THE EFFECTS OF MERCURIC CHLORIDE ON CULTURED ATLANTIC SPOTTED DOLPHIN (*Stenella plagiodon*) RENAL CELLS AND THE ROLE OF SELENIUM IN PROTECTION

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(ABSTRACT)

Marine mammals are known for their low susceptibility to mercury toxicity, and it was hypothesized that selenium may play a role in protection against mercury toxicity. To gain insight into the mechanisms of the low susceptibility of cetaceans, we investigated the *in vitro* effects (1) of mercuric chloride ($HgCl_2$) on the ultrastructure and cell death of Atlantic spotted dolphin renal cells (Sp1K cells), (2) of $HgCl_2$ on the cell proliferation and cell cycle status of Sp1K and Rhesus monkey renal cells (MK2), and (3) of sodium selenite (Na_2SeO_3) on cell proliferation and cell death of control and $HgCl_2$-treated Sp1K cells.

$HgCl_2$ affected multiple organelles and nuclei in Sp1K cells, and induced apoptosis in a time-and dose-dependent manner. Both ultrastructural changes and induction of apoptosis were milder than seen in other cell types in previous publications. In addition, Sp1K cells were able to proliferate at 25 μM $HgCl_2$ while MK2 cells were killed at 15 μM $HgCl_2$. An increase in percentage of cells in the G_0/G_1 phase in the cell cycle and a decrease in S, and G_2/M phase cells were seen in Sp1K cells exposed to more than 10 uM $HgCl_2$ more than 72 hours. MK2 cells showed cell cycle changes only at 24 hours exposure, and may be due to a sensitive subgroup. These data suggested that Sp1K cells were less suscepable than other cell types in a cell-specific way, which was independent of selenium protection.

Concurrent exposure to Na_2SeO_3 provided protection against the $HgCl_2$-induced decrease in cell proliferation of Sp1K. The protective effects were greater if Na_2SeO_3 and $HgCl_2$ were premixed, but disappeared if exposures did not overlap. Although pretreatments with Na_2SeO_3 alone did not provide protection, they increased the protection of selenium administered later. Furthermore, Na_2SeO_3 decreased $HgCl_2$-induced apoptosis. These data demonstrated the Na_2SeO_3 protection against $HgCl_2$ toxicity in Sp1K cells in terms of cell proliferation and apoptosis.

This study is the first report that reveals the existence of mercury-selenium antagonism in cultured cetacean cells. The data supported the hypothesis that selenium protection against mercury toxicity is, at least partially, through competition of binding sites and formation of mercury-selenium complex.

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PART I
GENERAL INTRODUCTION

MERCURY

Mercury is a metal whose elemental, metallic form is liquid at room temperature. Mercury exists naturally in the Earth's crust, soil, river, sea, rain water, air, and living organisms. Furthermore, it is widely used in the electrolytic production of chlorine, electronic apparatuses (batteries, fluorescent lights), paints, dental preparations, paper and pulp.

The concentrations of mercury constantly increase, not only in selected sectors of the environment and in animal tissues, but also in human tissues (Gaskin et al. 1979; Swain et al. 1992; Eide et al. 1993). In spite of the restrictions on mercury and environmental discharge in recent decades, human exposure to mercury is not totally diminished. The current human exposure to mercury can be classified into three categories according to the chemical form of mercury. (1) Inorganic mercury: Inorganic mercury is widely used in certain types of batteries and fluorescent lightbulbs. Humans can be exposed to the mercury when a battery shell or fluorescent lightbulbs break. Illegal mercury-containing skin creams have recently resulted in mercury poisoning in the U.S.-Mexican border states (Villanacci et al. 1996). (2) Elemental mercury: Mercury vapor is the main form of mercury in occupational exposures. In the general population, the major background source for human exposure to mercury vapor is dental amalgam, which includes mercury, silver, copper, and other metals (Clarkson 1997). Recently, liquid mercury has been placed in amulets or distributed around the house for religious purposes (Wendroff 1995). (3) Organic mercury: Methylmercury is a form of organic mercury. Humans intake of this form takes place predominately from fish and other marine foods. Mercury levels in human head hair had a positive correlation with the amount of fish in the diet and the amount of mercury in the fish species consumed in various countries around the world (Airey 1983b; Airey 1983a). In Greenland, seal liver was responsible for a high mercury intake (Johansen et al. 2000).

Mass mercury poisoning accidents from consumption of contaminated fish, such as Minamata disease in Japan, has focused public attention on marine mercury pollution (Eto 1997). Infants may be exposed to ethylmercury from vaccination with certain vaccines which use mercury-containing preservatives (Halsey 1999).

Organic mercury (such as methylmercury), and inorganic mercury (such as elemental mercury and mercuric chloride) have different toxic absorbaion routes and toxicities. Organic mercury is absorbed from the lungs, gastrointestinal (GI) tract, and through the skin due to its high lipid solubility. The main accumulation is in the calcarine cerebral cortex of the brain. Both the central and peripheral nervous systems are adversely affected. Inorganic mercury, in contrast, is poorly absorbed through the skin, but is absorbed from the lungs and GI tract. The major organ damage caused by inorganic mercury has been observed in the kidney. Inhalation of elemental mercury affects the lung. Both organic and inorganic mercury affect the GI, immune, hepatic, and cardiovascular system, and rarely the hematopoietic system (Osweiler 1996; Clarkson 1997; Graeme and Pollack 1998). Furthermore, mutagenicity and teratogenicity of mercury has been reported in fish, birds and mammals (Leonard et al. 1983).

MERCURY IN CETACEANS

Introduction

Since the 1950's, heavy metal residues in aquatic animals have attracted the attention of the public. At that time, the Japanese who lived near Minamata Bay contracted a syndrome known as Minamata disease, which affected the renal, neural, and reproductive systems, and progressed on to death. The etiologic agent was proven to be mercury in seafood contaminated by industrial pollution. Thus, in the field of marine biology, mercury was one of the earliest studied metals. According to reports from various countries, mercury contamination of the environment has been an increasing global problem.

This part of the General Introduction aims to describe the mercury concentrations in cetaceans from different areas, the factors affecting mercury accumulation, and the effects caused by the mercury. Mechanisms of mercury metabolism are also discussed.

Cetaceans acquire mercury mainly through prey ingestion, and accumulate mercury in the body by a biomagnification effect. All of the reports reviewed have documented mercury in the tested tissues (muscle, brain, kidney, liver, blubber, etc.) of every individual cetacean tested. Concentration differences are associated with the tissue, species, animal age (weight or length), sex, and diet. Mercury has been known to induce neurotoxicity (Cardellocchio 1995) and may cause liver disease (Kuehl and Haebler 1995), stranding of cetaceans (Leonzio et al. 1992) and may also act as a stressor (1989; Brody 1989) in cetaceans.

High concentrations of mercury in cetaceans are a result of their top position in the food web, as well as their different respiratory system compared to fish, the formation of non-toxic mercury-selenium complex (HgSe), and to the demethylation process (Leonzio et al. 1992; Palmisano et al. 1995).

How Cetaceans Acquire Mercury

In general, pollutants enter aquatic animals mainly through the following three ways: (1) Respiratory system: In fish, when the gills exchange gas with the water, the pollutants enter the gills and are distributed to other tissues. In cetaceans, air is inhaled by lungs which may take in pollutants evaporating at the water surface. (2) The integument: Some pollutants can be absorbed through the skin or mucous membrane. (3) Gastrointestinal system: The pollutants which exist in prey species (fish, squid, other marine mammals) may enter the predator's system through the gastrointestinal tract. Pollutants that cannot be metabolized or excreted will stay in the body leadign to accumulation of the pollutants in the tissue of predators, a condition known as biomagnification. This phenomenon results in increased pollutant concentrations as the position in the food web elevates. The importance of these three methods varies with the pollutants, the animal species, and the situation.

The highest mercury value of cetaceans has been reported in fish-eaters, such as *Tusiops truncatus* (Chvojka and Williams 1980). Conversely, the highest cadmium concentration has been recorded in the squid-eating species, *Kogia breviceps* (Martin and Flegal 1975). At the same time, the Franciscana dolphin, *Pontoporia blainvillei*, which eats both fish and squid as part of its diet, has mercury and cadmium concentrations lower than *T. truncatus* and *K. breviceps*, respectively (Marcovecchio et al. 1994).

Fetal and suckling cetaceans, which have not ingested other organisms in the sea, can acquire mercury from the mother via the placenta and milk, respectively. André et al. (1990) pointed out that methylmercury can be transfered to the fetus through the placenta in dolphins, and later Meador et al. (1993) reported mercury in the liver, kidney, and brain tissue in a female *Globicephala melaena*, as well as in her fetus. Nonetheless, Miyazaki (1994) argued that mercury was not transferred into the fetal organs through the placenta in appreciable quantities. Methylmercury is a lipophilic compound, and can be excreted through the milk (Cardellocchio 1995). Although the methylmercury levels in cetacean milk are unknown, in other terrestrial mammals the mercury concentration in the milk can be higher than in the maternal blood (1976).

Factors Affecting Mercury Concentrations in Cetaceans

In addition to the diet and reproductive factors mentioned above, other factors also contribute to the difference in mercury concentration, such as the species, the age (correlated to the length and weight), the tissue, and the ecological complexity.

The species

Mercury concentration is higher in bottlenose dolphins (*T. truncatus*) than in the common dolphins (*Delphinus delphis*) or white-sided dolphins, while cadmium, lead, manganese, and chromium concentrations are similar to or even less in the bottlenose dolphin (Kuehl and Haebler 1995). These differences in mercury may result from dietary, and/or metabolic differences.

The age

In several cetacean species, the mercury concentration increases with age (Leonzio et al. 1992). For example, a positive correlation between mercury level and age has been found in the liver of the striped dolphin (*Stenella coeruleoalba*) (Cardellocchio 1995), in the liver of bottlenose dolphins (Kuehl and Haebler 1995), and in the liver, kidney, muscle, and blubber of pilot whales (Muir et al. 1988).

The relationship between heavy metal concentration and age in the striped dolphin can be classified as the following: (1) Concentrations increase from birth to sexual maturity or older and then reach a plateau, (2) Concentrations of metals decrease from birth to sexual maturity or older and then level out, (3) Concentrations of metals increase with age, and (4) Concentrations remain constant with age, indicating that the accumulation rate of the metal is equal to the rate of increase in body weight. (Honda et al. 1983). Interestingly, the mercury accumulation in different organs follows a different pattern: the muscular mercury follows the first pattern, but renal mercury conforms to the second pattern (Miyazaki 1994).

The tissue

Inorganic mercury tends to accumulate in the liver and kidney. Organic mercury, conversely, cannot stay in the body very long since it is degraded into the inorganic form quickly. Before the organic mercury is excreted, it accumulates in the kidney (Osweiler 1996). Humphreys (1988) reported that 85-95% of mercury accumulated in the kidney seven days after exposure. Nonetheless, the mercury in cetaceans seems to accumulate more in the liver than in the kidney. This different accumulation may result from the coexistence of selenium (Fang 1977).

In cetaceans, the distribution of mercury in various tissues in a decreasing order is liver, kidney, and muscle (Leonzio et al. 1992; Marcovecchio et al. 1994). The reason that cetaceans can tolerate a high mercury concentration in liver without significant toxic effects is the formation of tiemannite (HgSe) (Martoja and Viale 1975).

The ecological complexity

Cetaceans can be considered as possible indicators for marine pollutants, which reflect the heavy metal contamination of the sea where they live (Marcovecchio et. al. 1994; Cardellocchio 1995). The dolphins in the Mediterranean Sea have a higher mercury level than similar species in the Atlantic Ocean (Cardellocchio 1995), and this can be explained the by the more severe pollution in Mediterranean.

Nonetheless, no linear relation exists between the contamination in the aquatic environment and mercury concentration in the cetacean. Honda (1990) has discussed the mercury level differences in the northwest Pacific and Antarctic Oceans. Assuming that the mercury concentrations in the surface water in both areas are the same, one unit, the accumulated mercury concentration of striped dolphins in the northwest Pacific is one million units, but in the Antarctic Ocean it is ten thousand units. The reason for the difference in accumulation rate is related to the different ecological complexity. The more complex food chain existing in the northwest Pacific contributes to the higher accumulation rate.

Metabolism of Mercury

The metabolism of mercury is the key to understanding why the cetaceans can tolerate such high mercury concentrations in the liver without harmful effects. Cetaceans cannot excrete mercury through the respiratory system as fish do, but they can take advantage of the formation of non-toxic HgSe (Leonzio et al. 1992). Palmisano et al (1995) described a two-stage mechanism for the demethylation accumulation process in dolphins.

The respiratory influence

The gills of fish are in contact with water all the time, and contaminants, including methylmercury, have a chance to be exchanged from the blood to teh water. Therefore, the contaminants in fish can remain in a stable concentration in the long term through dynamic equilibrium. Since cetaceans lack the ability to eliminate mercury through the respiratory system, their high mercury concentrations can reach ten tiems that of fish. The most important balance mechanism of this phenomenon is likely the *in vivo* selenium and mercury combination (Leonzio et al. 1992).

Mercury and selenium

The mercury and selenium accumulation at the same rate and a perfect 1:1 molar ratio has been reported in various cetacean species in different areas. The first description of this relationship was reported by Koeman et al in 1973. This equimolar ratio was found in striped dolphins and bottlenose dolphins near Italy (Leonzio et al. 1992; Palmisano et al. 1995), as well as in the bottlenose dolphins of the Gulf of Mexico (Kuehl and Haebler 1995).

The formation of HgSe from mercury and selenium in the liver of cetaceans was first observed by Martoja et al. in 1975. The HgSe is an insoluble and non-toxic compound, and plays an important role in preventing toxic effects from occurring during the high mercury concentrations in the cetacean liver, as has been observed in other animal species.

When age is also considered, Leonzio et al. (1992) observed a prevalence of selenium in young dolphins, and a prevalence of mercury in adult animals. The similar ratio difference with age is the same as the observation of striped dolphins in the Pacific by Itano et al. (1984). Since the selenium was a protective element against mercury toxicity, the protective effect could decrease with increasing age.

The two-stage mechanism

Palmisano et al. (1995) described a two-stage mercury accumulation mechanism. In the first stage, when the mercury concentration was low, the primary form of mercury was methylmercury. When the mercury accumulated over a threshold value, it entered the second stage: the methylmercury transformed to inorganic mercury through a demethylation process, then combined with selenium, which accumulated concurrently. The second stage was the dominant mechanism.

Above the threshold, the prevalent form of the mercury was a "nonlabile" form, with only a little mercury exiting in the labile form. For example, the Hg^{++} and $MeHg^{+}$ bound to cysteine residues are labile forms.

Conclusions

Mercury contamination in cetaceans is a global problem. Cetaceans obtain mercury mainly through the biomagnification effect. The mercury contamination level of the prey has a positive influence on the mercury level in the cetaceans. Female cetaceans can transfer the mercury to the fetus and calf through the placenta and milk, respectively.

The mercury concentration varies with the species, age, tissue, and ecologic complexity in terms of the food web. Fish-eaters tend to have higher mercury concentrations than the squid-eaters, because fish contains more mercury than squid. The mercury values have a positive correlation with the age, and this is associated with the bioaccumulation and decreased Se protection. Among the three most commonly tested tissues, the liver contains the highest mercury concentration, followed by the kidneys and

muscle. When the environment is considered, the more complex food chain contributes to a higher accumulation rate.

The metabolism of mercury mainly involves demethylation of organic mercury and subsequent formation of HgSe. The early form of accumulated mercury is methylmercury, which is converted to mercury and binds with Se to form HgSe when the mercury concentration surpasses a threshold. Mercury-selenium complex is non-toxic, and may be considered as a protective mechanism against mercury toxicity.

Mercury contamination is considered largely as an artificial environmental hazard. If mercury contamination is decreased, the mercury levels in cetaceans could decrease, because the change has been shown in captive cetaceans. After years in captivity, cetaceans from oceans with severe mercury contamination showed a decline in blood mercury concentration, and the longer they remained in captivity, the greater the reduction in mercury levels (Harrison and Bryden 1993).

HYPOTHESES AND SPECIFIC AIMS

The following specific hypotheses were tested in this project:

1. Mercuric chloride ($HgCl_2$) can damage Atlantic spotted dolphin renal cells (Sp1K cells) as assessed by altering cellular ultrastructure.
2. Mercuric chloride can decrease cell proliferation as a mechanism of toxicity.
    2.1 Mercuric chloride can decrease cell proliferation through changing the cell cycle.
    2.2 Mercuric chloride can decrease cell proliferation by inducing apoptosis.
3. Atlantic spotted dolphin renal cells are more resistant to the cytotoxicity of mercuric chloride than cultured Rhesus monkey renal cells (MK2 cells).
4. Selenium can protect cultured Atlantic spotted dolphin renal cells from the toxicity of mercuric chloride.
    4.1 Selenium protection takes place through formation of a mercury-selenium complex.
    4.2 Selenium protection is modulated through reduction of mercury-induced apoptosis.

The specific aims of the research were to determine:

Part II:

1. Whether $HgCl_2$ induces ultrastructural changes in Sp1K cells.
2. Whether $HgCl_2$ induces apoptosis in Sp1K cells.

Part III:

1. Whether $HgCl_2$ decreases cell proliferation in Sp1K cells.
2. Whether $HgCl_2$ decreases cell proliferation in MK2 cells.
3. Whether Sp1K cells are more resistant to a $HgCl_2$-induced decrease in cell proliferation than are cultured MK2 cells.
4. Whether $HgCl_2$ changes cell cycle status of Sp1K cells.
5. Whether $HgCl_2$ changes cell cycle status of MK2 cells.

6. The relationship between $HgCl_2$-induced changes in cell proliferation, cell cycle, and apoptosis.

Part IV:

1. Whether sodium seleniate (Na_2SeO_3) protects Sp1K cells from a $HgCl_2$-induced decrease in cell proliferation.
2. Whether Na_2SeO_3 protects Sp1K cells from $HgCl_2$-induced apoptosis.

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PART II

MERCURIC CHLORIDE-INDUCED SUBCELLULAR MORPHOLOGICAL CHANGES OF SP1K ATLANTIC SPOTTED DOLPHIN RENAL CELLS

BACKGROUND

The nephrotoxicity of mercury is well established, and the pathological changes in the kidneys after acute and chronic exposures to mercury have been extensively studied *in vivo* and *in vitro*. Mercury accumulates mostly in the kidneys of laboratory animals and humans after a single or chronic exposure to mercury (Madsen and Maunsbach 1981; Chen et al. 1983). Proximal tubules have been observed to be the most sensitive to the toxicity of mercury. Cellular injury was first observed in the pars recta, and with increasing dosage or exposure time, the pars convoluta was affected as well (Taylor 1965; Ganote et al. 1974; Siegel and Bulger 1975; Haagsma and Pound 1979; Madsen and Maunsbach 1981). During the whole course of mercury toxicity, the most severe changes usually occurred in the pars recta (Boti et al. 1979).

GROSS MORPHOLOGICAL CHANGES

Chronic exposures to mercuric chloride ($HgCl_2$) in rats significantly increased the size and weight of the kidney (Kempson et al. 1977; Madsen and Maunsbach 1981). The relative water content of the enlarged kidney was not different from that of control animals, so the increase of kidney weight was not due primarily to an increased water content (Madsen and Maunsbach 1981). Combined with the light microscopic findings, the increase in renal size and weight was due primarily to an increase in the volume of the proximal tubules as well as the combination effect of tubular dilatation and an approximately 30% increase in absolute tissue volume (Madsen and Maunsbach 1981).

The surfaces of the kidneys of rats administered $HgCl_2$ were granular and slightly spotted (Madsen and Maunsbach 1981), or extremely pale and mottled (Siegel and Bulger 1975). Unlike healthy kidneys, in which peeling of the renal capsule leaves a smooth

surface, kidneys of male rats subjected to more than 16 days of exposure to low dose (1.5 mg/kg) $HgCl_2$ had irregular depressions in the renal surface after stripping of the renal capsules (Haagsma and Pound 1979). The medulla had changed color from its normal pink to dark red (Kempson et al. 1977). A well-defined pale boundary was observed in the cortico-medulla zone (Kempson et al. 1977; Haagsma and Pound 1979), which later became a white necrotic band occupying the outer stripe of the medulla with variable extensions into the cortical zones (Haagsma and Pound 1979).

LIGHT MICROSCOPIC CHANGES

Cellular injury in rats was first seen in the pars recta, then in the pars convoluta. Calcium deposits were detected a few days after $HgCl_2$ injection *in vivo* (Siegel and Bulger 1975). The microscopic line between intact areas and damaged cells was quiet clear (Haagsma and Pound 1979). Glomeruli were normal for up to 4 days of exposure to 16 mg/kg $HgCl_2$ *in vivo* (Siegel and Bulger 1975).

Cellular debris, including degenerated cells and cell fragments, was found in the ascending part of the loop of Henle and distal convoluted tubules shortly after cellular damage was seen (Haagsma and Pound 1979). By the end of the first day, debris and casts were seen in more distal tubules, collecting ducts, and the urine of rats administered a single injection of 1.5 mg/kg $HgCl_2$ (Intraperitoneally) or 4 mg/kg $HgCl_2$ (subcutanesouly) (Siegel and Bulger 1975; Haagsma and Pound 1979). The tubules above the casts appeared dilated (Haagsma and Pound 1979).

An inflammatory reaction, which was characterized by the initial appearance of leucocytes, monocytes, and lymphocytes in the interstitial tissues, was observed 24 hours after $HgCl_2$ injection. (Haagsma and Pound 1979).

The basement membrane was basically intact (Krus 1976), but bare as a result of cell shedding after exposure to $HgCl_2$ (Siegel and Bulger 1975; Haagsma and Pound 1979). The surviving epithelial cells went through mitosis and re-covered the basement membrane (Haagsma and Pound 1979). Mercuric chloride also induced thickening of the

basement membrane in the kidney of crucian carp, *Carassius carassius* (Deung et al. 1978).

SUBCELLULAR CHANGES

Renal ultrastructural changes induced by $HgCl_2$ occurred prior to the histological changes observed by light microscopy (Haagsma and Pound 1979). These changes were dose- and time-dependent (Siegel and Bulger 1975). Furthermore, the sequence of the ultrastructural changes concurred with histochemical studies showing that $HgCl_2$ first affected the luminal plasma membrane and apical vacuoles, whereas mitochondria and other parts of the cell were changed in the later stages (Kempson et al. 1977).

Cell Membranes

Blebbing and disturbed microvilli were observed in brush borders immediately after the administration of mercury (Siegel and Bulger 1975; Kempson et al. 1977). In contrast, the basal plasma membranes were unaffected in the early stages, as revealed both in the morphology and in the activities of the basal membrane enzymes (Kempson et al. 1977).

Cytoplasmic blebs induced by $HgCl_2$ were observed at the luminal surface of the damaged brush borders, as revealed in scanning electron microscopy (SEM) (Siegel and Bulger 1975). These blebs resulted from reorganization of cytoskeletal proteins, and presumably from alterations in cytoskeleton-plasma interactions as well (Elliget et al. 1991). Mercuric chloride elevated the concentration of cytoplasmic free calcium^{2+} ($[Ca^{2+}]_i$) in the renal cells (Phelps et al. 1989), and reduced the abundance of F-actin, a cytoskeletal protein (Elliget et al. 1991). Since (a) the Ca^{2+}-activated proteinases, such as calpain, are known to affect cell membrane proteins and membrane-to-cytoskeleton actin attachment proteins, and (b) the disappearance of F-actin can be prevented by leupeptin, a calpain cytoskeletal inhibitor (Elliget et al. 1991), the following mechanism appears

possible: the $HgCl_2$-induced high $[Ca^{2+}]_i$ activated the calpain, which altered cytoskeleton proteins, including F-actin. Cellular membrane blebbing represented a combination effect of F-actin diminishment and other factors (Elliget et al. 1991).

Microvilli were extremely irregular and disoriented in the initial course of mercury toxicity, and they were internalized and lined with vacuoles by endocytosis (Ganote et al. 1974; Siegel and Bulger 1975) or fused with other microvilli (Ganote et al. 1974). By 12 hours after a single subcutaneous injection of $HgCl_2$ at 3 or 4 mg/kg body weight in rats, microvilli were often completely absent (Gritzka and Trump 1968; Kempson et al. 1977). The modified smooth luminal plasma membranes may have performed a protective function by preventing the escape of the cytoplasm (Kempson et al. 1977), although most cells eventually broke.

In spite of all of the morphological changes, such as the blebbing and the disappearance of microvilli and junctional complexes (Siegel and Bulger 1975), the chemical composition of the plasma membrane essentially remained the same under the influence of $HgCl_2$ (Kempson et al. 1977).

