). Upon inhalation of Hg vapor, Hg^0 is oxidized in blood to Hg^{2+} and taken up by erythrocytes (Templeton, 1999). It may under some circumstances be detoxified by reduction back to Hg^0 . Mercury is not otherwise biotransformed in the human body, and therefore speciation is mainly of interest in order to distinguish inorganic from organic forms, as these will reflect the compounds to which exposure has occurred (Campbell *et al.*, 1992).

The results of this study suggested that about 97.8% of mercury was labile to resin Ionac SR4; only 2.42% mercury was non-labile to resin Ionac SR4 in the MEM, which passed through resin. This may be attributed to the inorganic and organic components of MEM, or formed mercury complexes that are not as stable as those complexes formed at the surface of SR4 resin. Previous studies indicated that the chemical form of Hg in aquatic systems is strongly influenced by redox and pH conditions as well as by the concentration of inorganic and organic complexing

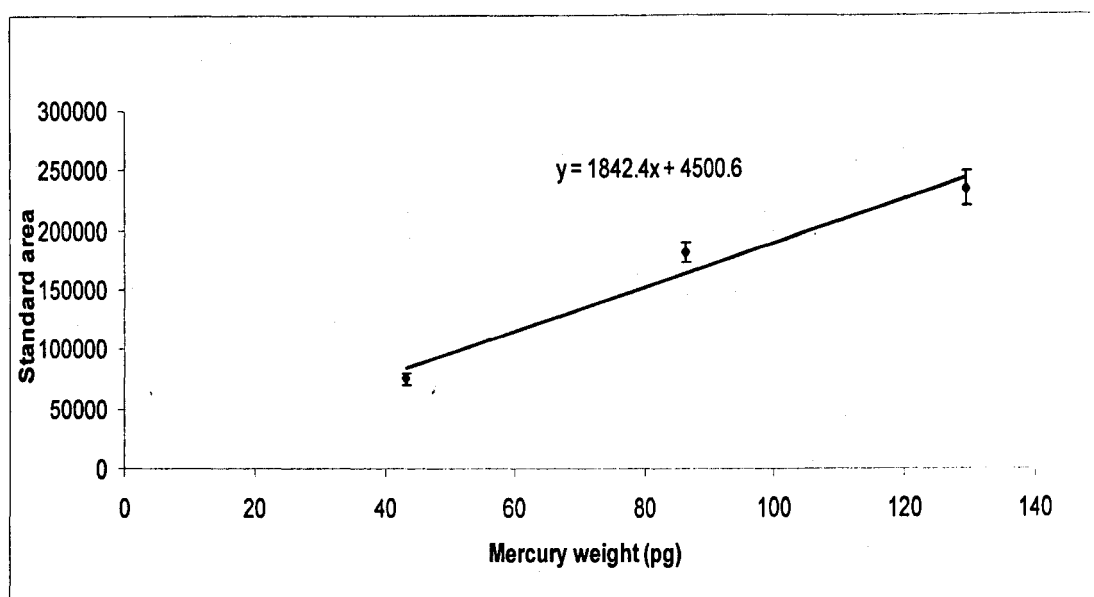


Figure 13. Mercury stand curve with CVAFS. Standard samples was injected and measured with three repetitions. Each point is the average of three repetitions. Equation came from regression analysis.

Table 4. Mercury measurement with CVAFS Mercury Detector 2500

Mercury measured with CVAFS			
Sample	Areas measured (Average)	Injected Weight (pg)	Hg weight calculated (pg)
1.0 uM Hg	3752874	2005.9	2034.506
Ionac SR4 treated	95256.4	unknown	49.205

agents (Ullrich *et al.*, 2001). The Hg^{2+} has a strong tendency to form complexes with soft ligands such as sulfur.