Mitochondria

Renal mitochondria went through various stages of morphological change following exposure to $HgCl_2$. Renal mitochondria became rounder and showed intracristal swelling for a short period of time (Taylor 1965; Gritzka and Trump 1968; Ganote et al. 1974; Siegel and Bulger 1975). Later, matrical swelling and intramitochondrial inclusions were observed as irreversible changes induced by $HgCl_2$ (Gritzka and Trump 1968; Siegel and Bulger 1975). In severely damaged cells, the contours of mitochondria were wavy and irregular (Gritzka and Trump 1968; Kawahara et al. 1975) or even ruptured (Kawahara et al. 1975) and the number of mitochondria decreased (Andreev 1991). Tubular myelin-like figures were found in some mitochondria (Gritzka and Trump 1968).

The two types of mitochondrial swelling, i.e., intracristal swelling and matrical swelling, are useful indicators for describing sequential morphological changes. Intracristal swelling happened early in the injury, and was transient (Gritzka and Trump 1968). Mitochondria were rounded with condensed matrical spaces and expanded intercristal space . Normal matrical granules were decreased first in density (Ganote et al. 1974) and then in number (Gritzka and Trump 1968), and the matrix was condensed (Gritzka and Trump 1968; Ganote et al. 1974; Siegel and Bulger 1975). Matrical swelling took place in the later intervals. At this stage, matrix granules were usually absent, and mitochondrial inclusions became apparent.

Three types of inclusions in mitochondria were observed in renal cells following exposure to $HgCl_2$: (1) flocculent density inclusions in mitochondria; (2) granular density inclusions; and (3) homogenous electron-dense inclusions (Gritzka and Trump 1968; Ganote et al. 1974; Madsen and Maunsbach 1981). The first two were seen after acute mercury intoxication; however, with chronic exposure to mercury, only the last type of inclusions was observed.

Flocculent density inclusions were considered a sign of irreversibly altered mitochondria. These amorphous inclusions appeared in the matrix (Ganote et al. 1974; Siegel and Bulger 1975) near the membranes (Gritzka and Trump 1968) and gradually enlarged. Although it has not been proven, the content was most likely denatured matrix material (protein) (Gritzka and Trump 1968; Ganote et al. 1974).

In contrast, granular density inclusions, which were also described as microcrystalline deposits, were located within the cristae. Granular inclusions could grow larger than the width of the cristae, while still surrounded by cristal membranes (Gritzka and Trump 1968; Ganote et al. 1974). This type of inclusion had a similar structure to calcium-loaded mitochondria (Greenawalt et al. 1964). Mercuric chloride is known to increase Ca^{2+} concentration (Phelps et al. 1989), and the development of a large number of granular inclusion bodies corresponded with the time of massive influx of Ca^{2+} (Siegel and Bulger 1975). Therefore, a reasonable assumption is that the granular

inclusion consisted of accumulated calcium (Gritzka and Trump 1968; Ganote et al. 1974; Andreev 1991).

The third type of inclusion (homogenous electron density inclusion) in the proximal tubules was only found after chronic exposure to $HgCl_2$. Its origin and composition remain unknown (Madsen and Maunsbach 1981).

Golgi Apparatus

The Golgi apparatus appeared normal in rat renal cells 3 and 6 hours after a subcutaneous injection of 4 mg $HgCl_2$/kg body weight (Gritzka and Trump 1968). Historically, researchers have not mentioned the renal Golgi apparatus following exposure to mercury, probably because early fragmentation reduced the Golgi apparatus indistinguishablely among the numerous single membrane vesicles (Gritzka and Trump 1968).

Lysosomes

The function and structure of renal lysosomes were affected in the early stage of acute mercury intoxication (Taylor 1965; Siegel and Bulger 1975; McDowell et al. 1976). Inhibited activities of renal lysosomal enzymes were observed in isolated lysosomal enzymes and in rats following chronic exposure to both methylmercury and $HgCl_2$ (Madsen and Christensen 1978). An increased number of lysosomes and changes in size were observed as early as one hour following exposure to 4 mg/kg $HgCl_2$ in rats *in vivo* (Siegel and Bulger 1975), while at the end stage, lysosomes and micobodies were rarely present (Siegel and Bulger 1975). In contrast, an increased number of lysosomes was not observed in chronic exposure to $HgCl_2$ (Madsen and Maunsbach 1981). However, a significant increase was observed in the relative volume of proximal tubule lysosomes following chronic exposure to both methylmercury (Fowler et al. 1975) and $HgCl_2$ (Madsen and Maunsbach 1981).

Endoplasmic Reticulum

Both rough endoplasmic reticula (RER) and smooth endoplasmic reticula (SER) were adversely affected by mercury in the early stages of exposure (Gritzka and Trump 1968). Swelling of the cisternae of both RER and SER was observed in all segments of the proximal tubules (Pfaller et al. 1990). Condensation of ER was also observed (Krus 1976).

In addition to dilatation and swelling of RER cisternae (Siegel and Bulger 1975), mercuric chloride also caused ribosomes to shed from the surface of RER. Consequently, the number of free ribosomes increased (Gritzka and Trump 1968).

On the other hand, SER proliferated and formed accumulated clumps in the early stages (Gritzka and Trump 1968; Ganote et al. 1974). The aggregated SER has been described as either compact with cleftlike spaces but lacking a regular reticular pattern (Ganote et al. 1974) or large spherical mass SER at the apex and base of the injured cells (Siegel and Bulger 1975). Later, the temporary proliferations of SER broke up into fragments (Gritzka and Trump 1968).

Ribosomes

The number of ribosomes was elevated in renal cells in the early course of $HgCl_2$ toxicity due to dilated RER shedding and polysome break up (Gritzka and Trump 1968). Ribosomes appeared as scattered single particles, and sometimes as clusters (Ganote et al. 1974). Later the number of ribosomes decreased, and they were randomly scattered in the cytoplasm. Eventually, they became unidentifiable (Gritzka and Trump 1968). In fibroblasts from the mice receiving $HgCl_2$, the electron density of ribosomes seemed to increase, and rosette-like polyribosomes were observed (Kawahara et al. 1975).

Nucleus

Nuclear changes varied among the cells receiving the same $HgCl_2$ treatment and usually corresponded with cytoplasmic changes in oncosis/necrosis (Gritzka and Trump 1968; Haagsma and Pound 1979). Normal nuclei were seen in swollen cells with only

small vacuoles (Haagsma and Pound 1979). When the vacuoles enlarged, nuclei were often displaced to the base of the cells (Siegel and Bulger 1975; Haagsma and Pound 1979) and showed clumped chromatin and interchromatic granules (Ganote et al. 1974; Siegel and Bulger 1975; Haagsma and Pound 1979). In severely damaged cells with eosinophilic cytoplasm, nuclei were pyknotic or karyolytic (Taylor 1965; Haagsma and Pound 1979). Furthermore, nuclear shrinkage was observed in the renal cells of fish *in vivo* (Dong et al. 1997), and micronuclei formation was revealed in skin fibroblasts of beluga whales *in vitro* (Gauthier et al. 1998) following exposure to $HgCl_2$.

In the early stages of exposure, chromatin tended to aggregate at the periphery of the nuclei (Gritzka and Trump 1968; Siegel and Bulger 1975; Haagsma and Pound 1979) and around nucleoli (Gritzka and Trump 1968), while interchromatic granules clustered near the center of the nuclei (Siegel and Bulger 1975). Nuclear membranes were interrupted. The intramembranous spaces were focally dilated, or the enveloping membrane was focally discontinued (Gritzka and Trump 1968). Enlarged, condensed nucleoli were often found in their regular oval or circular shape (Andreev 1991). In the nucleoplasm, homogenization and densification were observed in focal areas (Gritzka and Trump 1968).

In advanced cellular damage, fragmentation of chromatin and dissolution of interchromatic granules were observed (Gritzka and Trump 1968). Rupture of the nuclear membranes released the nuclear content. The nucleoplasm, which can be identified by its flocculent appearance, was evenly distributed (Gritzka and Trump 1968). Nuclear structure became denser and more distinct in many cells (Gritzka and Trump 1968; Andreev 1991) due to the dense fibrillar component and increased amount of granular components. Not uncommonly the granular components divided into two groups occupying two opposite sides of the nucleus. Multiple nucleoli within in a single nucleus, which resulted from fragmentation of the nucleoli, were also found (Andreev 1991). Sometimes the nucleoli were unidentifiable (Gritzka and Trump 1968).

In addition to oncosis and necrosis, apoptosis was also induced by $HgCl_2$ (Shenker et al. 1995; Goering et al. 1997) in renal cells (Duncan-Achanzar et al. 1996). In

apoptotic cells, the nucleus changed without significant changes in organelles. The typical apoptotic morphological changes were $HgCl_2$ concentration-dependent. Cultured porcine renal cells showed cell shrinkage and nuclear condensation following exposure to 35 μM $HgCl_2$ for 7 to 12 hours (Duncan-Achanzar et al. 1996).

CELLULAR DEATH CLASSIFICATION

Mercury-induced renal cellular damage has been classified into several forms: apoptosis, necrosis, apoptotic necrosis, and oncosis, depending on the form and concentration of mercury as well as exposure time (Nishisaka and Kishimoto 1994; Duncan-Achanzar et al. 1996; Goering et al. 1997). Researchers have used various criteria for distinguishing cell death forms. The conclusions from these publications may need to be reviewed carefully, since the quantification standards of these cell death types have been changed in recent years (Hockenbery 1995; Majno and Joris 1995; Levin 1998; Koester and Bolton 1999). For example, DNA ladders, which used to be considered as an unique character of apoptosis, have been observed in cells without morphologic characters of apoptosis (Ueda et al. 1995), and apoptotic cells may show no evidence of ladder-pattern of DNA degeneration (Oberhammer et al. 1992; Zakeri et al. 1993; Boix et al. 1997). Multiple methods are recommended to identify cell death types (Boix et al. 1997; Koester and Bolton 1999), and the best identification is by morphological change (Hockenbery 1995).

The new well-accepted standards of three major cell death types, apoptosis, oncosis, and necrosis, were defined by Majno and Joris in 1995. The most common microscopic criteria for apoptosis are cellular shrinkage, pyknotic chromatin, chromatin migration to the edge of nuclei, karyorrhexis, cell membrane budding (no swelling) and minimal to no swelling of organelles. Apoptotic cells may form apoptotic bodies, which may contain various organelles, including nuclear fragments. In contrast, oncosis is characterized by cellular swelling, karyolysis, cell membrane blebbing (which tend to swell and burst, usually excluding organelles), and organelle swelling. Cell membrane permeability increases in oncotic cells, and can be used as a diagnostic tool. When the

cellular damage progresses to an irreversible stage (such as karyolysis, pyknotic karyorrhexis, loss of cytoplasmatic structure, and fragmentation), the changes are defined as necrosis. Necrosis can result from either apoptosis or oncosis. (Majno and Joris 1995; Levin 1998)

Mercuric Chloride-induced Apoptosis and Oncosis in Renal Cells

A moderate concentration of $HgCl_2$ (35 μM) caused cultured porcine renal cells to become apoptotic, as determined by the morphological changes for apoptotic cells and the formation of a ladder pattern of DNA in gel electrophoresis (Duncan-Achanzar et al. 1996). Twenty μM $HgCl_2$ induced the expression of the c-fos gene, which is associated with apoptosis (Matsuoka et al. 1997). High concentration of $HgCl_2$ (100 μM), on the other hand, caused oncosis/necrosis (Duncan-Achanzar et al. 1996). The phonemenon that the same stimulant can cause both apoptosis and oncosis dependent on dosage is not unique for mercury.

OBJECTIVES OF THIS STUDY

Mercury is a major marine pollutant worldwide (Keckes and Miettinen 1970; Baldi 1986), and marine mammals accumulate high concentrations and body burdens of mercury due to their top position in the food web (Baldi 1986; Miyazaki 1994). Marine mammals display not only a low susceptibility to mercury (Bakir et al. 1973; Nordberg 1976; Itano et al. 1984; Rawson et al. 1995; Siebert et al. 1999), but also a different distribution of mercury in various organs compared to terrestrial mammals. The kidney acquires the second highest concentration of mercury, after the liver, in wild cetaceans which are constantly exposed to various environmental pollutants including mercury and selenium (Itano and Kawai 1981; Muir et al. 1988; Cardellocchio 1995; Dietz et al. 1996; Monaci et al. 1998; Dietz et al. 2000; Johansen et al. 2000).

In spite of the numerous reports of mercury in marine mammals, no publications have addressed the renal ultrastructural changes caused by mercury in cetaceans. Because all cetaceans are protected by law, and wild cetaceans are subjected to all marine

pollutants, cultured renal cells are the best material available for studying the effects of mercury on subcellular morphology. Mercury-induced renal cell ultrastructural changes have also been reported to be very similar *in vivo* and *in vitro* (Gritzka and Trump 1968). The purpose of the present work was to study the ultrastructural changes in cultured Atlantic spotted dolphin (*Stenella plagiodon*) renal cells following exposure to subcytotoxic concentrations of mercuric chloride, which is more nephrotoxic than organic mercury.

MATERIAL AND METHODS

Cell Culture

The Atlantic Spotted Dolphin *(Stenella plagiodon)* renal cell line, Sp1K, was purchased from the American Type Culture Collection (Rockville, MD) and cultured in a humidified incubator with 5% CO_2 at 37^{O}C. Minimal essential medium (MEM) with non-essential amino acids and L-glutamine (BibcoBRL, Grand Island, NY) including of 10% fetal bovine serum (FBS) (GibcoBRL, NY) was used as the growth medium. Cells were grown as adherent cultures in 50 cm^2 petri dishes (Falcon, Sonvrlle, NJ), except in the case of specific tests (see below). When performing a new experiment, the cells were detached from the petri dish by 0.05% trypsin and 0.02% ethylene-diamine-tetraacetic acid (EDTA) (Sigma, St. Louis, MO) in phosphate buffered saline (PBS).

Transmission Electron Microscopy

Cells were grown in 100mm^2 Permanox petri dishes (Nunc, Naperville, IL) and exposed to growth medium alone for 24 and 96 hours or 10 μM $HgCl_2$ for 96 hours or 20 μM $HgCl_2$ for 24 hours. Mercuric chloride (Sigma, MO) was dissolved, just before use, in the growth medium at 1000 μM for use as a stock treatment medium. Stock medium was further diluted to 10 or 20 μM $HgCl_2$ for use as a treatment medium. The growth medium alone served as the control. The growth medium was replaced by treatment media or control medium 24 or 96 hours before fixing the cells.

Cells were fixed in cold Karnovsky's fixative (2.5% glutaraldehyde and 2% formaldehyde) for 24 hours in the original petri dishes. Cells were then washed in 0.1 M sodium phosphate buffer, post-fixed in 1% OsO_4 in 0.1 M sodium phosphate for one hour, and washed again. Dehydration was performed in a graded ethanol series followed by propylene oxide. Specimens were infiltrated overnight with a solution made of equal volumes of prophylene oxide and Poly/Bed 812. Infiltration was completed by using 100% Poly/Bed 812 over the following night. Before oven-curing embedded samples at 60^{O}C, identified labels were added with 1ml of fresh Poly/Bed 812 to petri dishes. Cured samples were sawed into small pieces and mounted to appropriate labeled Beem capsules.

Thin sections (60-90 nm thick) were cut using a Leica Ultracut UCT microtome (Deerfield, IL), and collected on 200 mesh copper grids. Sections were stained with uranyl/acetone and lead citrate and viewed under a JEOL JEM-100 CXII transmission electron microscope (Tokyo, Japan) operating at 70KV.

Nucleic Acid Stain for Apoptosis

Apoptotic cells can be differentiated from live, oncotic and necrotic cells by the changes in cell size and cell membrane permeability. In contrast to live and apoptotic cells, which maintain their membrane integrity, oncotic and necrotic cells increase their cell membrane permeability. Live and apoptotic cells can be further distinguished from each other by cell size, which shrinks in apoptotic cells.

Seven-amino-actinomycin D (7-AAD) is a fluorescent dye, which specifically binds to GC regions of DNA. It has been documented that 7-AAD alone can replace the Hoechest 33342 plus 7-AAD method in which bright Hoechest 33342 density cells (non-living cells) can be further subdivided to 7-AAD negative cells (with intact cell membrane) and 7-AAD positive cells (having lost membrane integrity) (Schmid et al. 1994). In conjunction with a flow cytometer, cells stained only with 7-AAD can be classified into three groups: (1) live cells, which have a low 7-AAD density and normal cell size, (2) apoptotic cells, which have a medium 7-AAD density and are small, and (3) oncotic, necrotic, plus dead cells, which have a high 7-AAD density.

Cells were cultured in 75 cm^2 flasks, and exposed to growth medium alone, to 10 μM $HgCl_2$, or to 20 μM $HgCl_2$ for 12, 24, 48, or 72 hours before being harvested using trypsin and EDTA. In order to avoid artifacts induced by aging, overcrowding, and other unfavorable environmental situations in these cultures, all of the cells were harvested 6 days after seeding (before they reached 100% confluency). The total volume of treatment media and the PBS used to wash the cells was collected to obtain all the cells, including the detached cells. The cells were centrifuged at 1000 rpm (600 g) for 10 minutes, and the cell pellets were resuspended in the growth medium for cell counting. A total of $5x10^5$ cells were transferred to a 5 ml tube, centrifuged, and then collected as a

precipitate. One hundred µl of FACS buffer (0.1 g of sodium azide and 2 ml of fetal bovine serum in 98 ml PBS) and 100 µl 10 µg/ml 7-AAD per $5x10^5$ cells were added to the tubes, which were then vortexed. The cells were incubated on ice 15 to 30 minutes in the dark before flow cytometric analysis, which was accomplished with an Epics-XL flow cytometer (Beckman-Coulter, Miami, FL) equipped with an air cooled argon laser. The 7-AAD fluorescence was quantified under 650 nm emission and 488 excitation, and the cells were classified according the rules mentioned earlier.

Statistics

The experiment with the 7-AAD stain by flow cytometry had 10 data points for each exposure time and $HgCl_2$ concentration combination in 2 blocks. An analysis of variance (ANOVA) was performed. The statistical significance level employed was 0.05. The alpha was lowered to 0.0169 by the Bonferroni correction to bring the experiment-wide alpha level back to 0.05.

RESULTS

ULTRASTRUCTURAL MORPHOLOGIC CHANGES OF SP1K CELLS FOLLOWING EXPOSURE TO MERCURIC CHLORIDE ($HgCl_2$)

Control

Sp1K cells in the control groups (24 hour and 96 hour exposures to 0 μM $HgCl_2$) showed numerous pinocytotic bodies around the cell margins and scattered moderate swelling of rough endoplasmic reticula (RER) (Fig. 2.1). Elongated mitochondria had smooth outlines and parallel cristae. The nuclei had one or two nucleoli (Fig. 2.2).

24 hour Exposure to 20 μM $HgCl_2$

Cells exposed to 20 μM $HgCl_2$ for 24 hours showed various mild morphologic changes. The number of pinocytotic bodies did not change notably (Fig. 2.3). Clear secretory vacuoles in which the content may have been dissolved during tissue processing were occasionally observed (Fig. 2.4). Some cells revealed cytoplasmic vacuoles which were either bound in a single layer membrane or fused with cytoplasm. Large, irregularly shaped, degenerative cytoplasmic vacuoles containing membranous bodies and clusters of degenerative cytoplasmic fragments were observed in severely damaged cells (Fig. 2.5).

An increased number of electron dense particles was also observed in the cytoplasm (Fig. 2.6 and Fig. 2.7). Membrane-bound granulated inclusion bodies of moderate electron density were scattered throughout the cytoplasm (Fig. 2.6). Some RER were swollen (Fig. 2.8), while others were normal (Fig. 2.7). The Golgi bodies were ultrastructurally within normal limits (Fig. 2.8).

The organelle most significantly affected was the mitochondria. Their structure typically retained the normal length, but demonstrated rough, wavy outlines or were swollen and rounded with wavy outlines (Fig. 2.6 and Fig. 2.7). The cristae of the mitochondria were swollen, and intercristal spaces were irregular. Some mitochondria showed a dilated matrix or contained vacuoles (Fig. 2.6) and some mitochondria eventually transformed to membrane-bound, granulated inclusion bodies (Fig. 2.6).

Lysosome-like inclusions were found in very low numbers. These inclusions contained multiple membranes, RER, and other materials (Fig. 2.9). A few degenerated myelin-like figures were also found in multiphagic vacuoles (Fig. 2.5). The nucleus was homogenous in density (karyolysis) (Fig. 2.4).

96 hour Exposure to 10 µM $HgCl_2$

Increasing the exposure time produced additional and more severe ultrastructural changes. Cells exposed to 10 µM $HgCl_2$ for 96 hours displayed numerous cytoplasmic vacuoles, an increased number of lipid-like inclusions, abundant myelin-like figures and a decreased number of pinocytotic vacuoles (Fig. 2.10 and Fig. 2.11). The cytoplasmic vacuoles varied considerably in size and shape. The smallest vacuoles were half the diameter of the mitochondria, while the large ones reached 10 times the diameter of mitochondria. Conversely, the sizes of lipid-like inclusions were consistent, ranging between 1 to 2 times of the diameter of mitochondria. Another interesting change that was absent in the 24 hour exposure to 20 µM $HgCl_2$ was the appearance of myelin-like figures (Fig. 2.10). The myelin-like figures, whose name was suggested from the appearance of multiple concentric layers similar to the myelin, were presumably derived from damaged membranes, such as endocytoplasmic reticula or other cell membranes. These figures appeared with or without inclusions of cytoplasmic density.

The RER and mitochondria were swollen, and mitochondria contained swollen cristae (Fig. 2.12 and Fig. 2.13). Large multiphagic vacuoles, which contained myelin-like figures, swollen RER, swollen mitochondria and other organelles, were also found in the cytoplasm (Fig. 2.12). Multiphagic vacuoles had less electron density and more empty space than lysosomes, though both contained multiple organelles. Non-membrane bound granulated inclusion bodies of moderate electron density were present (Fig. 2.14).

Nuclear changes were diverse. In the less damaged cells, heterochromatin gathering around the nuclear membrane was observed. Sometimes the nucleos were not observed, and small high electron density granules appeared patchy in the nucleus (Fig. 2.14). In some cells nucleoli revealed considerable vacuolization (Fig. 2.15). In such

cells, all of the nucleoli had various sized vacuoles, and some nuclei had medium density, connected intranuclear vacuoles and pars amorpha as if the vacuole had burst through the nuclear membrane. Many smaller nucleoli-like particles (micronucleoli) with vacuoles were also observed (Fig. 2.15). Nuclear and cell membrane budding were rarely seen (Fig. 2.15). When present, nuclear buds contained a micronucleoli, while the cellular buds contained lipid-like inclusions, thus distinguishing them from blebs which are devoid of organelles and DNA fragments (Majno and Joris 1995).

MERCURIC CHLORIDE-INDUCED CELL DEATH IN SP1K CELLS

Concentration effects, exposure time effects, and concentration-exposure time interactions were observed in all three groups: (1) live cells, (2) apoptotic cells, and (3) oncotic, necrotic plus dead cells.

$HgCl_2$ decreased the percentages of live cells in a concentration-dependent manner (Fig. 2.16). After a 12 hour exposure, 20 μM $HgCl_2$ significantly decreased the ratio of live cells. After 48 and 72 hour exposures, live cells were significantly decreased from control at both the 10 and 20 μM $HgCl_2$ exposure tests.

The proportions of apoptotic cells were also elevated in a concentration-dependent manner. Low concentration (10 μM) $HgCl_2$ significantly increased apoptosis only at the longer exposure times of 48 and 72 hours. High concentration (20 μM) exposure to $HgCl_2$ significantly increased apoptosis at 12, 48, and 72 hour exposures. At the 72 hour exposure, the number of 20 μM $HgCl_2$ induced apoptotic cells was significantly increased as compared to the number of 10 μM $HgCl_2$ induced apoptotic cells. There appeared to be a trend that apoptosis increased with longer exposure time, but it was not tested statistically. Due to harvesting of the samples of the same exposure time in one day (block), separating the exposure time effect from the block effect was not possible. Theoretically, the time effect is unknown, since the changes could be due to day-to-day experiment variance (block effect).

For oncotic, necrotic, and dead cells, changes were largely similar to those of the apoptotic cells. Significant increases in the percentage of oncotic, necrotic, and dead

cells were seen at the 12 hour exposure to 20 μM $HgCl_2$, as well as at the 48 and 72 hour exposures to 10 and 20 μM $HgCl_2$. In addition to the 72 hour exposure (as seen in apoptotic cells), significant differences in percentage of oncotic, necrotic and dead cells between 10 and 20 μM $HgCl_2$ were also seen in the 48 hour exposure. This increase at the 48 hour exposure was primarily responsible for the difference of percentage of live cells between 10 and 20 μM $HgCl_2$ at the 48 hour exposure, since the apoptosis ratio was the same.

DISCUSSION

The present study demonstrated that multiple organelles in cultured Atlantic spotted dolphin renal cells were affected by the exposure to $HgCl_2$. The oncotic and necrotic morphologic changes were observed by a transmission electron microscope, and the apoptosis was detected by 7-AAD stain. Although comparison of the resistance of dolphin renal cells and terrestrial mammalian renal cells to $HgCl_2$ is complicated, the data suggest that Sp1K cells had lower frequencies of apoptotic cells when treated with $HgCl_2$ than other cell types.

ELECTRON MICROSCOPIC CHANGES

Past research into *in vivo* effects of $HgCl_2$ on renal cells of rats has documented swollen RER, shedding of ribosomes from RER, and clumped SER (Gritzka and Trump 1968; Ganote et al. 1974; Siegel and Bulger 1975). In the present *in vitro* study, changes in ER involved only swelling of RER. Consequently, the number of free ribosomes was not significantly increased in these Sp1K cells.

Most of the cells in this group still had granular cytoplasm, normal RER , swollen mitochondria, and pinocytotic bodies. In cells that demostrated karyolysis, a homogenous cytoplasm, very few organelles and multiphagic vacuoles containing myelin-like figures and electron-dense granules were observed.

Although the literature concerning other renal cells exposed to $HgCl_2$ does not report the decrease in the number of pinocytotic bodies. That was observed in Sp1K cells, Siegel et al. (1975) reported a decrease of the volume of the endocytotic apparatus in the renal cells of rats that were treated with $HgCl_2$.

Nuclear Changes

Karyolysis was seen in damaged Sp1K cells following a 24 hour exposure to 20 μM $HgCl_2$. In cells exposed to 10 μM $HgCl_2$ for 96 hours, nuclear changes, including patchy heterochromatin and vacuolization of nucleoli, were seen. The vacuolization of nucleoli has not been previously reported in cells treated with $HgCl_2$. The appearance of the nucleoli with multiple, various-sized vacuoles was similar to the documented changes of chromatin in ballooned keratinocytes of the tongue of oral hairy leukoplakia patients who were HIV positive (Guccion and Redman 1999), and in retinoblastoma at the late stage of necrosis (Cha et al. 2000). Nonetheless, the mechanisms of this change remain unknown. Micronucleoli were also observed in cells with vacuolated nucleoli. The micronucleoli were also vacuolated. Although micronucleoli have not been previously documented in $HgCl_2$-treated cells, micronuclei have been observed in both $HgCl_2$-and MeHg-treated cells (Al-Sabti 1995).

Cytoplasmic Vacuoles

The cytoplasmic vacuoles observed in Sp1K cells which were exposed to 10 μM $HgCl_2$ for 96 hours were bound within a single membrane. Pinocytotic vesicles and dilated mitochondria partly contributed to the formation of these vacuoles. The mitochondrial-derived vacuoles have also been reported in the proximal convoluted tubules of *Carassius carassius* (crucian carp) exposed to $HgCl_2$ (Deung et al. 1978). Due to the lack of microvilli on the plasma membrane of Sp1K cells, the vacuoles from cell membrane (pinocytotic vesicles) and mitochondria were not distinguishable from each other. In contrast, the vacuoles in renal cells from rats that were intravenously injected with $HgCl_2$ were morphologically divided into two groups: vacuoles with an inner fuzzy coat (derived from pinocytotic vesicles), and vesicles with smooth linings (derived from dilated endoplasmic reticulum (ER)) (Ganote et al. 1974). The Golgi apparatus has also been reported as a source of vacuoles (Gritzka and Trump 1968), but this was not observed in the present study.

Mitochondria

Mitochondria were one of the earliest affected organelles. Most altered mitochondria had matrical swelling and compressed intercristal spaces, and some mitochondria showed expansion of the intercristal space which eventually transformed the mitochondria into vacuole-like structure. Unlike renal mitochondria of rats that received 1.0 mg $HgCl_2$/kg body weight and whose matrix condensed (Ganote et al. 1974), mitochondria in this study retained a normal matrical electron density. Also differing from previous works involving *in vivo* systems (Gritzka and Trump 1968; Siegel and Bulger 1975), no inclusion bodies were found in the present study. The appearance of granular, dense inclusions (mitochondrial dense bodies) was associated with calcium accumulation. The difference in experiments with Sprague-Dawley rats that received the same dose of $HgCl_2$ (Gritzka and Trump 1968; Siegel and Bulger 1975) may be associated with the concentration of calcium in the system, which may vary with the diet of the animals. The absence of mitochondrial dense bodies in the present study may result from the difference between calcium concentration within the cells in the present versus previous studies, the suspectibility of the cell, and differences in *in vivo* versus *in vitro* experiments.

APOPTOSIS

The ultrastructural changes in cultured Sp1K cells following their exposure to $HgCl_2$ revealed that Sp1K cells went through oncosis and necrosis. Apoptosis induced by $HgCl_2$ was revealed by using 7-AAD dye in conjunction with a cell flow cytometer. However, ultrastructural morphologic changes of apoptotic cells were not seen in the present experiment. The differences between electron microscopic results and flow cytometric data may be due to (1) the low percentage of apoptotic cells and (2) the early detachment of apoptotic cells from the culture dish.

Apoptotic cells made up 1.32 % of total cells after a 24 hour exposure of 20 μM $HgCl_2$, and 4.28 % of cells after a exposure to 10 μM $HgCl_2$ for 72 hours. Even though a higher percentage of apoptotic cells was expected in the electron miscroscopic samples of

cells that received 10 μM $HgCl_2$ for 96 hours, the percentage most likely would still be very low. As a result of the natural limitations on cell quantities with the TEM method, the number of cells observed was very limited. The small area examined by TEM may simply have contained no apoptotic cells.

Detachment from the substrate is one of the earliest occurrences of apoptotic renal cells *in vivo* and *in vitro*. Epithelial renal cells *in vivo* detached early on from both the underlying matrix and other cells (Lieberthal et al. 1998), and cultured porcine renal cells detached from the culture flask before apoptosis (Duncan-Achanzar et al. 1996). Duncan-Achanzar et al. have shown that cultured porcine renal cells exposed to $HgCl_2$ revealed morphological evidence of apoptosis. Furthermore, the apoptotic morphology was only seen in floating cells, not in adherent cells. Because the current assessment by TEM targeted only cells that were attached to the petri dishes, the apoptotic cells may have been lost from the speculum.

Comparison of the present data with previously published studies is problematic for various reasons. For example, the percentage of apoptotic cells treated with $HgCl_2$ *in vitro* is affected not only by the sensitivity of the cells, but also by the culture conditions and sensitivity of the detection method. For example, increased serum concentration in the medium decreased apoptosis induced by mercury in a concentration-dependent manner (Shenker et al. 1997). Nonetheless, one report using mouse lymphocytes cultured in 10% FBS medium showed similar percentages of apoptotic cells in the control groups (Goering et al. 1997). About 33% of the mouse lymphocytes underwent apoptosis after an exposure to 10 μM $HgCl_2$ for 24 hours. In contrast, the present study found less than 5% of the dolphin renal cells exposed 10 μM $HgCl_2$ for 24 hours were apoptotic. Furthermore, even after a 72 hour exposure to 20 μM $HgCl_2$, the Sp1K cells had less than 20 % apoptotic cells. These data suggest that dolphin renal cells have a higher tolerance to $HgCl_2$ exposure than mouse cells in terms of induction of apoptosis.

Fig. 2.1 A portion of a Sp1K cell following a 24 hour exposure to medium only (control). Pinocytotic bodies (P) and mitochondria (M) were normal, while RER (arrow) showed moderate swollen. (X=17,600)

Fig. 2.2 The nucleus of a Sp1K cell after a 96 hour exposure to medium only (control). The nucleus (N) had intact nuclear membrane (NM) and two nucleoli (NC). The dense fibrillar component and granular component in the nucleoli are clearly shown. (X=9,300)

Fig.2.1

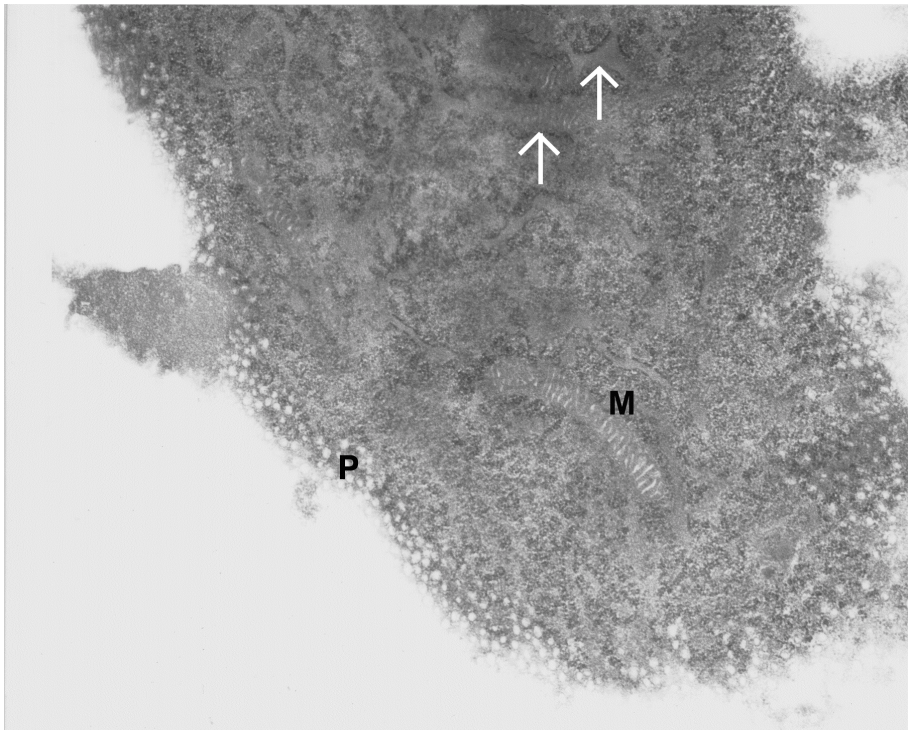


Fig. 2.2

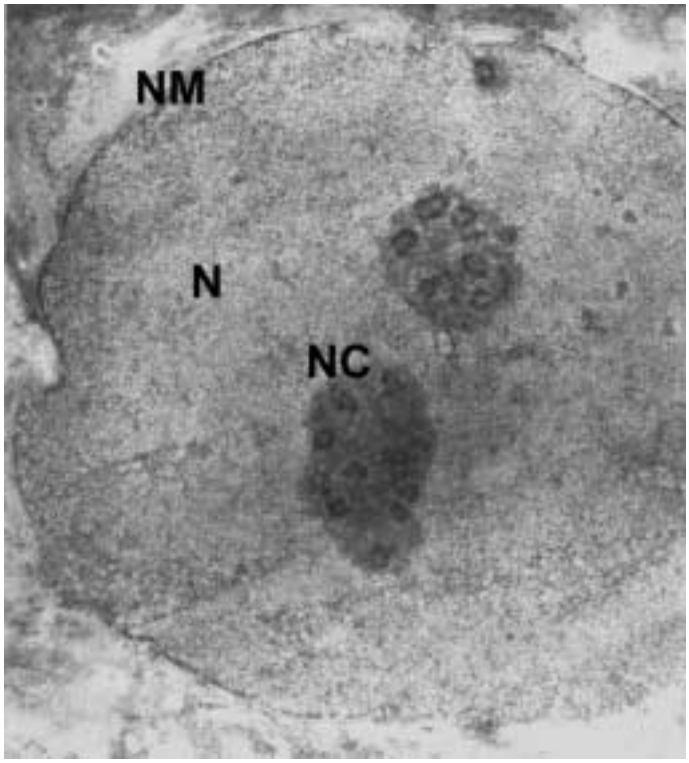


Fig. 2.3 Transmission electron micrograph of a portion of a Sp1K cell following exposure to 20 μM $HgCl_2$ for 24 hours. Pinocytotic bodies (P) were similar to control in number and shape. The contours of the mitochondria (M) were wavy, and cristae were irregular. (X=24,000)

Fig. 2.4 A portion of a Sp1K cell exposed to 20 μM $HgCl_2$ for 24 hours. The nucleus (N) presented a homogenous appearance (karyolysis) indicating irreversible damage. Secretory vacuoles (S) were identified and some fused with the cell membrane. (X=30,200)

Fig. 2.3

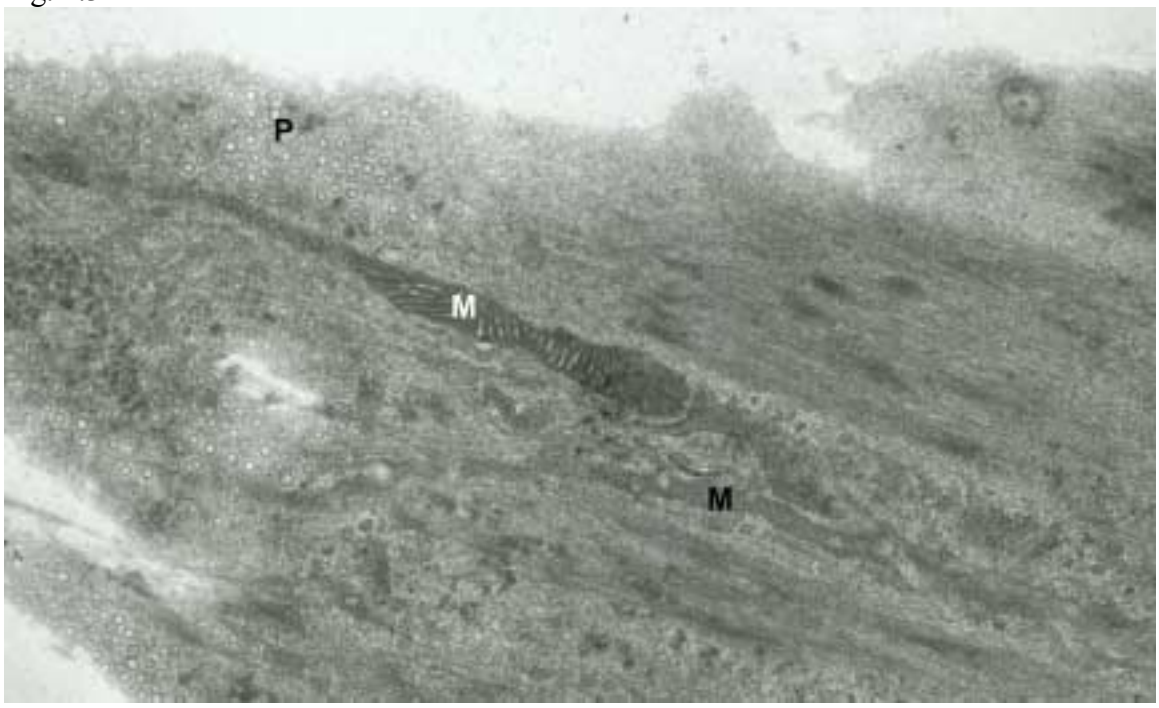


Fig. 2.4

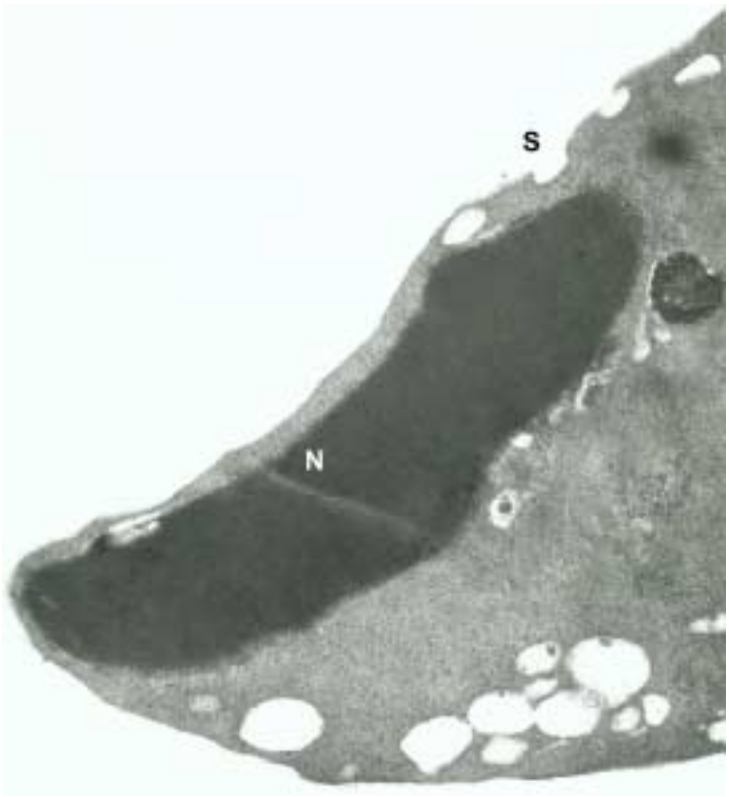


Fig. 2.5 Degenerative cytoplasmic vacuoles (D) and a degenerated myelin-like figure (MF) in a Sp1K cell after a 24 hour exposure to 20 μM $HgCl_2$. (X=35,600)

Fig. 2.6 A portion of a Sp1K cell exposed to 20 μM $HgCl_2$ for 24 hours. A membrane-bound granulated inclusion body (I) was observed, as well as multiple lipid-like inclusions (L). The mitochondria (arrows) were rounded with wavy outlines and irregular intercristal spaces. An expanded intercristal space in a mitochondrion (left arrow) may assume a large portion of the mitochondrion (middle arrow), and may eventually transform the mitochondrion into a membrane-bound granulated inclusion body (right arrow). (X=26,500)

Fig. 2.5

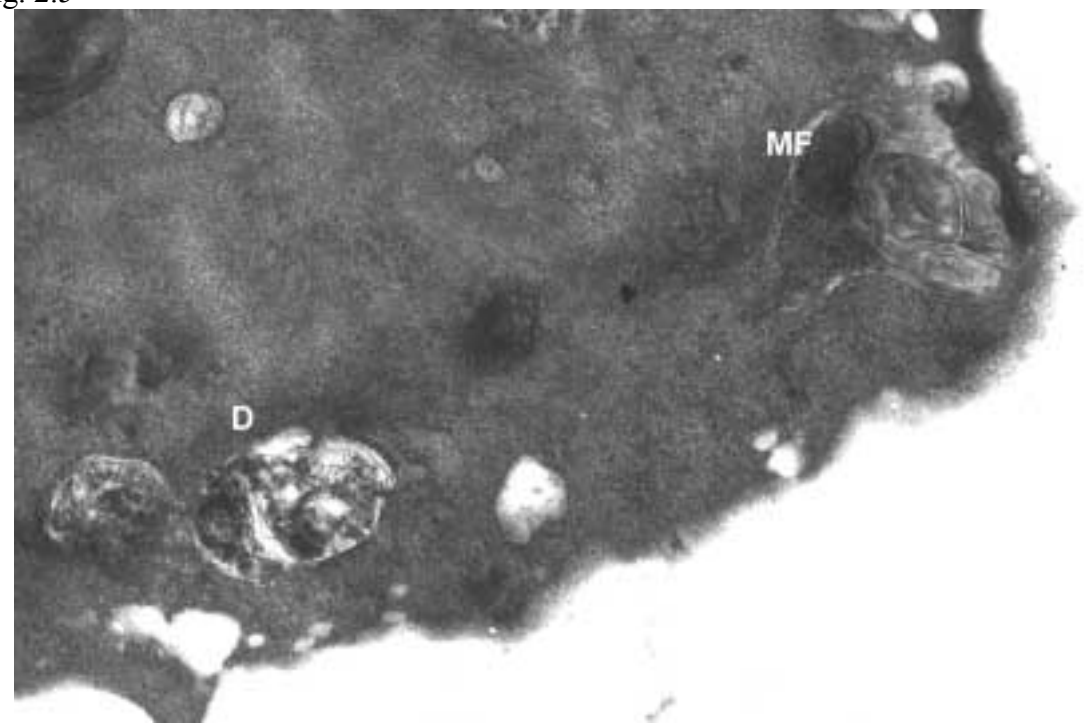


Fig. 2.6

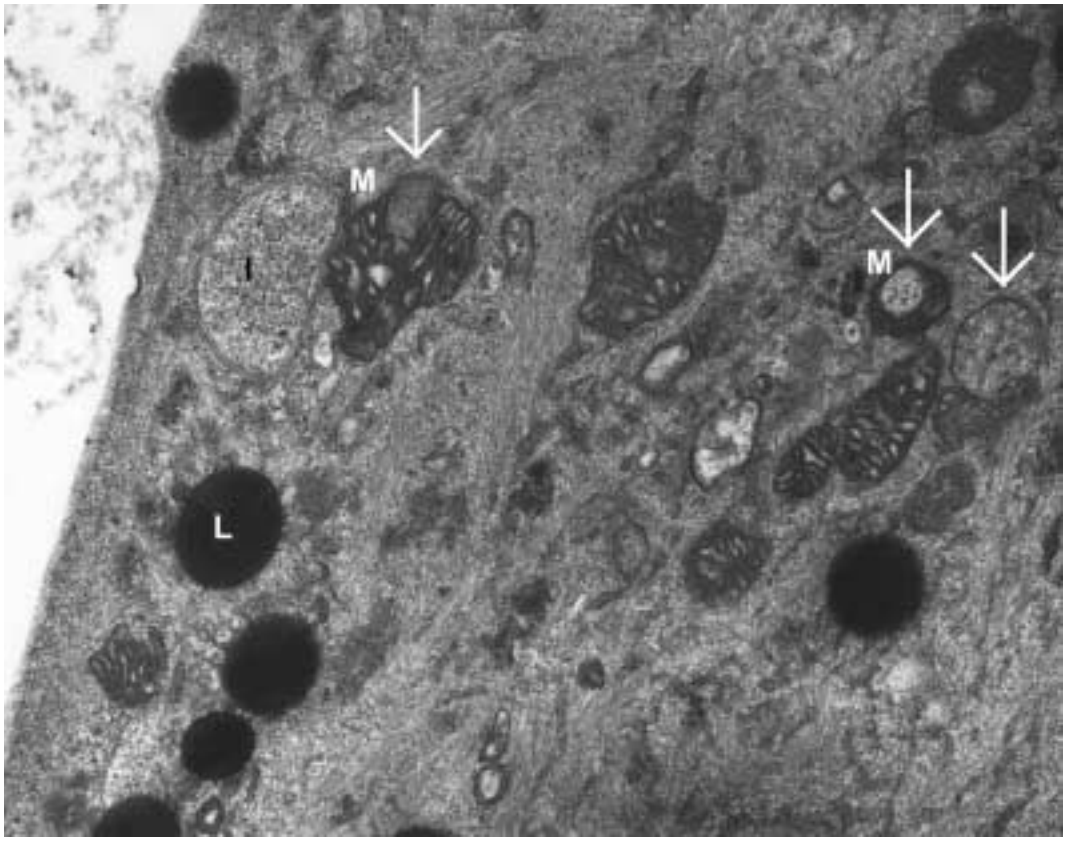


Fig. 2.7 A portion of a Sp1K cell exposed to 20 μM $HgCl_2$ for 24 hours. In this cell, RER (horizontal arrows) retained their normal long shape and attached ribosomes. Mitochondria (M) showed irregular cristae. (X=23,600)

Fig. 2.8 A portion of a Sp1K cell exposed to 20 μM $HgCl_2$ for 24 hours. Golgi apparatus (vertical arrows) remained within normal limits. Swelling was observed in RER (horizontal arrows), but the ribosomes were still attached. Cytoplasmic vacuoles (V) varied in size. (X=49,900)