Chapter 5. Conclusion

All forms of mercury are toxic to humans. There are two major forms of toxic effects of mercury, nephrotoxic effects and neurotoxic effects. Mercury can easily enter the body if its vapor is inhaled or if it is eaten in organic forms in contaminated fish or other foods. It can also enter the body when food or water contaminated with inorganic or organic mercury. The divalent ionic form of inorganic mercury primarily damages the kidney, while both organic and metallic mercury are known to penetrate into the central nervous system (CNS) and produces severe toxicity in this organ. A human epithelial cell line was selected in this study because it represents the portal of entry into the human systems. Deleterious effect of mercuric chloride may therefore indicate the need for careful monitoring of mercuric chloride in the aqueous environment.

Mercury is bound to the cell walls or membranes of microorganisms. The reactivity and toxicity of Hg^{2+} is higher than that observed for any divalent metal ion (Stohs & Bagchi, 1995). Inorganic mercury appears to move into the cells after damage to the membrane barrier. In this study, exposure to low concentrations of mercury ($5 \mu\text{M}$ and below) had essentially no significant effect on cell viability, as measured by crystal violet assay. But there was a statistically significant decrease in cell viability ($p < 0.01$) between controls and cells exposed to mercury at mercury concentrations above $10 \mu\text{M}$. Mercury concentrations of $10 \mu\text{M}$ and above led to a dramatic decrease in cell number.

These results indicated that exposure to mercuric chloride with concentration $> 10 \mu\text{M}$ was lethal to HEP2 cells.

DNA can be a target for mercury toxicity. Mercuric chloride is very potent at producing DNA damage in human and mammalian cells. Mercuric chloride not only induced DNA damage but also effectively inhibited DNA repair in contrast to X-ray irradiation. The single cell gel electrophoresis (SCGE) test or comet assay is a new test with widespread potential applications in genotoxicity testing and biomonitoring.

The comet assay in this study showed that even exposed to low concentration of mercury ($< 5 \mu\text{M}$), comet tails were formed in HEP2 cells. The percentage of cells forming comets increased as the concentration of mercury increased. At $2.5 \mu\text{M}$ HgCl_2 , a significant increase ($p < 0.01$) in the fraction of cells forming comet tails was observed. This indicated that low concentration exposure to mercuric chloride induced DNA damage in HEP2 cells consistent with apoptosis. Interestingly, higher concentrations of mercury produced less comet formation, and yet cells were killed in the presence of mercury above $10 \mu\text{M}$ as determined by the cell viability assay. This likely can be attributed to the fact that high mercury concentrations kill cells by a mechanism independent of DNA damage or before DNA damage occurs.

Apoptosis (programmed cell death) is a distinct genetic and evolving biochemical pathway essential for all multicellular organisms. An acridine orange / ethidium

brimode assay was used to define the extent of apoptosis and necrosis in HEp2 cells exposed to various concentrations of mercury for 24 hours. The results showed evidence of apoptosis at all mercury concentrations peaking at 5 μ M. The extent of necrosis increased with increasing mercury concentrations and was indeed the primary cell death mechanism at concentrations above 5 μ M. The results support the view at low mercury concentrations; cell death occurs by a mix of apoptosis and necrosis while at high concentrations of mercury, cell death occurs primarily by necrosis. This is consistent with the comet assay results where DNA damage is most extensive at lower concentrations of mercury, where there is more cell death by apoptosis

Speciation affects the bioavailability and toxicity of elements and so is important in toxicology and nutrition. The ecological and toxicological effects of Hg are strongly dependent on the chemical form (species) present. The results of this study suggested that the predominated form of mercury of HgCl_2 in MEM was labile to resin Ionac SR4. About 97.8% of mercury was absorbed by Ionac SR4 resin; only 2.42% mercury was non-labile to resin Ionac SR4, which passed through resin. These results suggested that mercury labile to resin Ionac SR4 is predominate in the MEM medium and induced DNA damage killing HEp2 cells when exposed to concentration > 5 μ M for 24 hours.

In the future, further studies should be conducted in the apoptosis mechanisms of HEp2 cells exposed to mercury. Mercury speciation is very important to illustrate the DNA

damage induced by mercury, because the ionic mercury and organic mercury have different toxicity. More work should be carried out in mercury speciation.

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