Fig. 2.7

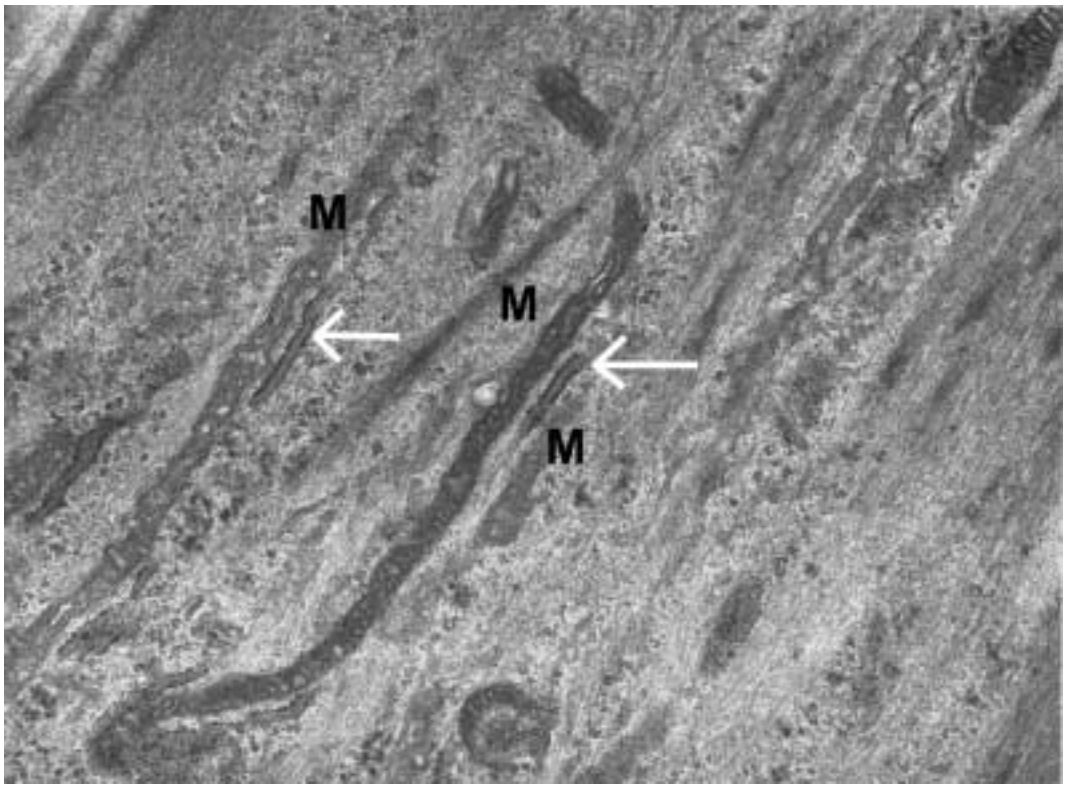


Fig. 2.8

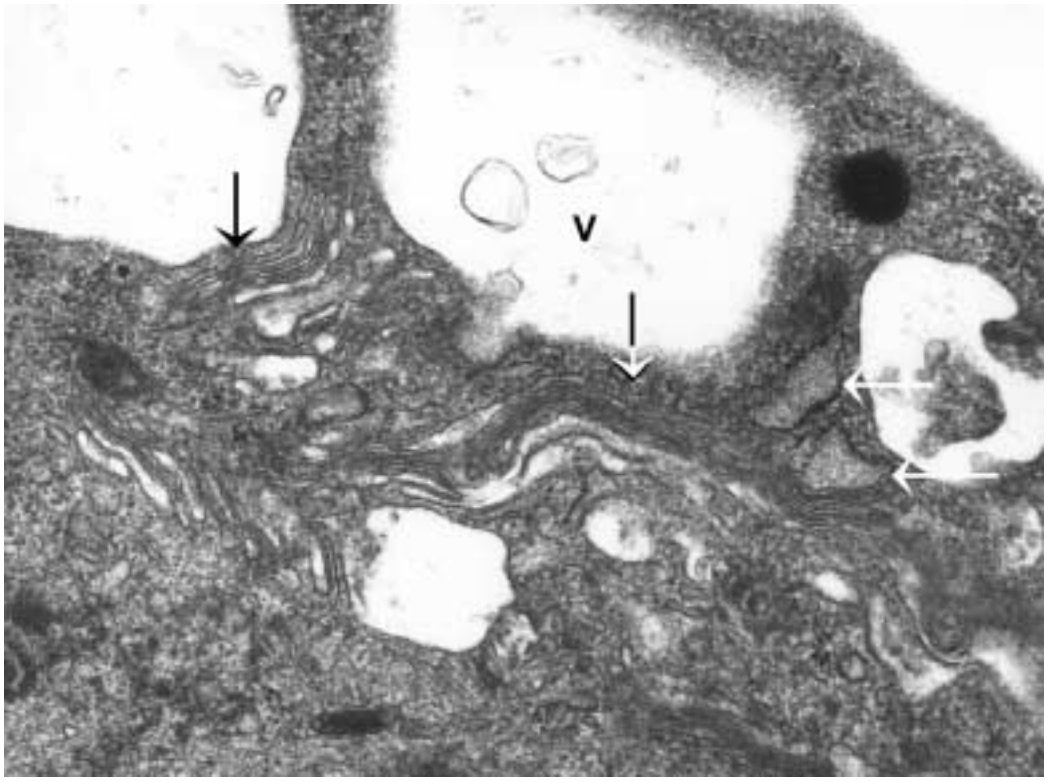


Fig. 2.9 A portion of a Sp1K cell exposed to 20 μM $HgCl_2$ for 24 hours. Lysosome-like inclusions (arrows) contained swollen RER, membranes, and other materials. Mitochondria (M) varied in shape, and the contrast between the matrix and cristae was decreased. (X=51,200)

Fig. 2.9

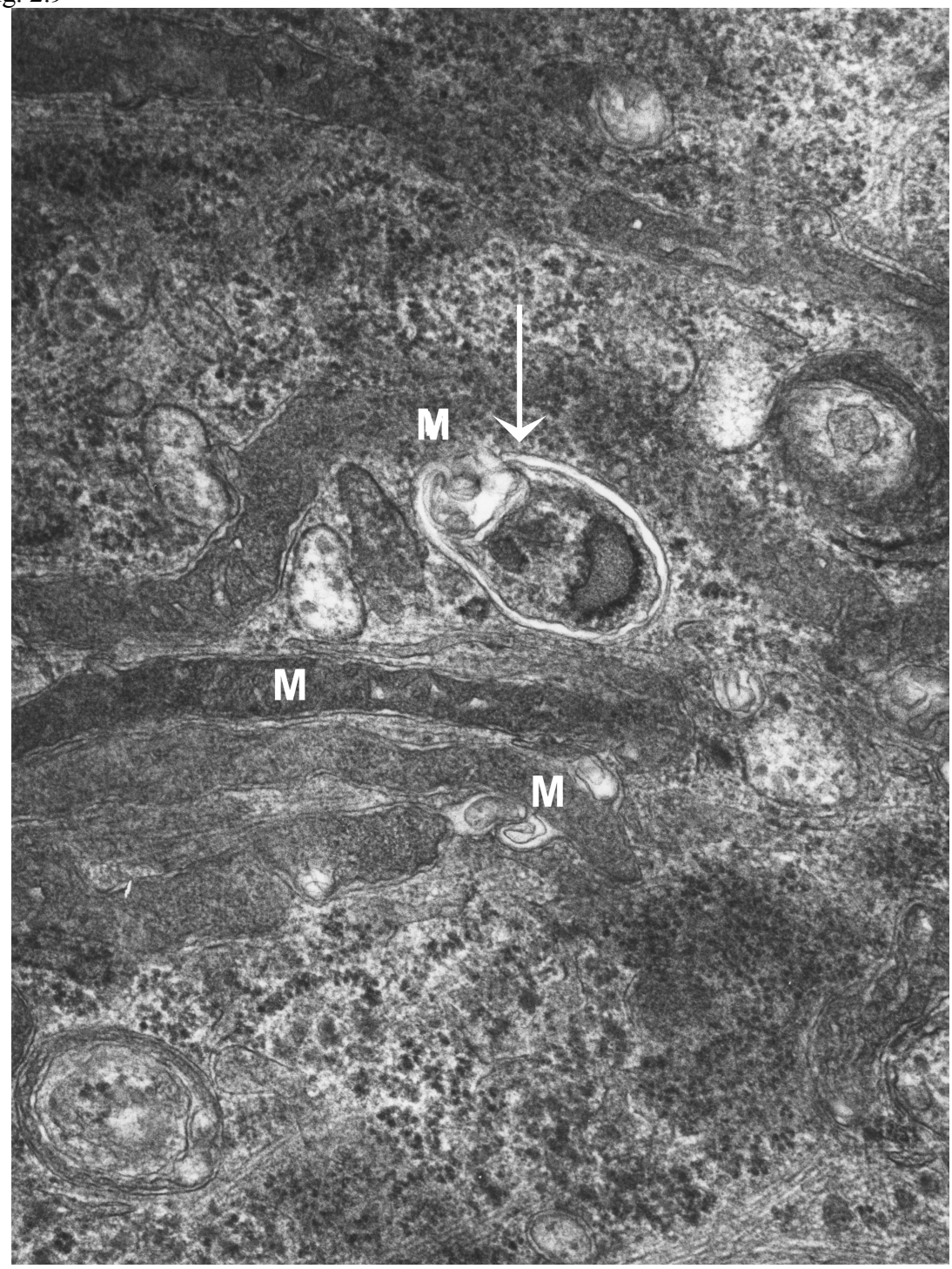


Fig. 2.10 A portion of a Sp1K cell following a 96 hour exposure to 10 μM $HgCl_2$. Cytoplasmic vacuoles (V) and myelin-like figures (MF) were scattered in the cytoplasm, and RER (arrow) were swollen. (X=18,000)

Fig. 2.11 Lipid-like inclusions (L) and cytoplasmic vacuoles (V) in a Sp1K cell following a 96 hour exposure to 10 μM $HgCl_2$. (X=12,900)

Fig. 2.10

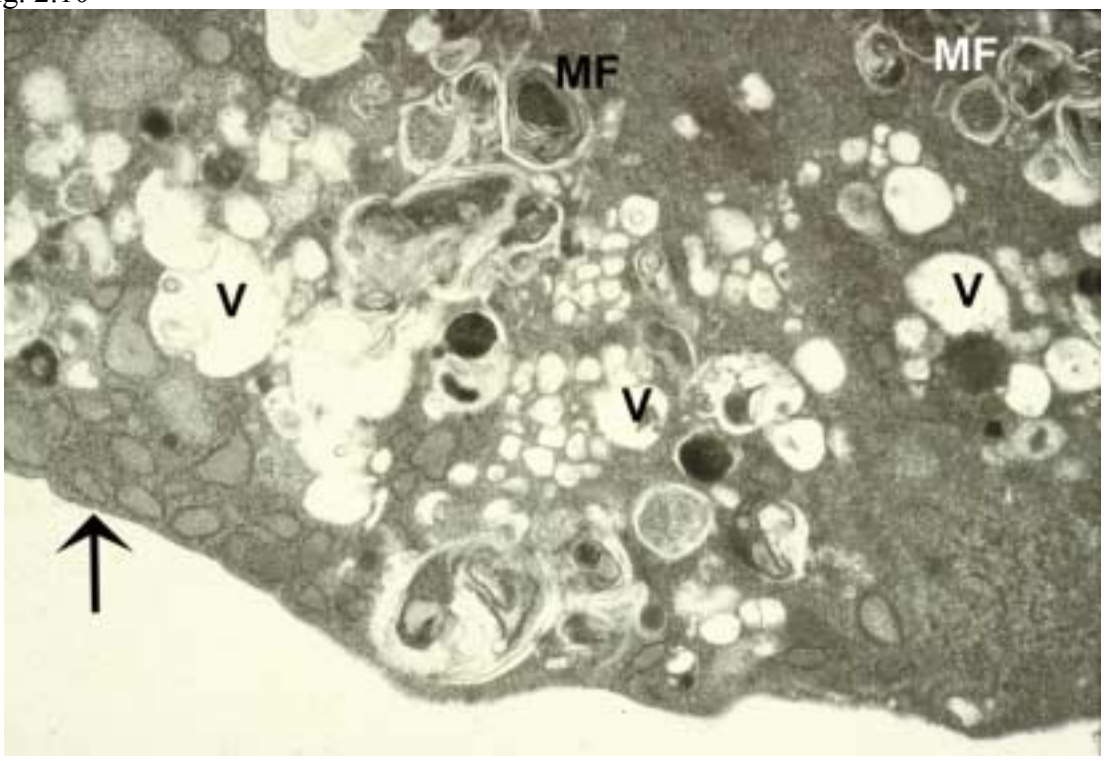


FIG. 2.11

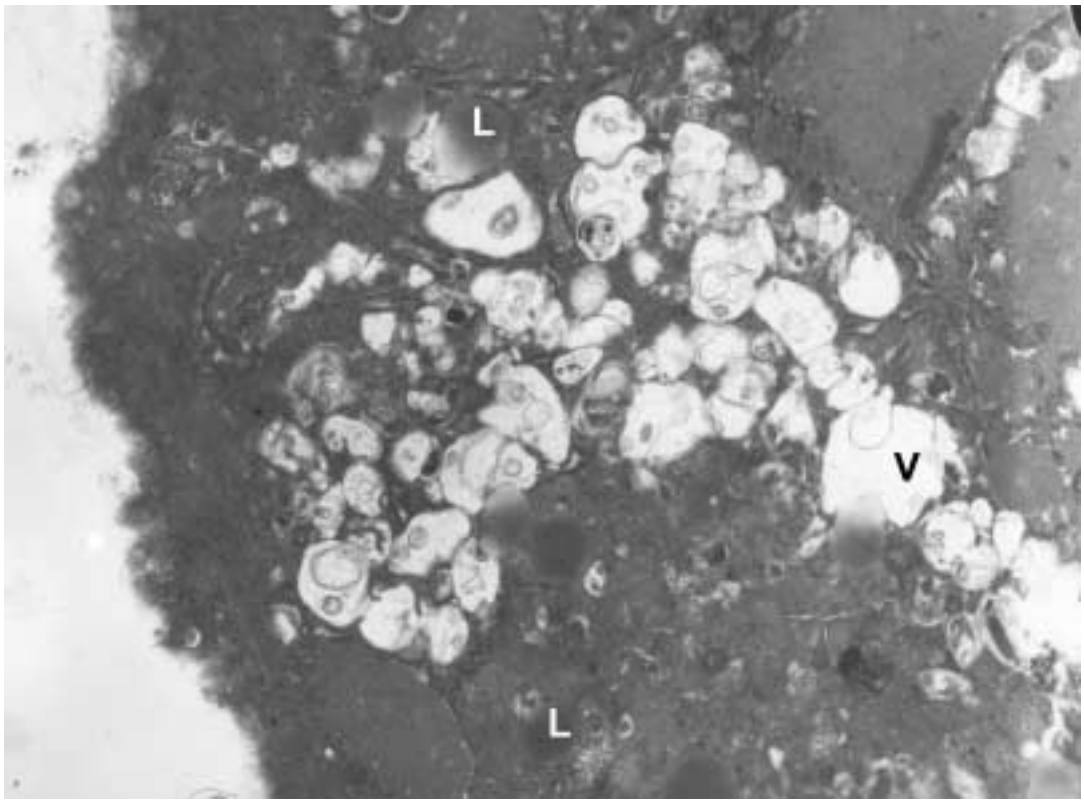


Fig. 2.12 Multiphagic vacuoles (P) and swollen RER (arrow) in a Sp1K cell which was subjected to a 96 hour exposure of 10 μM $HgCl_2$. (X=19,900)

Fig. 2.13 Swollen mitochondria (M) in a Sp1K cell subjected to a 96 hour exposure of 10 μM $HgCl_2$. (X=24,600)

FIG. 2.12

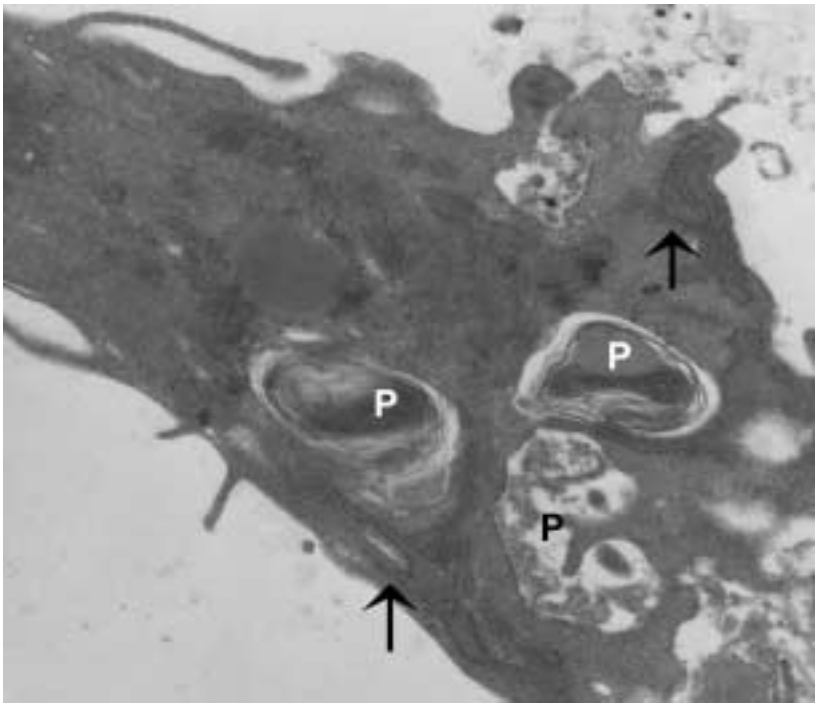


FIG. 2.13

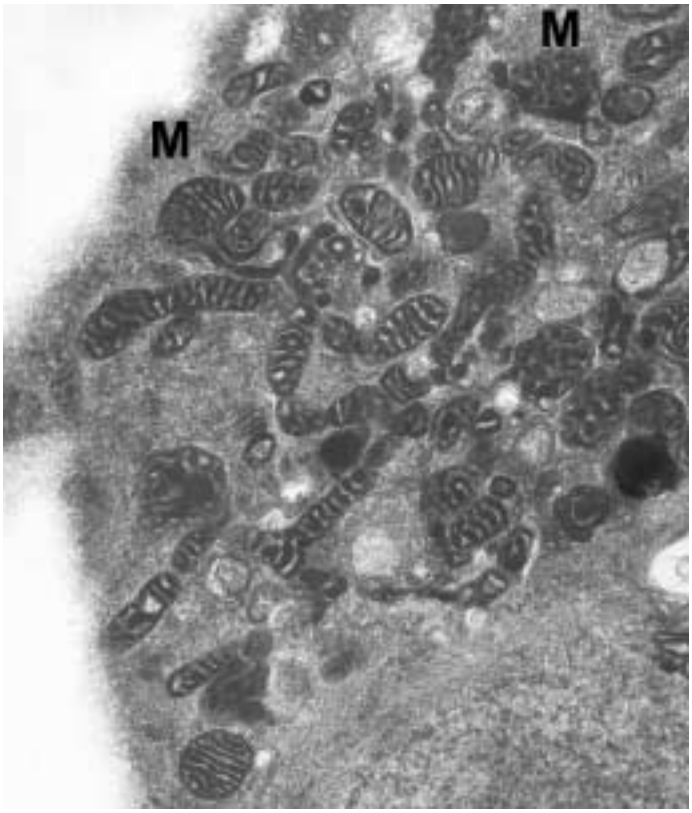


Fig. 2.14 A portion of a Sp1K cell exposed to 10 μM $HgCl_2$ for 96 hours. The nuclei (N) were bounded by the nuclear membrane (NM), which remained morphologically within normal limits. Nucleoli were not seen, but patchy heterochromatin (vertical arrow) was observed. In the cytoplasm, increased numbers of lipid-like inclusions (L) and multiphagic vacuoles (MP) were presented. Non-membrane-bound granulated inclusion bodies (horizontal arrow) were also observed. (X=7,800)

Fig. 2.14

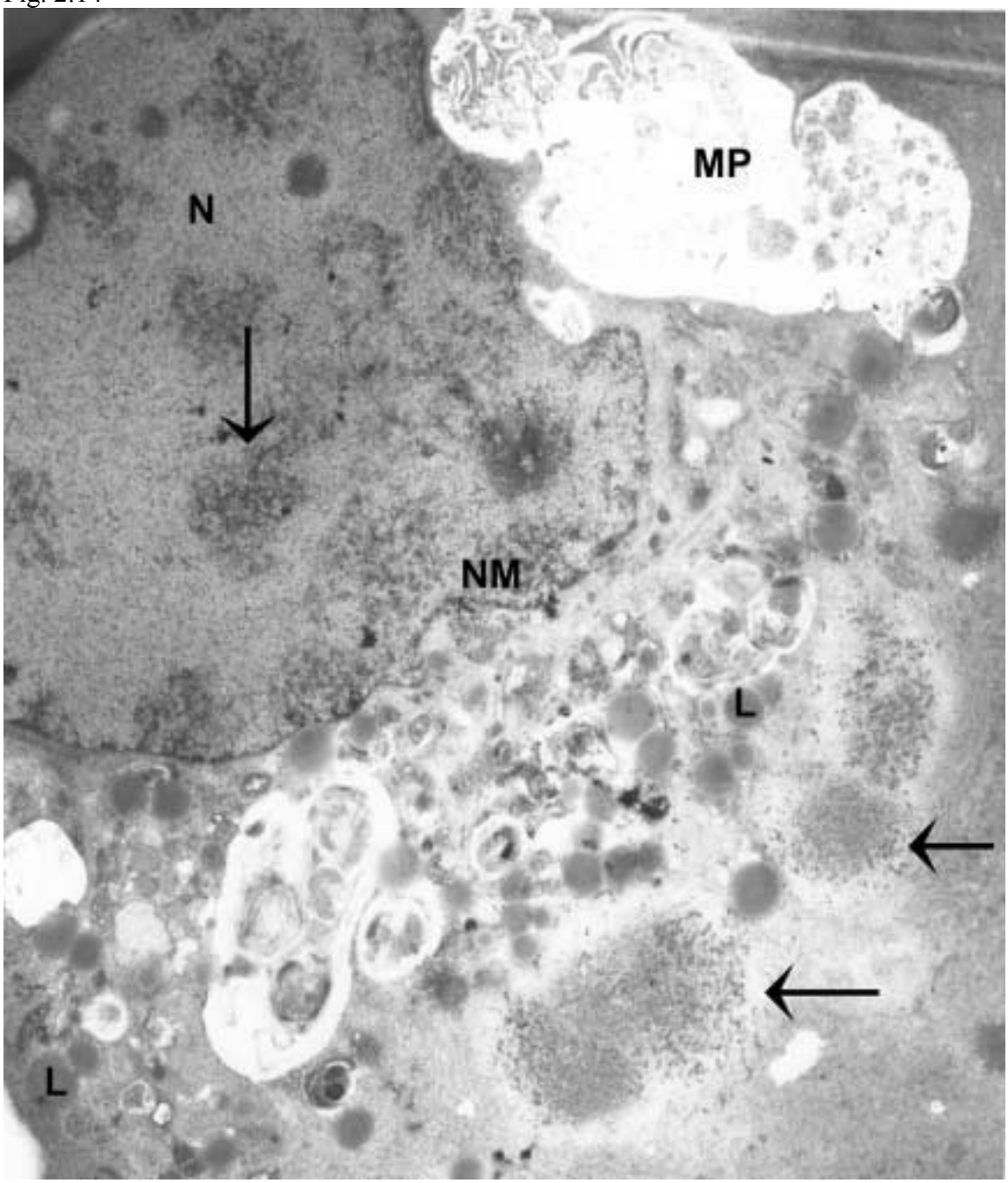


Fig. 2.15 A portion of a Sp1K cell following a 96 hour exposure of 10 μM $HgCl_2$. The nucleus (N) is visible in the upper part of the micrograph, though the nuclear membrane (NM) is not clear. Buds of the nucleus (vertical arrow) and of the plasma membrane (horizontal arrow) were identified by the inclusion within them of nucleoli and lipid-like inclusions (L), respectively. Nucleoli (NC) presented multiple vacuoles, and an expelling vacuole (arrow head in left nucleolus). Micronucleoli (triangle) also showed vacuoles. (X=9,200)

Fig. 2.15

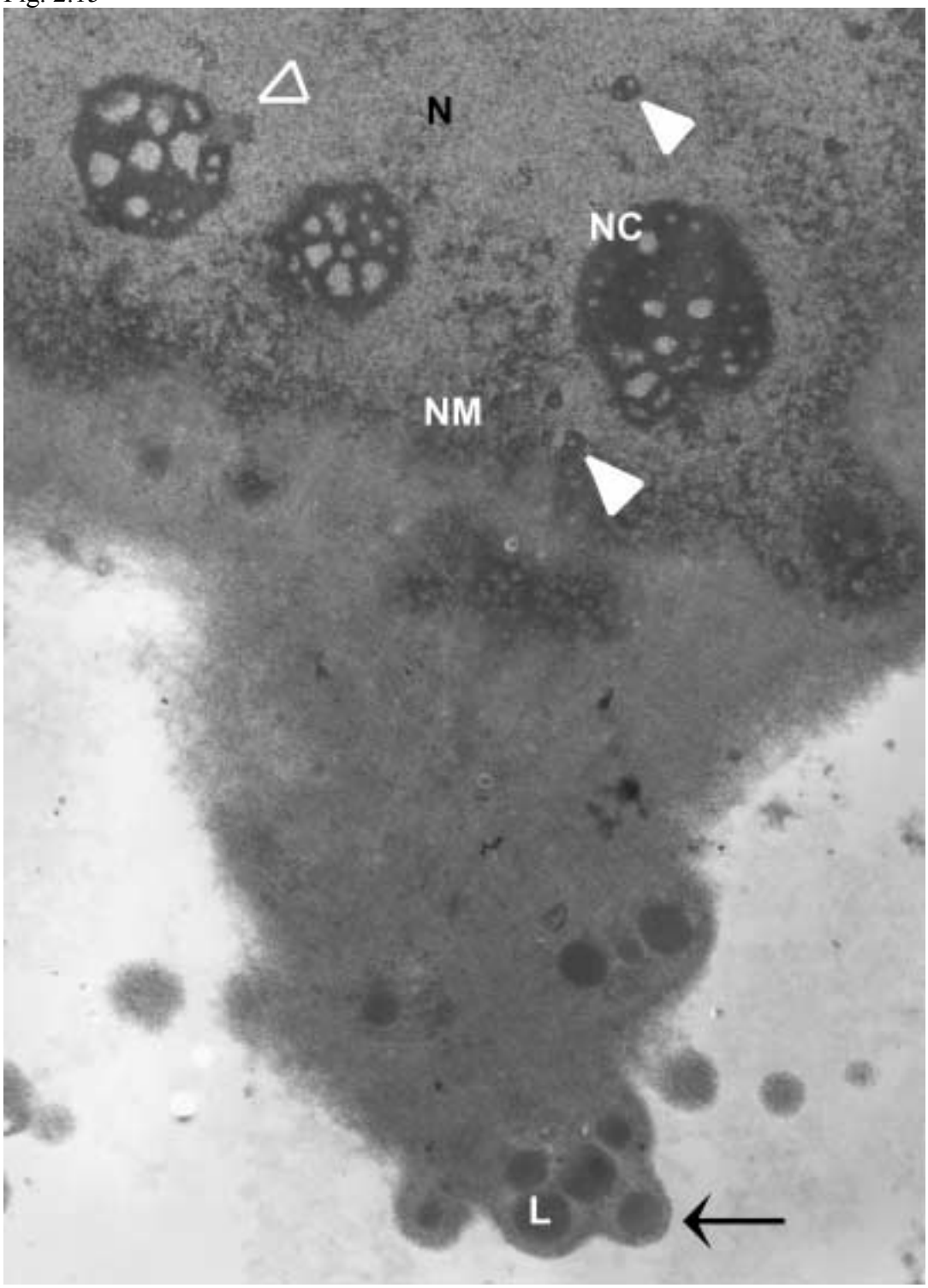
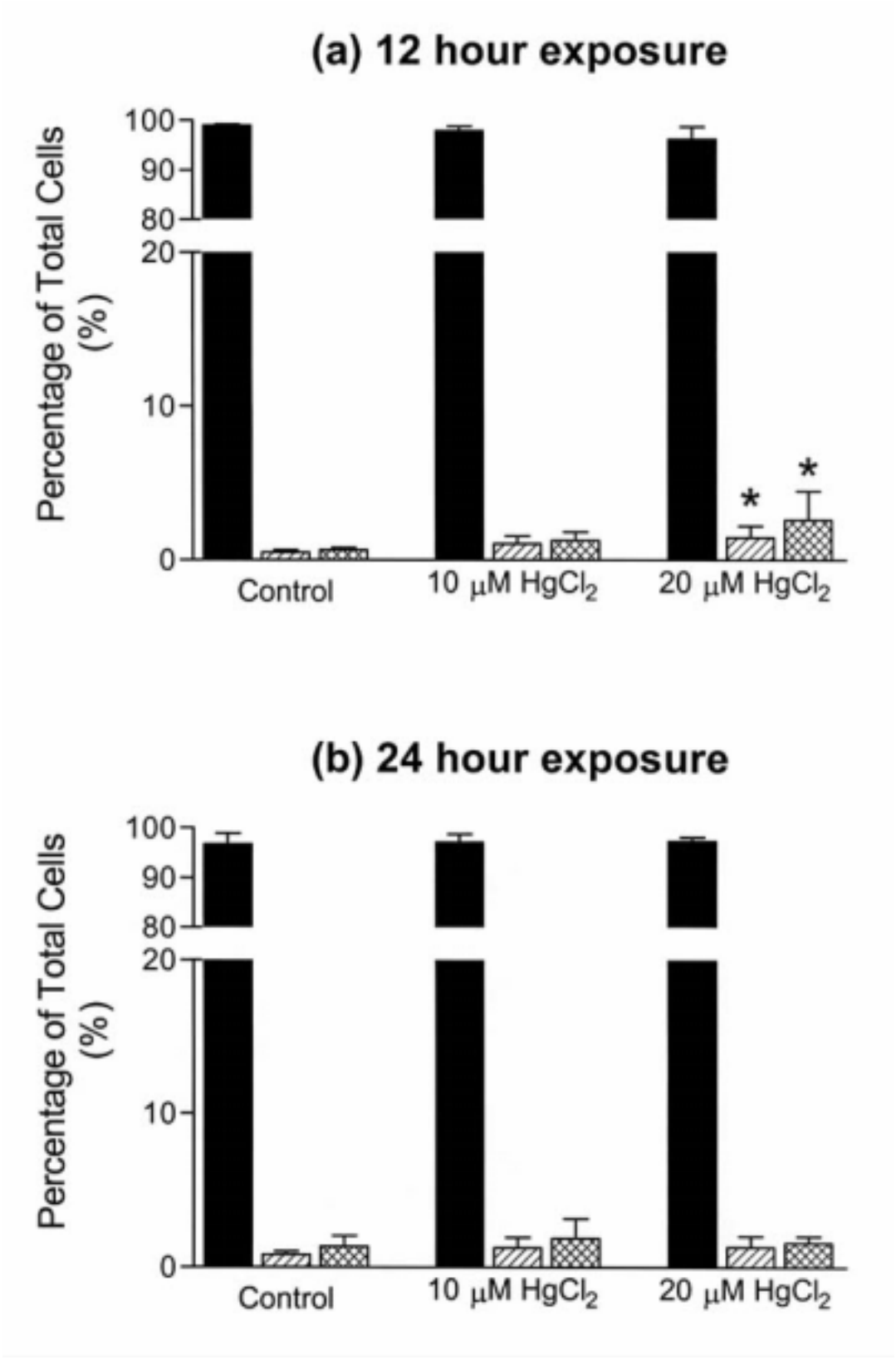
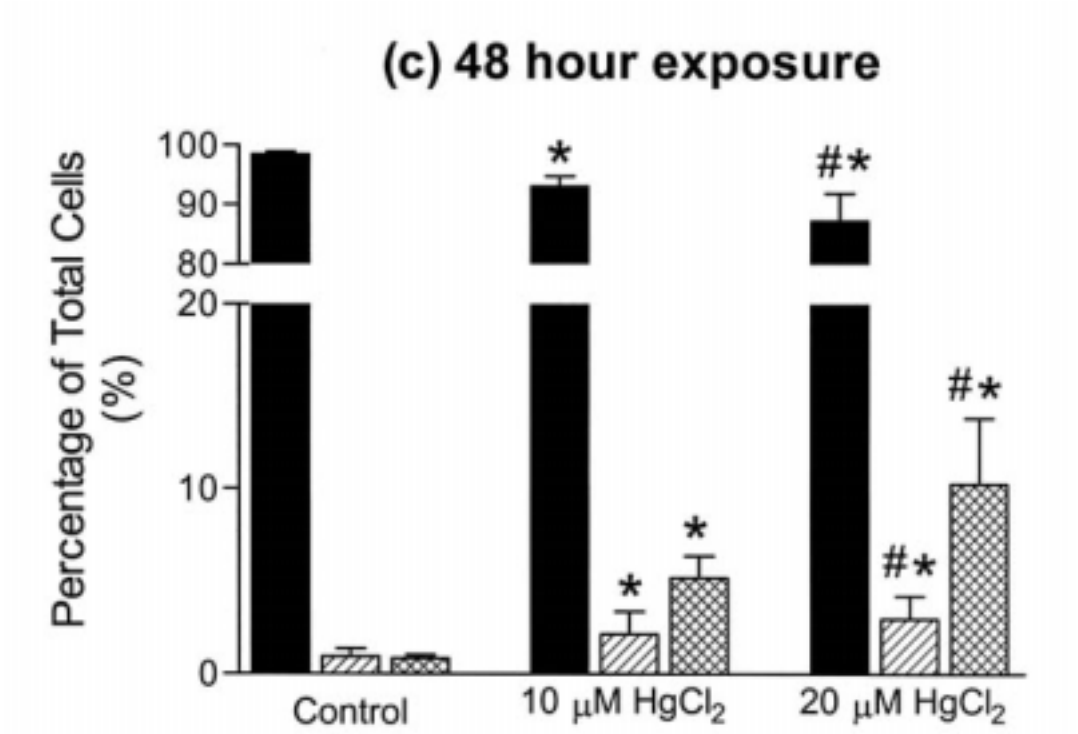
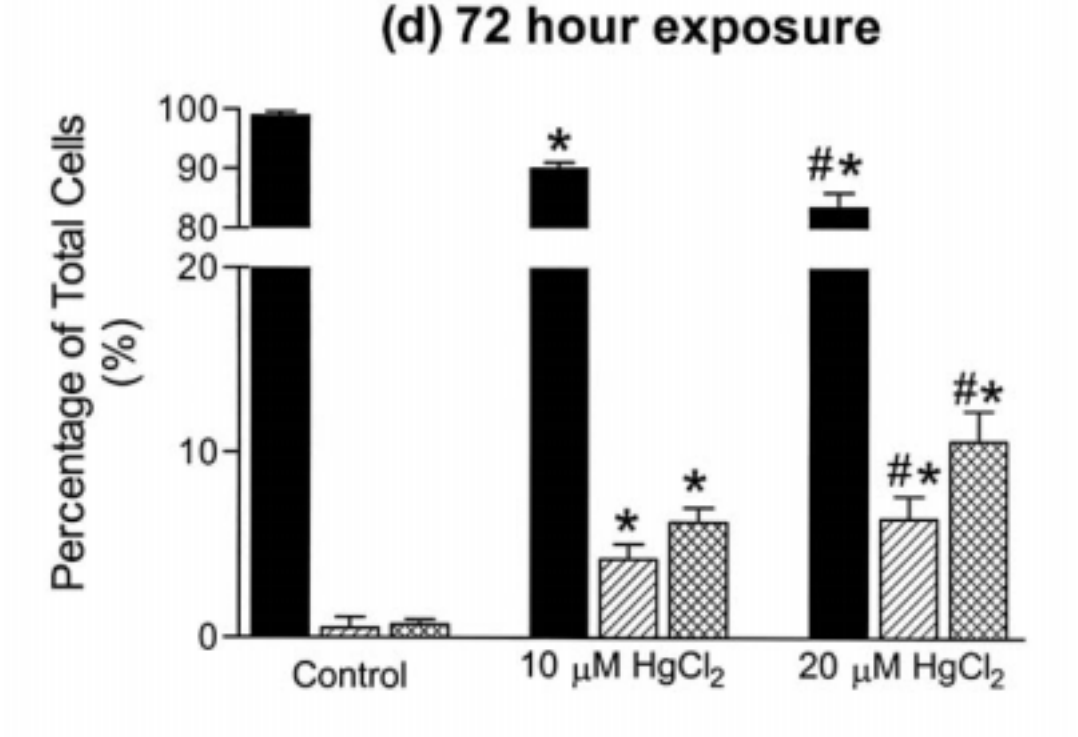


Fig. 2.16 Cell injury classification in Sp1K cells exposed to $HgCl_2$. Cells were treated with 0, 10, or 20 μM $HgCl_2$ for 12 hours (Fig. 16 (a)), 24 hours (Fig. 16 (b)), 48 hours (Fig. 16 (c)) or 72 hours (Fig. 16 (d)). ■ = normal cells. ▨ = apoptotic cells. ▩ = oncotic, necrotic, and dead cells. Y error bars represent standard error. Statistical difference is indicated by ✶ when either concentration is compared to medium-only control, and as # when the 20 μM exposure is compared to 10 μM exposed.


(a) 12 hour exposure
Percentage of Total Cells (%)
100
90
80
20
10
0
*
*
Control
10 μM HgCl2
20 μM HgCl2
(b) 24 hour exposure
Percentage of Total Cells (%)
100
90
80
20
10
0
Control
10 μM HgCl2
20 μM HgCl2


(c) 48 hour exposure
Percentage of Total Cells (%)
100
90
80
20
10
0
*
#*
Control
10 μM HgCl2
20 μM HgCl2


(d) 72 hour exposure
Percentage of Total Cells (%)
100
90
80
20
10
0
*
#*
Control
10 μM HgCl2
20 μM HgCl2

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PART III

MERCURIC CHLORIDE-INDUCED CHANGES IN CELL PROLIFERATION AND CELL CYCLE OF ATLANTIC SPOTTED DOLPHIN RENAL CELLS AND RHESUS MONKEY RENAL CELLS

BACKGROUND

Mercury Nephrotoxicity

Mercury, especially inorganic mercury, is well known as a nephrotoxicant. Inorganic mercury (such as mercuric chloride, $HgCl_2$) is more nephrotoxic than organic mercury (such as methylmercury, MeHg) *in vivo*, but methylmercury is generally more cytotoxic to cultured cells than is inorganic mercury (Aleo et al. 1992; De Flora et al. 1994). This may be partially due to the facts that (a) higher concentrations of inorganic mercury accumulate in kidneys of animals exposed to mercury (Miettinen et al. 1972; Magos et al. 1974), (b) the toxicity of methylmercury to the kidney *in vivo* may result from its demethylation to inorganic mercury instead of a direct toxic effect (Klein et al. 1973; Rodier et al. 1988), and (c) *in vitro* inorganic mercury accumulates less and more slowly in cells due to its low lipophilicity (Aleo et al. 1992).

The following mechanisms of inorganic mercury-induced nephrotoxicity have been suggested: (1) Ischemia: Mercuric chloride ($HgCl_2$) decreases renal blood flow (Wolfert et al. 1987; Girardi and Elias 1995). Therefore, ischemia or hypoxia has been proposed as a mechanism of nephrotoxicity of mercury. (Wolfert et al. 1987; Girardi and Elias 1995; Clarkson 1997) The diminished circulation may be due to vasoconstriction, since mercuric chloride decreases tubular reabsorption of sodium (Girardi et al. 1989) and a decrease of circulating sodium can cause vasoconstriction through the renin-angiotensin system. (2) Cytotoxicity: Direct toxicity to epithelial cells of the proximal tubules is one possible mechanism (Clarkson 1997). More details as this possibility are presented in the following section "Cytotoxicity". (3) Autoimmune Reaction: Another possible mechanism may involve autoimmunity. Chronic exposure to foreign toxicants can induce

an autoimmune response that produces antibodies targeting the basement membrane of the glomerulus. (Clarkson 1997; Moszczynski 1997)

The most sensitive part of kidney to mercury toxicity is the proximal tubule. The mercuric chloride-induced histochemical changes in this region progress from the lower segment of the upper, proximal convoluted tubules in the outer cortex to the terminal portion of the proximal tubules in the inner cortex (Taylor 1965). However, cellular injury can be first observed in the pars recta, and with increasing dosage or exposure time, the pars convoluta is affected (Taylor 1965; Ganote et al. 1974; Siegel and Bulger 1975; Madsen and Maunsbach 1981). In very closely monitored cases, both the pars recta and pars convoluta went through same subtle changes together initially. Later, more severe damage was observed in the pars recta, while the damage in the pars convoluta remained very minimal (McDowell et al. 1976).

The mechanisms of selective, straight proximal tubular injury following $HgCl_2$ exposure include: (1) an increase of tubular concentration of $HgCl_2$ in the urine as it progresses to the pars recta (Siegel and Bulger 1975); (2) renin-angiotensin-induced ischemic injury as a feedback response to decreased tubular reabsorbance of sodium (Haagsma and Pound 1979); (3) secretion or selective accumulation of $HgCl_2$ in the pars recta (McDowell et al. 1976); and (4) a higher sensitivity to $HgCl_2$ -induced toxicity in proximal straight tubular cells than in proximal convoluted cells, as demonstrated in cultured isolated cells (Trifillis et al. 1998).

Mercury Cytotoxicity

The mechanisms of cytotoxicity of mercury remain relatively unknown. Mercury has high affiliation to sulfhydryl groups of proteins, and results in alterations in many renal enzyme activities, such as in glutathione peroxidase, glutathione reductase, superoxidase (Nielsen et al. 1994; Hussain et al. 1999), alkaline phosphatase, 5'-nucleotidase, acid phosphatase, alpha-glycerophosphate dehydrogenase (NAD-independent), malic dehydrogenase, succinic dehydrogenase, latic dehydrogenase, glucose-6-phosphate dehydrogenase and glucose-6-

phosphatase. (Zalme et al. 1976). Mercuric chloride *in vitro* has been reported to alter production of heat shock protein and immunoglobulin, energy charge ratio, adenine nucleotides (Daum et al. 1993; Oshima et al. 1997), lysosome function (Christensen et al. 1991), and it may also induce derangement of the cytoskeleton (Elliget et al. 1991). Mercuric chloride-treated animals also present with increased lipid peroxidation (Fukino et al. 1984) and free radical production. However, the cascade of mercury-induced cellular level changes has not yet been elucidated.

In vivo, mercuric chloride at the cellular level induced blebbing and defoliation of the brush border membrane of proximal tubules as one of the earliest changes (Kempson et al. 1977; Fukino et al. 1984). The cell membrane blebbing was associated with elevation in localized cytosolic Ca^{2+} concentration (Ikeda et al. 1998), and a massive inflow of calcium resulting in cell death following exposure to $HgCl_2$ (Kempson et al. 1977; Fukino et al. 1984). In addition to the morphological changes, activities of plasma membrane-associated enzymes changed early and before the alterations in the activities of enzymes of mitochondria or endoplasmic reticula (Zalme et al. 1976). Data suggested that the interaction between mercury and cell membrane proteins of the brush border could be the initial step of mercury cytotoxicity (Zalme et al. 1976). This is in agreement with Ganote et al. and Zalme et al.'s findings (Ganote et al. 1974; Zalme et al. 1976).

Mercury also induced nuclear and genetic changes, such as a decrease in DNA synthesis, production of DNA fragmentation, impairment in DNA replication (De Flora et al. 1994; Sekowski et al. 1997; Takenaka et al. 1997), DNA single strand breaks, and an inhibition in repair of DNA strand breaks caused by X-rays (Cantoni and Costa 1983). Mutations, sister-chromatid exchanges, chromosomal aberrations (Verschaeve et al. 1984; De Flora et al. 1994), chromosomal banding, (Vershaeve and Susanne 1978), c-mitosis (Vershaeve and Susanne 1978; Leonard et al. 1983) as well as changes in RNA and protein synthesis (Gruenwedel et al. 1981; Kishimoto et al. 1990; Zucker et al. 1990) were also documented following exposure to mercury. Many of these alterations may interfere with cell cycle progression and cell growth.

Cell Proliferation and Cell Cycle Changes Induced by Mercury

Inhibition of growth in exponentially growing cell populations *in vitro* and interference of cell cycle progression are often used as indicators of cytotoxicity (Mummery et al. 1983). These parameters change before gross evidence of mercury poisoning can be observed (Howard and Mottet 1986), and therefore are sensitive indicators of mercury toxicity.

Both inorganic and organic mercury decrease cell growth and/or cell proliferation (Mummery et al. 1983; Howard and Mottet 1986; Kishimoto et al. 1990; Daum et al. 1993; Ou et al. 1997), as well as a reduction cellular and embryonic viability (Aleo et al. 1992; Shenker et al. 1992; Shenker et al. 1993; Oshima et al. 1997). The cell cycle changes depend on the forms of the mercury, the durations of exposure, and possibly the methods of detection (Costa et al. 1982; Howard and Mottet 1986; Vogel et al. 1986; Kishimoto et al. 1990; Zucker et al. 1990).

The cell cycle is usually divided to four phases: mitosis (M) phase, DNA synthesis (S) phase, gap between M and S (G_1) and gap between S and M (G_2). Two major checkpoints in the cell cycle regulate the fate of the cell. The checkpoint in the G_1 phase determines if the cell should stay in G_1 phase, go to G_0 phase, undergo apoptosis, or go to the S phase. In the S phase, cells start to synthesize new DNA, and the DNA content increases until it becomes tetraploid, which is in the G_2 phase. The other checkpoint is in the G_2 phase, which determines if the cell is ready for mitotic division. Once a cell starts mitotic division, the tetraploid content returns to diploid. Theoretically, the ratio of the cells in different phases, G_1: S: G_2 is quiet stable for a given cell under given conditions.

Methylmercury affects the cell cycle in a time- and dose-dependent manner. Acute, low dose exposure prolonged the G_1, and decreased the G_1/M transition rate, while the G_1 effect became undetectable with chronic exposure (Vogel et al. 1986). The G_1-phase effect may result from the inhibition of protein synthesis caused by MeHg synthesis (Gruenwedel et al. 1981; Kishimoto et al. 1990; Zucker et al. 1990). Furthermore, the most frequently observed cell cycle changes were accumulation of cells in G_2/M with or

without S phase changes (Howard and Mottet 1986; Vogel et al. 1986; Zucker et al. 1990; Ponce et al. 1994). This might be explained by the binding of tubulin, and inhibition of microtubule (a major component of the mitotic spindle) formation (Miura et al. 1984; Vogel et al. 1985).

Mercuric chloride, on the other hand, specifically blocked the S phase (Costa et al. 1982; Kishimoto et al. 1990), which may have been due to decreased DNA replication (Costa et al. 1982). Cells treated with $HgCl_2$ had increased probability of being in the G_1 to S phase transition; therefore, the percentage of cells in the G_1 phase decreased with increased percentage of the S phase cells (Mummery et al. 1983). Daum et al (1993) reported that 200 μM $HgCl_2$ reduced mouse lymphocyte entry into G_{1a}, G_{1b} and S/ G_2. Furthermore, a negative correlation was observed between mercury concentrations and percentages of B-cells entering the later phases of the cell cycle. At 100 μM of methylmercury, all phases of the cell cycle were blocked (Zucker et al. 1990).

In the present investigation, cultured Atlantic Spotted Dolphin (which has high physical tolerance to mercury) renal cells were used to investigate the effects of mercuric chloride on cytotoxicity, compared with Rhesus monkey (a closer relative of human beings) renal cells. Cell proliferation was continuously monitored by using alamarBlue ™, and the distribution of cells in the three phases (G_0/G_1, S, G_2/M) of cell cycle was evaluated by nuclear dye with the flow cytometer after exposure to mercuric chloride.

MATERIALS AND METHODS

Cell Culture

The Atlantic Spotted Dolphin Renal Cell line, Sp1K, and Rhesus Monkey Renal Cells, MK2, were both purchased from the American Type Culture Collection (Rockville, MD) and cultured in a humidified incubator with 5% CO_2 at 37^{O}C. Minimal essential medium (MEM) with non-essential amino acids and L-glutamine (BibcoBRL, Grand Island, NY) consisting of 10% fetal bovine serum (FBS) (GibcoBRL, NY) for Sp1K cells or 5% of FBS for MK2 cells was used as growth medium. Both cell types were grown as adherent cultures in 50 cm^2 petri dishes (Falcon, Franklinlake, NJ), except in the case of specific tests (see below). When performing a new experiment, the cells were detached from the petri dish using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in phosphate buffered saline (PBS).

Cell Proliferation Rate

The cultures were seeded with Sp1K cells or MK2 cells in the growth medium as described above in 96-well flat bottom plates (Nunc, Denmark). Treatments were started 24 or 36 hours after seeding. The growth medium was pipetted off and replaced by a fresh test medium in which the cells were grown for another 6 days in the presence of various concentrations of $HgCl_2$.

$HgCl_2$ (Sigma) was dissolved in the growth medium at 1000 µM as a stock solution, which was freshly prepared. The stock solution was further diluted to test concentrations. Mercury toxicity was affected by the serum concentraion in the medium, so the test medium of Sp1K and MK2 cells in this experiment was both including 10% FBS. Sp1K cells were exposed to 0, 10, 25, 50, 100 or 500 µM $HgCl_2$ in 10% FBS medium, and MK2 cells were treated with 0, 10, 15, 20, 25, 30, 40, 50, and 100 µM $HgCl_2$ in 10% FBS medium.

AlamarBlue™ (Accumed International Inc., Westlake, OH), a fluorescent/colorimetric indicator dye, was added to the medium at a final concentration of 10% (v/v). The absorbance of the plates were read immediately after adding the dye,

and later at 12 or 24 hour intervals. When cells grow, the metabolic activities transform alamarBlue™ from the nonfluorescent, blue (maximum absorbency wavelength at 600 nm) oxidized form to the fluorescent, red (maximum absorbency wavelength at 570 nm) reduced form. Specific absorbance (specific OD) was attained by subtracting the absorbance at 600 nm from the 570 nm absorbance, determined by a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA).

Cell Cycle

The distribution of cells in various phases of the cell cycle were determined by evaluating relative DNA composition. Cells were classified into four groups: (1) cells in G_0 and G_1 phases, (2) cells in S phase, (3) cells in G_2 and M phases, and (4) dead cells and cell debris. The proportion of live cells in each cell cycle phase (G_0/G_1, S, G_2/M) was obtained by using the following equation: percentage of cells in a given phase / (percentage of cells in G_0/G_1, S, and G2/M phases).

Sp1K cells were seeded in 75 cm^2 flaskes, and MK2 cells were cultured in 50cm^2 petri dishes. Sp1K cells were treated with $HgCl_2$ at 0, 5, 10, 15, and 20 μM for 12, 24, 48, and 72 hours. It was a complete blocking design with 4 blocks. MK2 cells were exposed to $HgCl_2$ at 0, 5, and 10 μM for 12, 24, 48, and 72 hours. After exposure to $HgCl_2$, $5x10^4$ cells per sample were collected and centrifuged to form a pellet. The cells were resuspened in 100 ul each of a propidium iodine (PI) buffer (PBS containing 50ug/ml PI, 1% FBS, and 0.1 % sodium azide) and Vindelov's solution (0.01M Tris, 10mM NaCl, 700U RNAse A, 50ug/ml PI, and 0.1% of Igepal-40). Samples were incubated at 4^OC overnight to insure that cellular DNA was saturated with fluochrome, then analyzed on an Epics-XL flow cytometer (Beckman-Coulter, Miami, FL) at excitation 488nm and emission 625nm.

Statistical Analysis

Each experiment had between 4 and 6 data points at each time and $HgCl_2$ - concentration combination. An analysis of variance (ANOVA) was performed. The

statistical significance level employed was 0.05.

The data for cell proliferation (as measured by the absorbance of alamarBlue^{TM}) of Sp1K cells exposed to $HgCl_2$ were analyzed by one-way ANOVA. At each time point, all the data were compared by using the Tukey comparison. The averages of each $HgCl_2$ concentration over the whole experimented period were also compared by the Tukey comparison.

The analysis of cell proliferation data of MK2 cells in 10% FBS medium following exposure to $HgCl_2$ was first analyzed as single measurements at each time point. The alpha was lowered to 0.003125 by the Bonferroni correction to bring the alpha level overall back to 0.05. The data were compared to the medium only (control) at each time point. Then the data were analyzed as repeated measures. This analysis was only applied to the data obtained after more than 24 hours after the administration of $HgCl_2$ and alamarBlue^{TM}. Analyses of repeated measures using GLM procedure in SAS system, including polynomial tests, and analysis of individual treatment regression were performed. The slope of each regression line was compared by the Tukey comparison.

The data for cell distribution in the phases of the cell cycle were analyzed by two-way ANOVA. The analysis of the data of cell cycle distribution of Sp1K cells following exposure to $HgCl_2$ was conducted using the Tukey comparison. The alpha of the cell cycle distribution of MK2 cells following exposure to $HgCl_2$ was lowered to 0.00294 by the Bonferroni correction to bring the experiment-wide alpha level back to 0.05.

RESULTS

Cellular Proliferation of Sp1K Cells Following Exposure to $HgCl_2$

Effects of $HgCl_2$ concentration, time, and $HgCl_2$-time interaction were revealed in the cell proliferation of Sp1K cells after exposure to $HgCl_2$ (Fig. 3.1 (a) and (b)). High concentrations of $HgCl_2$ inhibited the cell proliferation, and the decreases were more apparent when cells were exposed longer to $HgCl_2$. Furthermore, the decrease in cell proliferation caused by $HgCl_2$ was greater by the end of experiment, indicating an interaction between time and $HgCl_2$ concentration.

Starting at 24 hours after exposure to $HgCl_2$ and alamarBlue™, proliferation of cells at all tested concentrations of $HgCl_2$ (10, 25, 50, 100, and 500 μM) were significantly decreased when compared to the cells exposed to the medium only (control). The readings from cells exposed to 10 and 25 μM $HgCl_2$ were the same at 24 hours, but readings from 10μMwere higher then 25μMafter 48 hours. Cells exposed to 100 μM and 500 μM $HgCl_2$ shared resutls statistically idental to each other at all time points. The data suggested that 100 and 500 μM $HgCl_2$ killed the Sp1K cells within 24 hours.

Cell proliferation data over the whole experimented time (120 hours) were grouped into four sets using the Tukey comparison. Cells exposed to the medium only, 10 μM $HgCl_2$, and 25 μM $HgCl_2$ had the highest, the second highest, and the third highest proliferation, respectively. The cells that received higher concentrations of $HgCl_2$ (50, 100, and 500 μM) had low proliferation rates, and further, were similar to each other at all measurements.

Cellular Proliferation of MK2 Cells Following Exposure to $HgCl_2$

At each time point, the specific absorbance of each treatment was compared to the medium only (control) using one-way ANOVA. All treatments were the same at 0, 1, and 8 hours after the administration of $HgCl_2$ and alamarBlue™, except the reading from the 40 μM $HgCl_2$ group, which was significantly lower than the control at 0 hour (Fig. 3.2 (a) and (b)). Since the later reading showed that they were the same, it was most likely due

to the small difference of the amount of dye which was detected at this time point. At 24 hours the reading of 100 μM $HgCl_2$ was significantly lower than the control. At 48 hours the readings of both 50 and 100 μM $HgCl_2$ were lower than the control. At 72 hours and above, all the treatments (15, 20, 25, 30, 40, 50, and 100 μM $HgCl_2$) were lower than control. The data suggested that higher concentrations of $HgCl_2$ decreased cell proliferation more than lower concentrations.

The data analyzed as repeated measurements showed $HgCl_2$ concentration effects, time effects, and interactions between $HgCl_2$ concentration and time. Higher concentrations of $HgCl_2$, longer reading times, and the combination of high $HgCl_2$ concentration and long reading time all caused greater inhibition of cell proliferation. The polynomial tests indicated that the data fit a linear model. The Tukey comparison of slopes showed that the slopes of the control and 10 μM $HgCl_2$ were different from each other and the rest of the treatment groups, and the slopes of the rest of the treatment groups (15, 20, 25, 30, 40, 50, and 100 μM $HgCl_2$) were the same. The cells that received medium only had the highest cell proliferation, and the cells exposed to 10 μM $HgCl_2$ had the second highest cell proliferation. The cells that received more than 15 μM $HgCl_2$ died.

Distribution of Sp1K Cells in the Cell Cycle Following Exposure to $HgCl_2$

No changes were observed in the cell distribution within the cell cycle for Sp1K cells receiving 0, 5, 10, 15, or 20 μM $HgCl_2$ for 12, 24, or 48 hours (Fig. 3.3).

Time-dependent changes in the cell cycle were only observed between the 72 hour treatment and the control groups. At every tested $HgCl_2$ concentration (including 0 μM $HgCl_2$ -control), higher percentages of cells in the G_0/G_1 phases, and a lower proportion of cells in the S and G_2/M phases were observed when compared to cells exposed to matched concentrations of $HgCl_2$ for 12 hours (except the percentage of cells in the S phase of cells exposed to medium only for 72 hours).

Within the 72 hour treatment group, higher concentrations of $HgCl_2$ induced higher portions of cells in the G_0/G_1 phases, and lower percentages of cells in the S and

G_2/M phases. The increase in the G_0/G_1 phases and the decrease in the G_2/M phases were observed in cells which received higher than 10 μM $HgCl_2$ (included), as compared to cells treated with medium only (control) for 72 hours. The decrease of cells in the S phase became significant for cells treated with 15 or 20 μM $HgCl_2$.

Distribution of MK2 cells in the Cell Cycle Following Exposure to $HgCl_2$

Exposure time-dependent alterations in cell cycle were observed following exposure to all concentrations of $HgCl_2$, including 0 μM $HgCl_2$ (control) (Fig. 3.4). Exposure to 24, 48, and 72 hours of tested concentrations of $HgCl_2$ significantly increased the number in G_0/G_1 phase (except the 72 hour exposure to medium only) and decreased the proportion of S phase cells, as compared to the same phase of cells at 12 hour exposure of matched $HgCl_2$ concentration. Changes in the percentage of G_2/M phase cells were seen at 24 hours of exposure to 0 and 5 μM $HgCl_2$ only.

A mercury concentration effect was only observed in the 24 hour exposure group. Cells exposed to 5 μM $HgCl_2$ had a higher proportion of cells in the G_0/G_1 phase, and fewer cells in the G_2/M phase. At 20 μM $HgCl_2$, the number of cells in the G_2/M phase was significantly higher when compared to G_2/M phase cells treated with medium alone (control).

DISCUSSION

The present study evaluated the effects of $HgCl_2$ on cell proliferation and cell cycle status of Sp1K cells and MK2 cells. The cell proliferation data from both types of cells under the same culture conditions allowed us to compare their tolerance to $HgCl_2$. Changes in the cell cycle status were observed at non-cytotoxic concentrations for Sp1K cells, and they could be directly related to the influence of $HgCl_2$. Data for cell cycle status of MK2 cells following exposure to $HgCl_2$ were also presented.

Inhibition of cell proliferation induced by mercury has been previously reported in various cell types (Mummery et al. 1983; Howard and Mottet 1986; Kishimoto et al. 1990; Daum et al. 1993; Ou et al. 1997), but direct comparison among these experiments is complicated by the tissue/cell types, test methods (cell counting, protein amount, etc.), and culture conditions. For example, a high temperature enhanced the toxicity of mercury (Kishimoto et al. 1990), and a high concentration of serum in the culture medium decreased the effects of mercury.

Atlantic spotted dolphin renal cells (Sp1K cells) and Rhesus monkey renal cells (MK2 cells), which were grown in the same medium, were used to compare the inhibition of cell proliferation caused by $HgCl_2$. Both cell cultures showed inhibition in cell proliferation when exposed to 10 μM $HgCl_2$ and higher concentrations of this toxicant. However, Sp1K cells were able to keep growing at 25 μM $HgCl_2$, while MK2 cells could not survive after exposure to 15 or more μM $HgCl_2$. The data suggested that Sp1K cells had a higher tolerance to $HgCl_2$ toxicity , at least partially, in a cell specific manner, which was independent from the selenium protection or other possible mechanisms.

$HgCl_2$-induced changes in cell distribution in particular phases of the cell cycle were determined by cell flow cytometry. Portions of Sp1K cells in G_0/G_1, S, and G_2/M phases of the cell cycle were compared at non-cytotoxic exposures, identified by no significant increase in the dead cell percentage under the highest tested concentration of $HgCl_2$ (20 μM) for the longest exposure time (72 hours) (see Part 1).

Sp1K cells showed a concentration-dependent increase in the percentage of cells in G_0/G_1 phase of the cell cycle, and a decrease in S and G_2/M phase cells only at the 72

hours exposure, when the medium-only-control also showed a higher portion of G_0/G_1 phase cells and lower portion of G_2/M phase cells, as compared to the 12 hour exposure control. The changes in medium-only-control indicated that culture in the same medium for 3 days was a suboptimal condition, and cells may tend to stay in the G_0/G_1 phase. Cells at non-optimal conditions may be more susceptible to $HgCl_2$ toxicity, which is in agreement with the finding of Kishimoto et al. (1990).

MK2 cells, on the other hand, showed similar $HgCl_2$-induced cell cycle changes at 24 hour exposure, but not at 12, 48, or 72 hour exposures. Although cell cycle status differed for medium-only-control at different exposure times, there was no clear trend for the changes. Furthermore, exposure time effects and day-to-day experimental variation, (block effect) were not distinguishable due to treating and harvesting cells for the same exposure time on the same day. The $HgCl_2$-induced increase of G_0/G_1 phase cells and decrease in G_2/M phase cells at 24 hour exposure could be contributed to the block effect or a subgroup being more sensitive to $HgCl_2$ toxicity. It is possible that those sensitive cells proliferated less effectively, and the longer exposure groups, which included less sensitive cells, showed more resistance to $HgCl_2$-induced cell cycle changes.

However, the cell cycle changes caused by $HgCl_2$ in Sp1K and MK2 cells responded differently from the published literature. $HgCl_2$ was known to decrease G_1 or G_0/ G_1 phase cells by facilitating the G_1 to S phase transition, and reducing cell entry into G_1 phase (Mummery et al. 1983; Daum et al. 1993). In contrast, an increase of percentages of G_0/G_1 phase cells, which was observed in Sp1K and MK2 cells under the influence of $HgCl_2$, was seen in cells with an acute low dose exposure to MeHg (Vogel et al. 1986). The different cell cycle changes with the same chemical may have contributed to the differences in exposure time, concentration (Vogel et al. 1986; Carlson 2000), and cell types (Ou et al. 1997).

The increased percentage of G_0/G_1 phase cells, with or without decreased proportions of S and G_2/M phase cells, was also observed in (a) cultured human neuroblastoma cells following exposure to phenylmethylsulfonyl fluoride (PMSF), which inhibited proteases important to cell cycle control (Carlson 2000), and (b) cultured

NIH3T3 cells with mutated adenomatous polyposis coli (APC) gene, which over-expressed hDLG, the human homolog of the *Drosophila lethal (1) discs large-1 (dlg)* tumor suppressor protein (Ishidate et al. 1999).

The $HgCl_2$ effects on increasing G_0/G_1 phase and decreasing S and G_2/M phase Sp1K and MK2 cell populations most likely occurred directly in the G_0/G_1 phase, since an inhibition of cells during the first G_2/M phase would deplete the population of cells in the new G_0/G_1 and S phases (Ponce et al. 1994). Increased proportions of G_0/G_1 phase cells could be due to accumulation of cells in the first gap phase (G_1) or a resting state (G_0). The blocking in the G_1 phase could result from failing to pass G_1 checkpoints in the cell cycle, and may be associated with p53, bcl2, and other cell cycle regulatory genes. The entry to G_0 could be induced by a lack of growth factors or inhibition of protein synthesis. Inhibition of protein synthesis was also one of the courses of increased G_0/G_1 phase cells. Since these tests were not conducted in the presented study, further investigation is needed for elucidating the mechanism of observed changes of cell cycle states.

The relatively late and small changes in the cell cycle did not fully explain the previously observed cell proliferation inhibition. Conversely, the increase in apoptosis induced by $HgCl_2$ in Sp1K cells corresponded to the data of cell proliferation for both time and concentration findings. The data suggested that the decrease in cell proliferation was mainly through apoptosis rather than cell cycle changes.

In short, the data suggested that (1) $HgCl_2$ decreased cell proliferation in Sp1K and MK2 cells through apoptosis rather than cell cycle changes, and (2) $HgCl_2$ increased the percentage G_2 of Sp1K and MK2 cells in the G_0/G_1phase and decreased the proportions in S and /M phase cells. Furthermore, the cell cycle alterations were only observed in cells in suboptimal conditions, as revealed by cell cycle changes in the matched control. The selenium-independent high resistance to $HgCl_2$ in Sp1K cells, as compared to MK2 cells, may explain, partially, the high *in vivo* physical tolerance to the toxicity of mercury in cetaceans.

Fig. 3.1 Cell proliferation, as measured by absorbance of alamarBlue^{TM}, of Sp1K cells exposed to $HgCl_2$. Values are the average of 18 data points within 3 complete blocks. Y error bars in Fig. 3.1 (b) represent standard errors, and the Y error bars that were smaller than the symbols are not visible.

Fig. 3.1 (a)

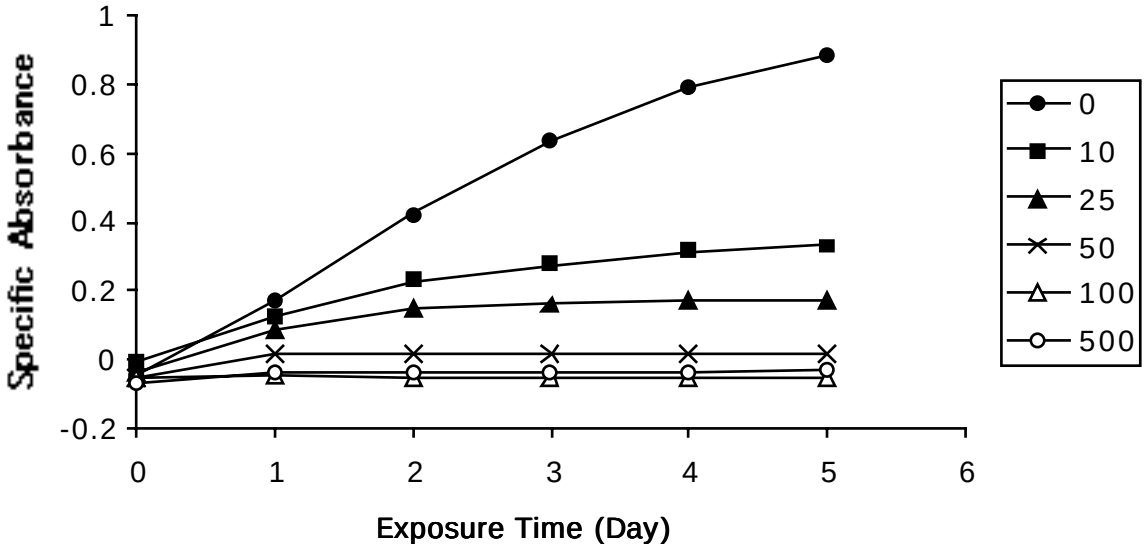

Specific Absorbance
1
0.8
0.6
0.4
0.2
0
-0.2
0
1
2
3
4
5
6
Exposure Time (Day)
0
10
25
50
100
500

Fig. 3.1 (b)

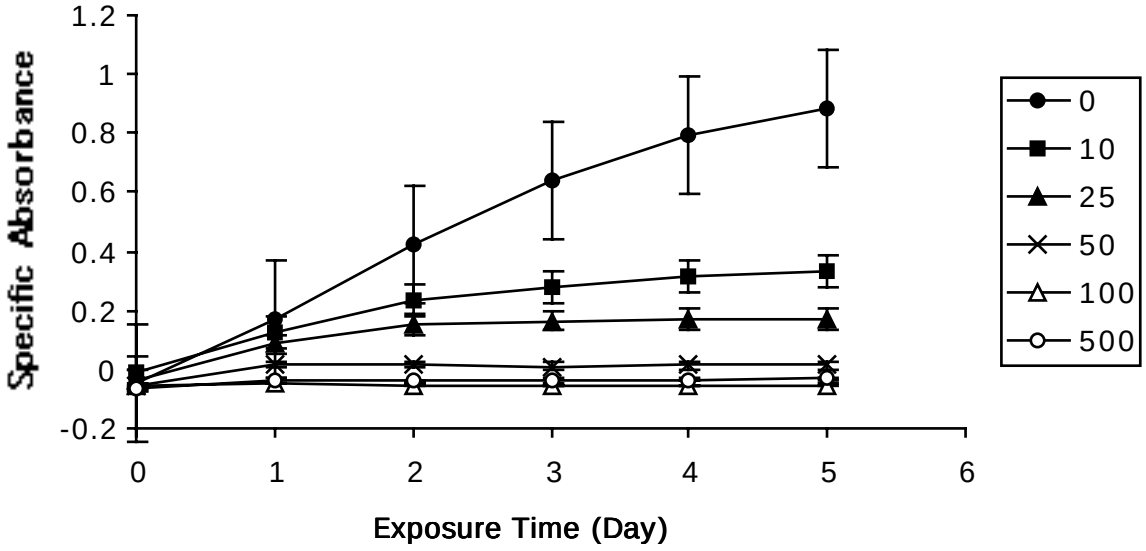

Specific Absorbance
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
1
2
3
4
5
6
Exposure Time (Day)
0
10
25
50
100
500

Fig. 3.2 Cell proliferation, as measured by absorbance of alamarBlue^{TM}, of MK2 cells exposed to $HgCl_2$. Values are the average of 6 data points. Y error bars in Fig. 2.2 (b) represent standard errors, and the Y error bars that were smaller than the symbols are not visible.

Fig. 3.2 (a)

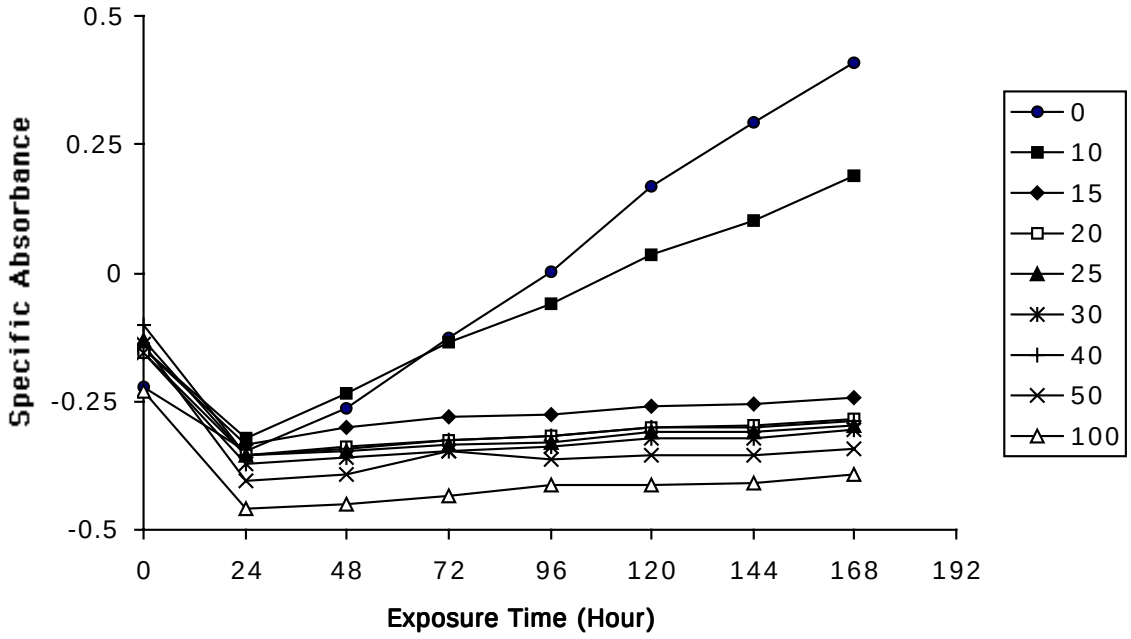

0.5
0.25
0
-0.25
-0.5
Specific Absorbance
0
24
48
72
96
120
144
168
192
Exposure Time (Hour)
0
10
15
20
25
30
40
50
100

Fig. 3.2 (b)

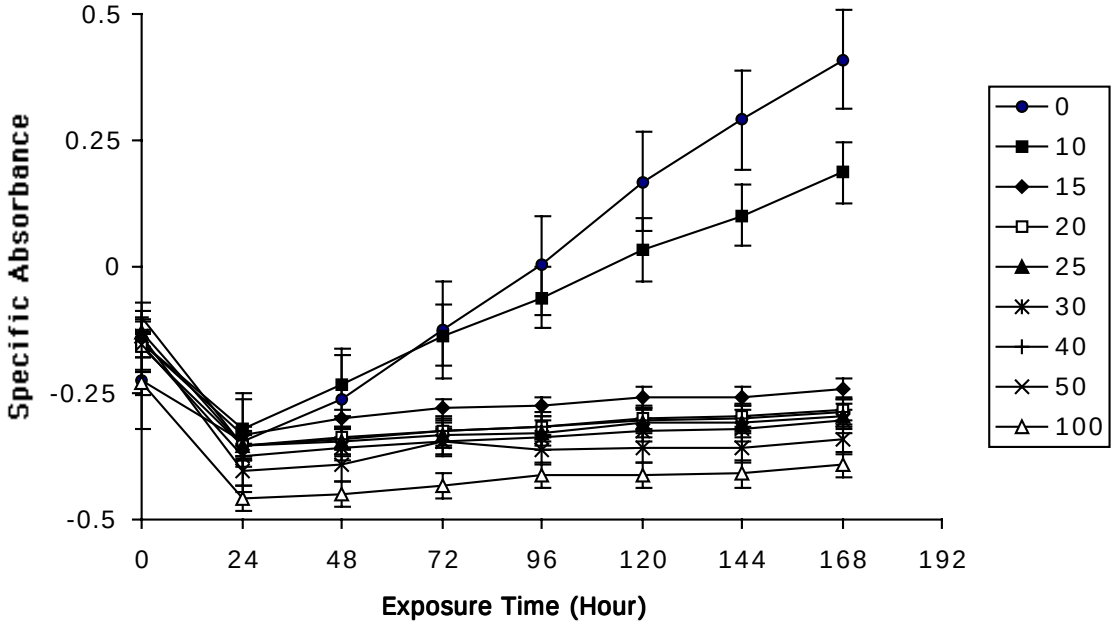

0.5
0.25
0
-0.25
-0.5
Specific Absorbance
0
24
48
72
96
120
144
168
192
Exposure Time (Hour)
0
10
15
20
25
30
40
50
100

Fig. 3.3 The distribution of cells within different phases of the cell cycle for Sp1K cells following exposure to $HgCl_2$. Values are the average of 4 data points within 4 complete blocks + the standard error. Statistical difference ($p < 0.05$) is indicated by * when cells at 5, 10, 15, or 20 μM $HgCl_2$ is compared to the matched medium-only control, and as # when cells at 24, 48, or 72 hour exposure compared to the matched 12 hour exposure time control.  = cells in G_0/G_1 phase.  = cells in S phase.  = cells in G_2/M phase.

Fig. 3.3

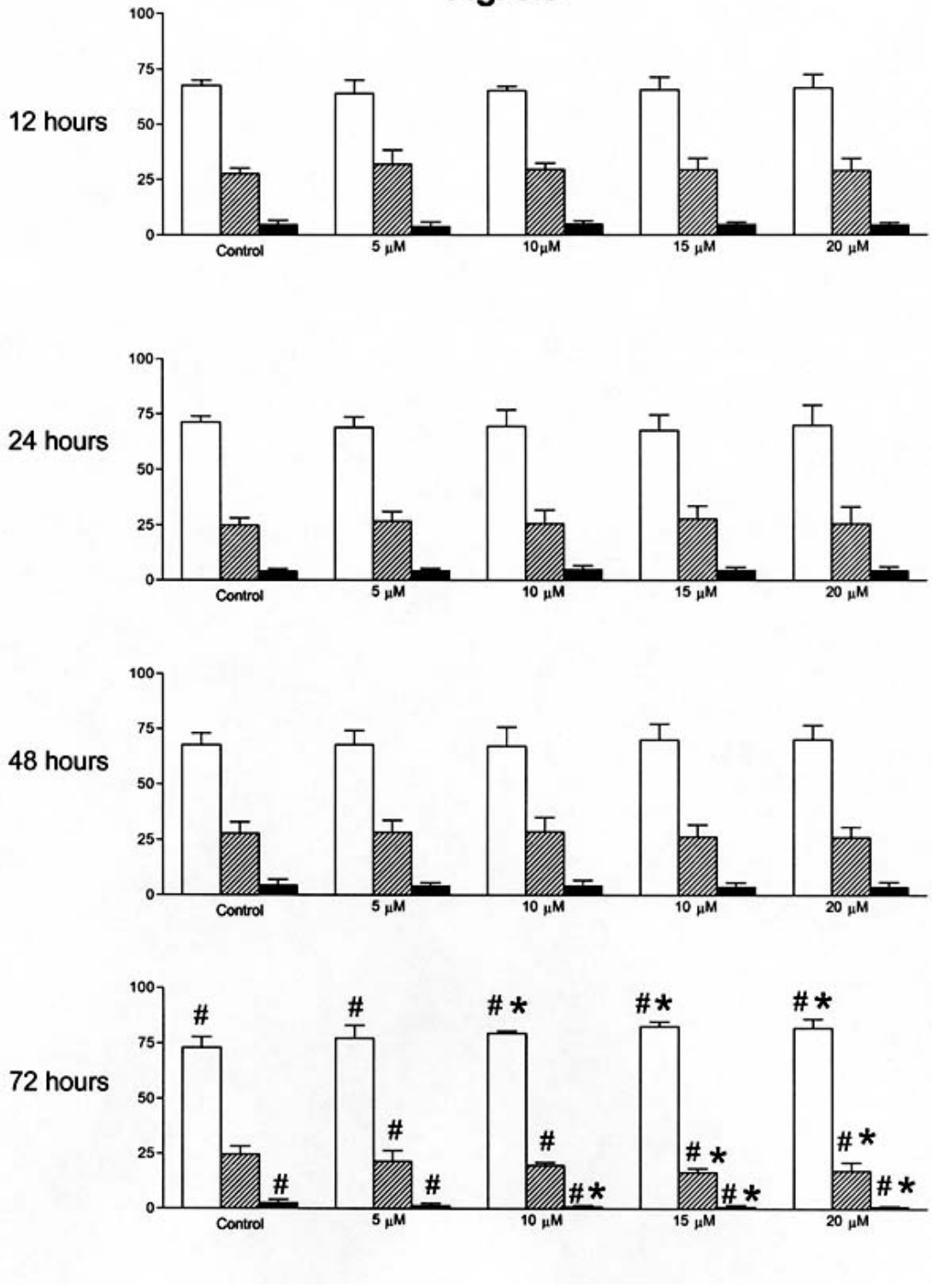
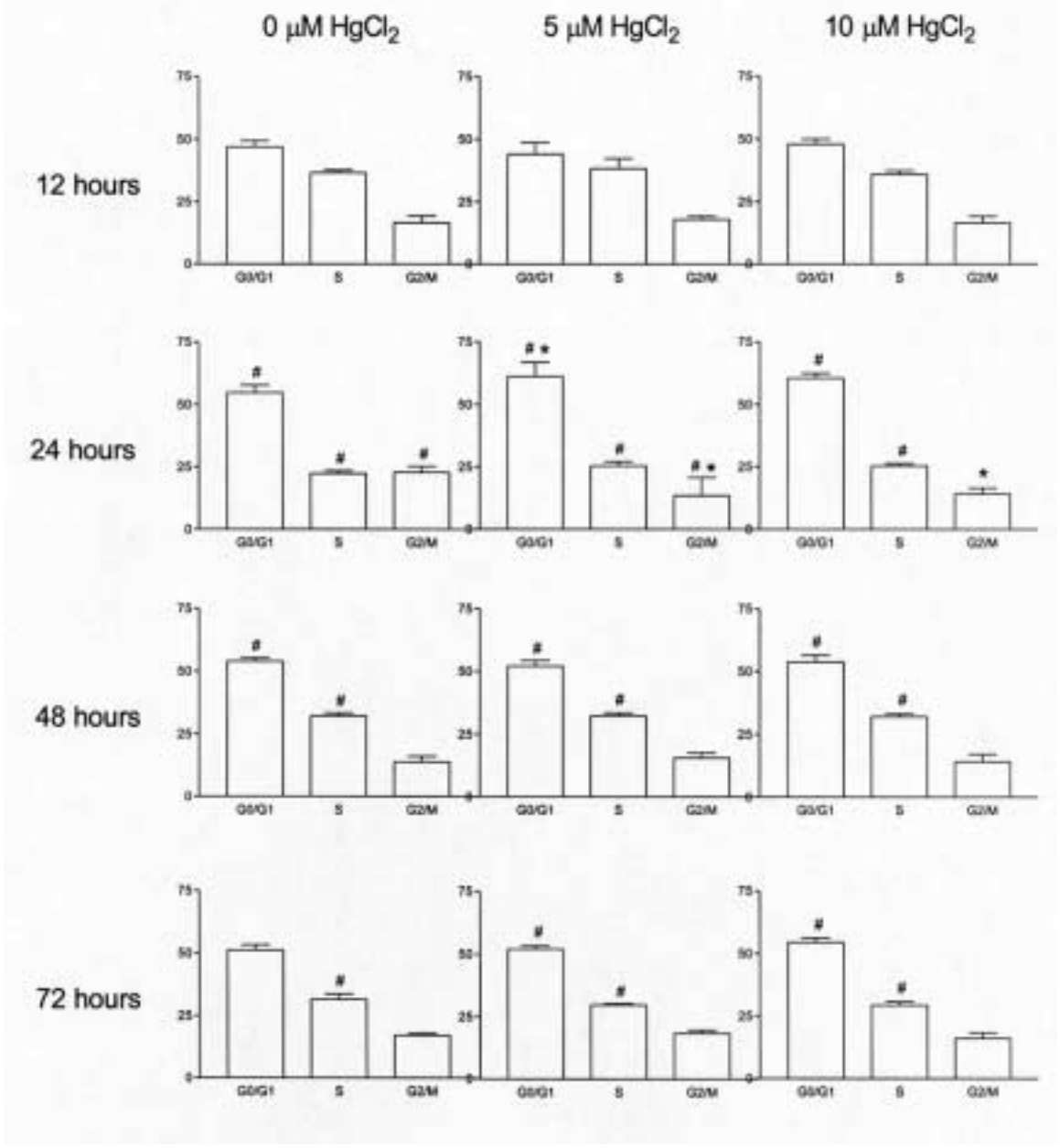

12 hours
24 hours
48 hours
72 hours
100
75
50
25
0
Control
5 μM
10 μM
15 μM
20 μM
#
#*

Fig. 3.4 The distribution of cells within different phases of the cell cycle for MK2 cells following exposure to $HgCl_2$. Values are the average of 5 data points + the standard error. Statistically significant difference ($p < 0.05$) is indicated by * when 5 or 10 μM is compared to the matched medium-only control, and as # when 24, 48, or 72 hour is compared to the matched 12 hour exposure time.

Fig. 3.4


0 μM HgCl2
5 μM HgCl2
10 μM HgCl2
12 hours
24 hours
48 hours
72 hours
G0/G1
S
G2/M

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PART IV

PROTECTIVE EFFECTS OF SELENIUM AGAINST MERCURY TOXICITY IN CULTURED ATLANTIC SPOTTED DOLPHIN (*Stenella plagiodon*) RENAL CELLS

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ABSTRACT

Marine mammals are known for their low susceptibility to mercury toxicity, and selenium may play a role in this protection against mercury intoxication. To gain insight into mechanisms by which selenium might inhibit mercury toxicity in cetacean cells, we investigated the effects of sodium selenite on cell proliferation and cell death (including apoptosis, oncosis, and necrosis) of control and mercuric chloride-treated Atlantic spotted dolphin renal cells (Sp1K cells). Concurrent exposure to 80 μM Na_2SeO_3 provided full protection against the decrease in cell proliferation induced by 20 μM $HgCl_2$. Pretreatment with Na_2SeO_3 increased the protective effects of selenium administered later in conjunction with mercury, but pretreatment alone did not provide protection against mercury given alone. Furthermore, Na_2SeO_3 administered after the exposure to $HgCl_2$ did not protect cells. These data suggest that the coexistence of Na_2SeO_3 and $HgCl_2$ was essential for the protective effects of Na_2SeO_3 against the toxicity of $HgCl_2$ in Sp1K cells, and may involve selenium-mercury binding. This is supported by the results of an experiment in which earlier pre-mixed mercury and selenium solutions were less cytotoxic than freshly mixed solutions. Furthermore, $HgCl_2$ induced apoptosis in Sp1K cells, as revealed by nuclear specific dye (7-AAD) incorporation and cell flow cytometry, and this was prevented by the concurrent exposure to Na_2SeO_3. Inhibition of mercury-induced apoptosis in marine mammal cells, provided by selenium, may contribute to the *in vivo* protection. This study is the first report which addresses the mechanism of mercury-selenium antagonism in cultured cetacean cells at the cellular level.

BACKGROUND

Due to a position high in the food chain, marine mammals accumulate high concentrations of mercury which are associated with the concentrations of mercury in their prey (Monaci et al. 1998) and which increase with age (Palmisano et al. 1995). Since cetaceans have limited ability to eliminate mercury (Nigro and Leonzio 1996), mercury concentrations may be 10 to 100 times that in tuna or swordfish, which are on the same trophic level and which have a similar average life span (Baldi 1986). The relative biomagnification factor for dolphins may be as high as approximately 500, whereas top fish predators with similar levels of dietary mercury have a relative biomagnification factor of about 30 (Nigro and Leonzio 1993).

Dolphins display low suspectibility to methylmercury (MeHg) toxicity. Human threshold burdens for the onset of MeHg-induced paresthesia and death were 0.49 and 3.92 mg/kg, respectively (Bakir et al. 1973), but mature striped dolphins have demonstrated methylmercury concentrations from 2.83 to 3.88 mg/kg (Itano et al. 1984). For the bottlenose dolphin, *Tursiops truncatus*, the minimal body burden that produces mild symptoms was 2 mg/kg, which is about 7 times the human threshold for symptoms (0.285 mg/kg) (Nordberg 1976; Rawson et al. 1995).

Demethylation of methylmercury and protection by selenium have been suggested as possible mercury detoxification mechanisms in cetaceans. Evidence of demethylation has been observed in harp seals orally administered methylmercury (Ronald et al. 1977), and has been strongly suggested in cetaceans. Almost all of the mercury acquired by cetaceans is methylmercury, based on the fact that mercury in fish is generally more than 80% methylmercury (Cappon and Smith 1982), and evidence of absorption of methylmercury in the stomach. However, in adult dolphins methylmercury averages only 30% of the total mercury in the whole body (Itano et al. 1984).

Protection against mercury toxicity by selenium is species- and tissue-specific (Cuvin-Aralar and Furness 1991). *In vivo*, this effect depends upon the chemical forms of mercury and selenium as well as upon the molar ratios of the elements supplied to the animals (Komsta-Szumska and Chmielnicka 1977; Cuvin-Aralar and Furness 1991).

Equimolecular accumulation of mercury and selenium in marine mammals was first suggested as a mechanism to reduce toxicity of mercury in marine mammals by Koeman in 1973. Further studies have shown that electron dense granules in marine mammal tissues were mercury selenide (tiemannite), which may serve as a storage end product of mercury detoxification and contribute to the adaptation to dietary mercury toxicants (Rawson et al. 1995; Nigro and Leonzio 1996).

However, demethylation and the formation of completely inert tiemannite (HgSe) do not rule out other detoxification mechanisms, such as competition for binding sites between mercury and selenium and selenium inhibition of oxidative damage from mercury (Cuvin-Aralar and Furness 1991; Palmisano et al. 1995). Accordingly, the mechanism of high physical tolerance to mercury in cetaceans requires more investigation, especially at the cellular level, as the previous studies have been limited to cultured cetacean fibroblasts or immunocytes. (Betti and Nigro 1996; De Guise et al. 1996; De Guise et al. 1998)

Since the kidney is the major organ that stores mercury, and the nephrotoxicity of MeHg *in vivo* may result from its demethylation to inorganic mercury instead of a direct effect (Klein et al. 1973; Rodier et al. 1988), the interactions of $HgCl_2$ and Na_2SeO_3 were studied in cultured Atlantic spotted dolphin renal cells. These data include findings on apoptosis, and cell proliferation rate.

MATERIALS AND METHODS

Cell Culture

The Atlantic Spotted Dolphin (*Stenella plagiodon*) renal cell line, Sp1K, was purchased from the American Type Culture Collection (Rockville, MD) and cultured in a humidified incubator with 5% $C0_2$ at $37^{O}C$. This cell line has been previously used in our toxicity studies (Pfeiffer et al. 2000). Minimal essential medium (MEM) with non-essential amino acids and L-glutamine (BibcoBRL, Grand Island, NY) including 10% fetal bovine serum (FBS) (GibcoBRL, NY) was used as growth medium. For each new experiment, adherent cells were detached from the petri dish by 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in phosphate buffered saline (PBS).

Cell Proliferation

Cultures were seeded with 600 cells in 200 µl of growth medium per well in 96-well flat bottom plates (Nunc, Denmark). Treatments were started 24 or 36 hours after seeding. Cells were treated by replacing growth medium with a test medium for an additional 6 days in the presence of various test agents. The experiments conformed to a complete block design with four blocks, except in the premixing experiment (see below), which had two blocks.

$HgC1_2$ (Sigma) and Na_2Se0_3 (Sigma) were dissolved in the growth medium just prior to use, except in the premix experiment.

AlamarBlue™ (Accumed International Inc., Westlake, OH) was added to the medium at a final concentration of 10% (v/v), immediately followed by the first reading and later readings at 12 or 24 hour intervals. When cells grow, metabolic activities reduce alamarBlue™ from the blue, oxidized form (maximum absorbency wavelength at 600 nm) to the red, reduced form (maximum absorbance wavelength at 570 nm). Specific absorbance (specific OD), directly proportional to the cell number, was attained by subtracting the absorbance at 600 nm from the 570 nm absorbance, determined by a

SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA). In order to eliminate the effects from media difference and air oxidation, all specific ODs from treatment media without cells were subtracted from specific ODs from the same treatment groups with cells when possible.

Mercuric Chloride Exposure Protocol for Cell Proliferation Test

In the experiment with concurrent exposure to mercury and selenium, cells were exposed to both $HgCl_2$ and Na_2SeO_3 for various periods. The $HgCl_2$ concentrations investigated were 0 (control) and 20 μM, and the Na_2Se0_3 concentrations were 0, 10, 20, 40, and 80 μM. The durations of exposure were 0, 2, 4, 8, 12, and 24 hours. After the exposure periods, the media were replaced by 10% alamarBlue™ medium.

To investigate the effects of chemical binding between mercury and selenium, cells were exposed for 24 hours to premixed mercury and selenium solutions which were premixed for various periods. $HgCl_2$ and Na_2SeO_3 media were made separately in advance and mixed for durations of 0, 4, 8, 12, 24 hours (treatment media) before use. After a 24 hour exposure to the treatment media, the media were replaced by 10% alamarBlue™ medium.

To evaluate effects of pretreatment with selenium, cells were exposed to 0, 20, or 80 μM selenium for 24 hours at either 0, 12, or 24 hours before the experimental tests. Experimental tests consisted of exposure to growth medium only (control), exposure to 20μM $HgCl_2$, and to 20μM $HgC1_2$ plus 80 μM Na_2SeO_3. Ten percent alamarBlue™ was simultaneously administered.

In order to investigate if selenium could inhibit or repair existing damage caused by mercury, the selenium effects subsequent to mercury exposure were tested. Cells were treated with 0 or 20 μM $HgC1_2$ for 24 hours and then the medium was replaced by 10% alamarBlue™ medium. Na_2Se0_3 was added at 0, 12, 24, 36, or 48 hours after the $HgC1_2$ treatment was initiated, for a final concentration of 0, 20, or 80 μM of Na_2Se0_3. Therefore, there were (a) a 24 hour concurrent exposure to mercury and selenium for cells

which received selenium immediately after mercury exposure was started (delay 0 hour), (b) a 12 hour concurrent exposure for cells in the 12 hour delay group, and (c) no concurrent exposure for the remaining groups (24, 36, and 48 hour delay).

Nucleic Acid Stain for Apoptosis

Seven-amino-actinomycin D (7-AAD) is a fluorescent dye, which specifically binds to guanine-cytosine (GC) regions of DNA. It has been documented that 7-AAD alone can substitute the Hoechest 33342 plus 7-AAD method in which bright Hoechest 33342 density cells (non-living cells) can further be subdivided as 7-AAD negative cells (with intact cell membrane) and 7-AAD positive cells (with damaged membrane integrity) (Schmid et al. 1994). By flow cytometery, 7-AAD stained cells can be classified into three groups: (1) live cells, with low 7-AAD density and normal cell size, (2) apoptotic cells, which show moderate 7-AAD density and small cell size, and (3) oncotic, necrotic plus dead cells, which show high 7-AAD density.

An estimated total of 22.5 x 10^4 cells was cultured in 75 cm^2 flasks in the growth medium for 4 days, and then cultured in the treatment medium for 72 hours. The treatment media were (1) growth medium alone (2) 20 µM $HgC1_2$ (3) 80 µM Na_2Se0_3 and (4) 20 $HgC1_2$ plus 80 µM Na_2SeO_3 dissolved in growth medium. Each treatment had three replicates per block. There were 3 blocks performed on different days in this experiment.

After a 72 hour exposure to the treatment media, cells were harvested with trypsin and EDTA. All of the treatment media, and the PBS used to wash the cells, were collected to obtain all of the cells, including floating cells. Cells were centrifuged at 100 rpm for 10 minutes, and the cell pellets were re-suspended in the growth medium for cell counting. A total of $5x10^5$ cells was transferred to a 5 ml tube and centrifuged, and collected as precipitate. One hundred µl of FACS buffer (0. 1 g of sodium azide and 2 ml of fetal bovine serum in 98 ml PBS) and 100 µl 10ug/ml 7-AAD (Sigma) per $5x10^5$ cells

were added, and the tubes were vortexed. Cells were incubated on ice 15 to 30 minutes in the dark before flow cytometric analysis.

Flow cytometric analyses were accomplished with an Epicx-XL flow cytometer (Beckman-Coulter, Miami, FL) equipped with an air cooled argon laser. The 7-AAD fluorescence was assessed at 650 nm emission and 488 nm excitation.

Trypan blue and a hemocytometer were used to estimate the numbers of cells in suspension. The volume of suspension needed for 7-AAD stain and the numbers of total cells in each sample were calculated. By light microscopy cells with bright cytoplasm are vital cells, whose intact cell membrane excludes the dye, and cells with blue content and bright outlines are dead cells. The percentages of vital cells and dead cells were calculated.

Statistical Analysis

Data are presented as means ± standard error from four data points. Differences between treatment groups were evaluated statistically by using analysis of variance for multiple group comparisons. A P value less than 0.05 was considered statistically significant. After the analysis of variance, the mean for each set of data was statistically compared with the means of the medium alone and $HgCl_2$ alone, each as a control, using the Tukey comparison.

RESULTS

ALAMARBLUE™ ASSAY FOR CELL PROLIFERATION

Concurrent exposure to $HgCl_2$ and Na_2SeO_3

The selenium effect was more significant at 20 μM $HgCl_2$ than at 0 μM $HgCl_2$ after one hour following the introduction of alamarBlue™. $HgCl_2$- exposure and Na_2SeO_3- exposure effects were observed 12 hours following the introduction of alamarBlue™. Both metals had more influence on cellular growth when the exposure period was longer. After 24 hours, the inhibition of cell proliferation was most significant for cells exposed to the higher concentration of $HgCl_2$ plus lower concentrations of Na_2SeO_3 after longer exposures.

The effects of simultaneous exposure to $HgCl_2$ plus Na_2SeO_3 were most apparent in the 24 hour exposure group (Fig. 4.1). Concurrent exposure to 80 μM Na_2SeO_3 plus 20 μM $HgCl_2$ induced no change from the control. There were no significant differences between the media-only-control and 20 μM $HgCl_2$ plus 20 or 40 μM Na_2SeO_3 groups, although 20 μM $HgCl_2$ plus 20 or 40 μM Na_2SeO_3 results were not statistically different from 20 μM $HgCl_2$ alone.

Premixing $HgCl_2$ and Na_2SeO_3 Prior to Application

If direct chemical binding between mercury and selenium, forming a less toxic complex, were to contribute to selenium protection against mercury toxicity, then mixing $HgCl_2$ plus Na_2SeO_3 prior to treatments may reduce the mercury toxicity.

Shorter premix times and lower concentrations of Na_2SeO_3 both were associated with greater decreases in cell proliferation, as measured by alamarBlue™, than the no-premix and high concentration of Na_2SeO_3 groups, respectively (Fig. 4.2). Specifically, the specific absorbance for the premix 12 hour group was significantly greater than for the non-premix group, but premixing for shorter periods of time (4, or 8 hours) yielded the same result as the non-premix group. The differences between 20 versus 80 μM Na_2SeO_3 were seen only in the premix 12 hour group.

Effects of Pretreatment with Na_2SeO_3 on Cells Exposed to $HgCl_2$ and $HgCl_2$ plus Na_2SeO_3

The effects caused by the various concentrations of Na_2SeO_3 prior to the treatments were designated as "preSe" effects, and the time between the end of the preSe exposure and initiation of treatments was designated as "gap".

PreSe effects were seen in all treatment groups (Fig. 4.3). Pretreatment with a high concentration of Na_2SeO_3, resulted in a greater cell proliferation than for cells exposed to a low concentration of Na_2SeO_3 before the treatments. Furthermore, the preSe effects were most obvious in cells subsequently exposed to both mercury and selenium, and least in cells subsequently exposed to medium only. For cells exposed to both mercury and selenium, preSe at 20 and 80 μM doses induced changes that were significantly different from preSe at 0 μM at all gaps. In contrast, cells that received 20 μM $HgCl_2$ showed differences between preSe 80 μM and preSe 0 μM at all gaps, but differences between preSe 20 μM and preSe 0 μM were observed only at the 24 hour gap group after the treatments were administered for 36 hours. For cells receiving medium only, there were only differences between preSe 80 μM and 0 μM at gap 12 and gap 24, and differences between preSe 20 μM and 0 μM at gap 24.

Interactions between preSe and gap, and gap effects were also revealed (Fig. 4). Longer gap times were associated with less proliferation. The high concentration of preSe provided an even better protection when the gap was shorter. Gap effects were seen at preSe 20 and 80 μM. For all three treatments, significant differences were seen between the 12 hour gap and 0 hour gap at preSe 80 μM and between 24 hour gap and 0 hour gap at preSe 20 and 80 μM. Only cells treated with both mercury and selenium showed differences between the 12 hour gap and 0 hour gap at preSe 20 μM treatments. Unlike the differences among the preSe effects, which showed up gradually with time, the differences between gaps were seen within 12 hours after the treatments were initiated.

Selenium Exposure Subsequent to Mercury Exposure

Selenium effects were seen only in the 0 and 12 hour delay groups. Both 20 μM and 80 μM Na_2SeO_3 initiated a significant increase in cell growth in the 0 and 12 hour delay groups; furthermore, in the 0 hour delay group, the reading from alamarBlue™ (an indicator of cell growth) of cells that were exposed to 80 μM Na_2SeO_3 was significantly higher than for cells treated with 20 μM Na_2SeO_3 (Fig. 4.4). These results suggested that there was a greater Se effect in the non-delayed group than in 12 hour delay group.

A longer delay time was associated with significantly decreased cell growth in cells receiving the same concentration of selenium (Fig. 4.4). A 36 and 48 hour delay significantly decreased cell growth at all concentrations of Na_2SeO_3. A 12 hour delay significantly reduced cell growth at 20 and 80 μM Na_2SeO_3 exposures, while a 24 hour delay reduced cell growth at all concentrations of Na_2SeO_3.

NUCLEIC ACID STAIN FOR APOPTOSIS

Exposure to $HgCl_2$ 20 μM for 72 hours significantly decreased the percentages of normal cells, and increased the percentages of apoptotic cells, as well as of oncotic, necrotic, and dead cells (Fig. 4.5). A 72 hour exposure to Na_2SeO_3 80 μM, or to $HgCl_2$ 20 μM plus Na_2SeO_3 80 μM, did not induce changes in any of the percentages of the degenerative categories compared to the medium only control. When compared to the cells receiving only $HgCl_2$ 20 μM, cultures exposed to $HgCl_2$ 20 μM plus Na_2SeO_3 80 μM displayed significantly more normal cells, less apoptotic cells, and less oncotic, necrotic and dead cells (Fig. 4.5). Thus, a selenium protection against mercury-induced apoptosis was shown in this experiment.

Interestingly, the total numbers of cells (data not shown) as counted by a hemocytometer were decreased significantly in all three conditions: 20 μM $HgCl_2$, 80 μM Na_2SeO_3, and 20 μM $HgCl_2$ plus 80 μM Na_2SeO_3. Selenium failed to increase the total cell number, not only in cells treated with $HgCl_2$, but also in cells receiving Na_2SeO_3 alone. The percentages of dead cells (data not shown), characterized with trypin blue in the cytoplasm, were the same in all treatments.

DISCUSSION

These results demonstrated that Na_2SeO_3 antagonized the inhibition of cell proliferation and the induction of apoptosis induced by $HgCl_2$ in cultured Atlantic Spotted Dolphin renal cells. This is the first proof of selenium antagonism against mercury intoxication in cetacean cells *in vitro*, and may be applicable to dolphins *in vivo* in the natural environment as selenium is present in their ingested prey (Nigro and Leonzio 1996).

Concurrent exposure to Na_2SeO_3 and $HgCl_2$ decreased the toxicity of $HgCl_2$ in cetacean cells. This is in agreement with other *in vivo* and *in vitro* data (Parizek and Ostadalova 1967; Berlin 1978; Alexander et al. 1979; Aoshima and Kasuya 1980; Naganuma and Imura 1983b). However, the molar ratio for optimal protection varied. In Sp1K cells, a Hg: Se molar ratio lower than 1:2 did not provide protection, and a Hg: Se molar ratio 1: 4 provided full protection as measured by cell growth and apoptosis frequency. In contrast, the maximum protection was seen at a ratio of $HgCl_2$: Na_2SeO_3 = 10: 1 in cultured rat cerebrum (Aoshima and Kasuya 1980), and at 1:1 in human whole - blood cultures, and in rats *in vivo* (Parizek and Ostadalova 1967; Morimoto et al. 1982). Therefore, although cetacean cells were more resistant to mercury toxicity in a selenium-independent manner (Betti and Nigro 1996), they may also require relatively more selenite for protection than other cell types and animals.

The formation of a complex containing equimolar Hg and Se (Naganuma and Imura 1981; Morimoto et al. 1982) from $HgCl_2$ and Na_2SeO_3 is one of the possible mechanisms of selenium protection. Our results illustrating that premixed $HgCl_2$ and Na_2SeO_3 was associated with lower toxicity indicated that chemical binding might play a role in selenium protection. However, the relative importance of pure chemical binding between these two metals is unclear. Alexander (1979) proposed that specific cell-dependent processes instead of a direct chemical reaction was responsible for the selenium protection against mercury toxicity. The basis of this hypothesis was (a) cell specific protection, and (b) a molar ratio as low as 1:12 molar ratio of selenite to MeHg

can elicit protection, and one mole of selenite cannot strongly bind more than 2 moles of MeHg.

The order of administration of mercury and selenium affected the antagonism in a cell specific manner. When mercury was administrated prior to selenium, (1) there was no protection for dolphin renal cells *in vitro* or mice *in vivo* (Naganuma et al. 1984), (2) there was protection for cultured hematoma cells (Alexander et al. 1979), (3) it decreased the final amount of Hg and Se incorporated into RBC in rats (Naganuma and Imura 1983a) and (4) it inhibited the release of dimethyl selenide (Parizek and Ostadalova 1967). Furthermore, pre-exposure to selenium (1) decreased the toxicity of $HgCl_2$ in murine macrophage cells (Christensen et al. 1991), (2) decreased the final amount of Hg and Se incorporated into RBC in rats (Naganuma and Imura 1983a), (3) did not protect dolphin renal cells from $HgCl_2$ toxicity, but enhanced the Na_2SeO_3 protection against $HgCl_2$ in dolphin renal cells, (4) canceled the protection against $HgCl_2$ toxicity in mice (Naganuma et al. 1984) and (5) enhanced the toxic effect of dimethyl selenide which had been administrated prior to mercuric acetate in rats (Parizek and Ostadalova 1967). The effects from the changes in time of administration may result from different interactions from the simultaneous exposure to mercury and selenium (Naganuma and Imura 1983a).

The decrease of apoptosis following exposure to $HgCl_2$ by Na_2SeO_3 in dolphin renal cells suggests a new direction to elucidate the mechanism of marine mammal tolerance to mercury. Mercury-induced apoptosis has been demonstrated in various *in vivo* and *in vitro* systems (Goering et al. 1997; Matsuoka et al. 1997; Akhand et al. 1998), but has not previously been reported in cetaceans. Comparing the mercury toxicity in terms of apoptosis between experiments is complicated by wide range of conditions (such as serum in the medium and testing methods) which could affect the percentage of cells in apoptosis. However, Sp1K cells (cultured in 10% FBS medium and exposed to 20 μM $HgCl_2$ for 72 hours) had a lower percentage of apoptotic cells than mouse lymphoma cells (cultured in 10% FBS medium and exposed to 10 μM $HgCl_2$ for 12 hours) (Goering et al. 1997). It is possible that the resistance of Sp1K cells to $HgCl_2$, in terms of cellular growth, is partially due to the resistance to apoptosis.

The present data support the notion that selenium is protective against mercury toxicity by formation of a mercury-selenium complex and competition for binding sites (Cuvin-Aralar and Furness 1991). Na_2SeO_3 and $HgCl_2$ may bind to the same binding sites which modulate adverse effects on cells by mercury, but not by selenium or a mercury-selenium complex. The complex may form from free mercury and selenium, but not from bound metals. After concurrent exposure to selenium and mercury, some binding sties were occupied by selenium or the complex, inhibiting the mercury toxicity. Alternatively, the complex may not bind but still remove free, reactive mercury. When mercury and selenium were premixed, more non-toxic complex was formed than in fresh mercury plus selenium solutions.

Pretreatment with selenium enhanced protection provided by simultaneous treatment with Na_2SeO_3 and $HgCl_2$, but was not sufficient to provide protection against $HgCl_2$ without simultaneous exposure to Na_2SeO_3. Pretreatment with selenium may increase the free intracellular selenium concentration , effectively increasing the selenium:mercury ratio within the cell. However, the intracellular selenium achieved by pretreatment alone can not completely prevent binding of mercury to critical targets.

Other mechanisms for selenium protection against mercury toxicity (redistribution of mercury to less sensitive organs, conversion of toxic forms of mercury to less toxic forms, and prevention of oxidative damage) (Cuvin-Aralar and Furness 1991) cannot be ruled out. More investigation will be needed to elucidate the existence of these possible mechanisms in cetaceans *in vivo* and *in vitro*.

The present investigation demonstrated selenium protection against mercury intoxication in cultured Atlantic spotted dolphin renal cells, and suggested a selenium-independent low susceptibility to mercury toxicity. The changes in cell proliferation under a variety of exposures to $HgCl_2$ and Na_2SeO_3, or premixed $HgCl_2$ and Na_2SeO_3 supported the hypothesis that the protective effect of selenium is in part from competition for binding sites and the formation of a non-toxic mercury-selenium complex. This phenomenon may contribute to the observed *in vivo* relative tolerance of cetaceans to mercury toxicity.

Fig. 4.1 Cell growth of Sp1K cells that were concurrently exposed to $HgCl_2$ and Na_2SeO_3 for 24 hours. Values are the average of 4 data points. Y error is the standard error. A statistically significant difference in specific absorbance, an indicator of cell growth, is shown as ✶ ($P<0.05$) when compared to medium only control, and shown as # ($P<0.05$) when compared to cells exposed to $HgCl_2$ 20 µM. Control = medium only. Hg only = $HgCl_2$ 20 µM. Se 10 = $HgCl_2$ 20 µM plus Na_2SeO_3 10 µM. Se 20 = $HgCl_2$ 20 µM plus Na_2SeO_3 20 µM. Se 40 = $HgCl_2$ 20 µM plus Na_2SeO_3 40 µM. Se 80 = $HgCl_2$ 20 µM plus Na_2SeO_3 80 µM. Exposure times (0-48 hours) are indicated by bar shading.

Fig. 4.1

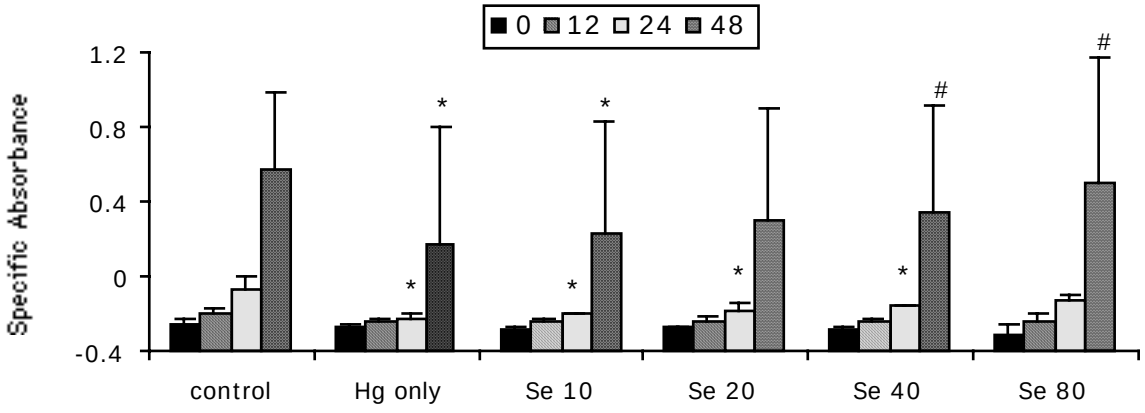

0 12 24 48
Specific Absorbance
1.2
0.8
0.4
0
-0.4
control
Hg only
Se 10
Se 20
Se 40
Se 80
*
#

Fig. 4.2 Cell growth of Sp1K cells exposed to premixed 20 μM $HgCl_2$ and 80 μM Na_2SeO_3. Statistically significant differences are shown as ✶ ($P<0.05$) when the pre-mix groups were compared to cells exposed to 20 μM $HgCl_2$ only. Control = medium only. Hg only = $HgCl_2$ 20 μM. Premix 0 hr = $HgCl_2$ 20 μM plus Na_2SeO_3 80 μM mixed at the time of treating cells. Premix 4 hr = $HgCl_2$ 20 μM plus Na_2SeO_3 80 μM premixed 4 hours. Premix 8 hr = $HgCl_2$ 20 μM plus Na_2SeO_3 80 μM premixed 8 hours. Premix 12 hr = $HgCl_2$ 20 μM plus Na_2SeO_3 80 μM premixed 12 hours. Numbers by code for bar shading indicate number of hours after the administration of alamarBlue™.

Fig. 4.2

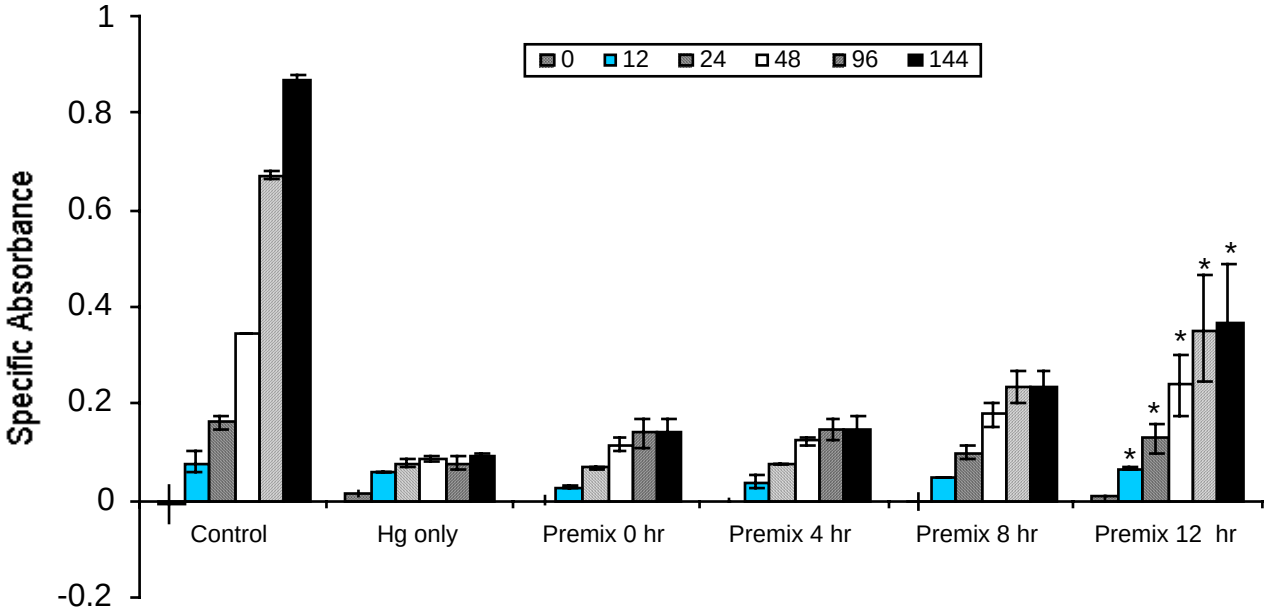

Specific Absorbance
1
0.8
0.6
0.4
0.2
0
-0.2
0
12
24
48
96
144
Control
Hg only
Premix 0 hr
Premix 4 hr
Premix 8 hr
Premix 12 hr
*
*
*
*
*

Fig. 4.3 Cell growth of Sp1K cells that were pretreated with selenium prior to concurrent mercury and selenium treatment. Open signs represented cells that were not pretreated with Na_2SeO_3. Closed signs represented cells that pretreated with 20 µM Na_2SeO_3, (Fig. 4.3 (a)) or with 80 µM Na_2SeO_3, (Fig. 4.3 (b)). ◇ = no pretreatment of Na_2SeO_3, exposed to $HgCl_2$ 20 µM plus Na_2SeO_3 80 µM. Δ = no pretreatment of Na_2SeO_3, medium only (control). □ = no pretreatment of Na_2SeO_3, exposed to $HgCl_2$ 20 µM. ● = pretreated with Na_2SeO_3 0 hour prior to $HgCl_2$ 20 µM plus Na_2SeO_3 80 µM. ▲ = pretreated with Na_2SeO_3 12 hours prior to $HgCl_2$ 20 µM plus Na_2SeO_3 80 µM. ◆ = pretreated with Na_2SeO_3 24 hours prior to $HgCl_2$ 20 µM plus Na_2SeO_3 80 µM.

Fig. 4.3 (a)

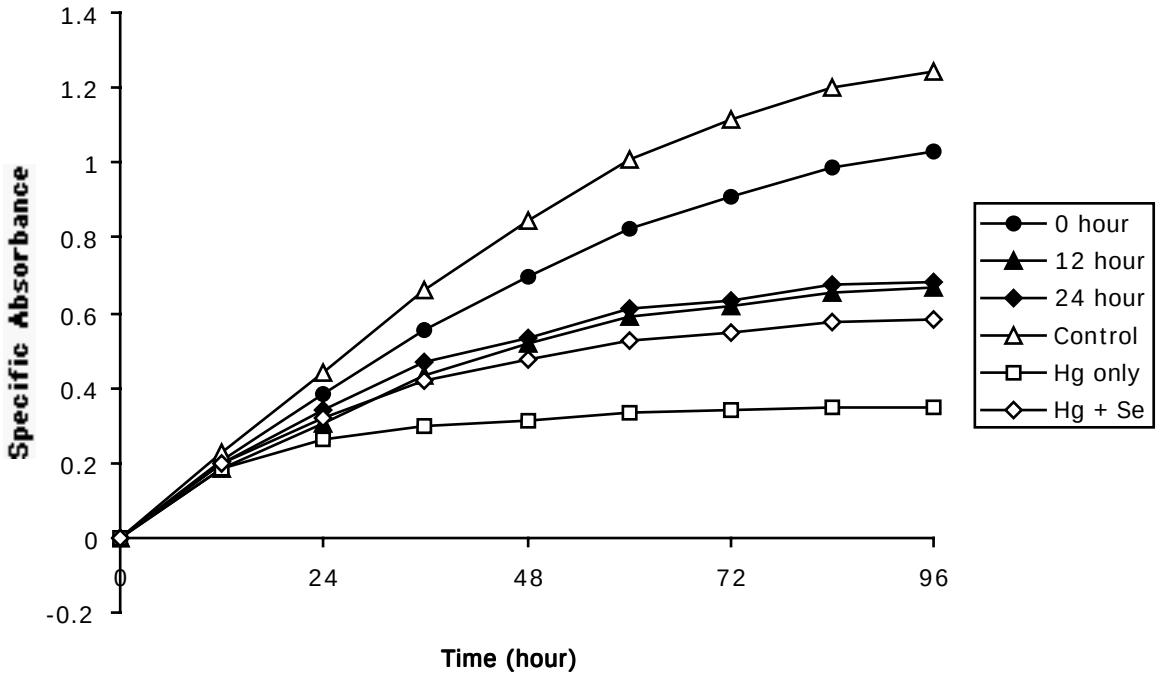

1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
24
48
72
96
Specific Absorbance
Time (hour)
0 hour
12 hour
24 hour
Control
Hg only
Hg + Se

Fig. 4.3 (b)

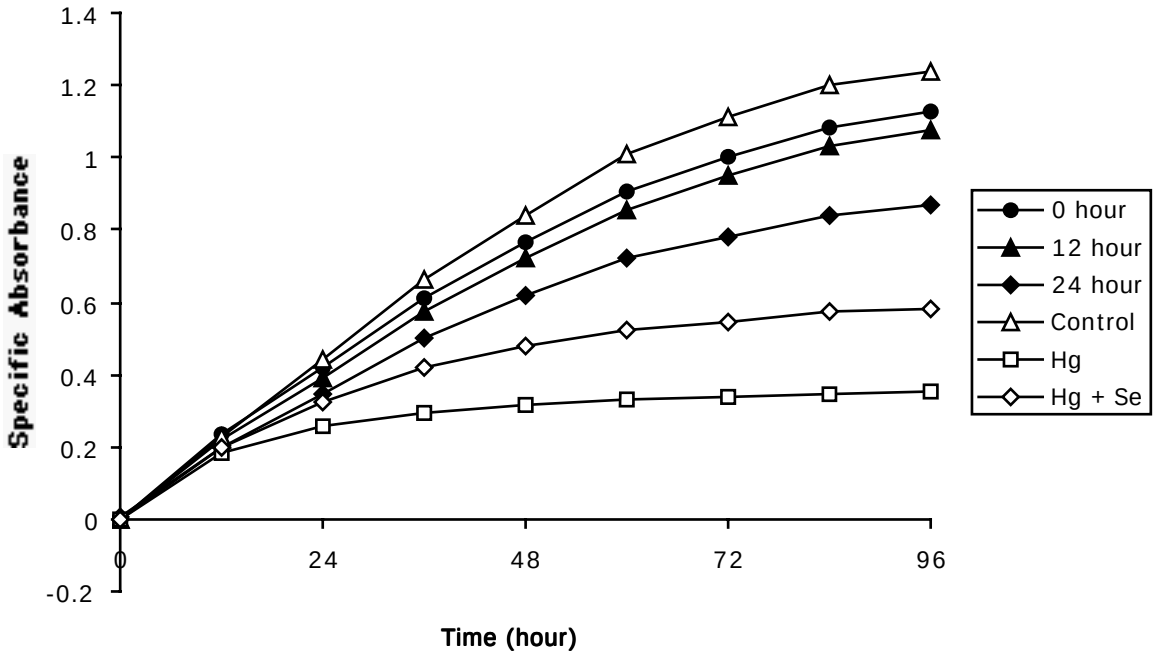

1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Specific Absorbance
0
24
48
72
96
Time (hour)
0 hour
12 hour
24 hour
Control
Hg
Hg + Se

Fig. 4.4 Cell growth of Sp1K cells that were treated Na_2SeO_3 20 μM (open signs) or Na_2SeO_3 80 μM (closed signs) following exposure to $HgCl_2$ 20 μM. ✳ = no Na_2SeO_3 treatment (control). ▲△ = received Na_2SeO_3 0 hours after $HgCl_2$. ● ❍ = received Na_2SeO_3 12 hours after $HgCl_2$. ■□ = received Na_2SeO_3 24 hours after $HgCl_2$.

Fig. 4.4

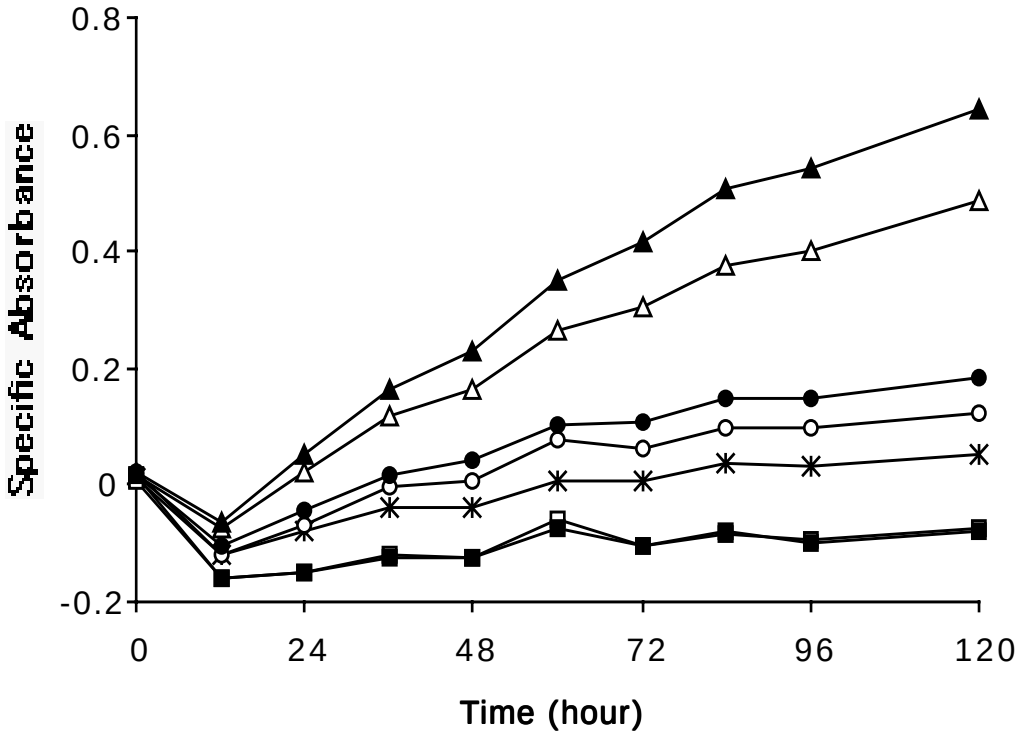
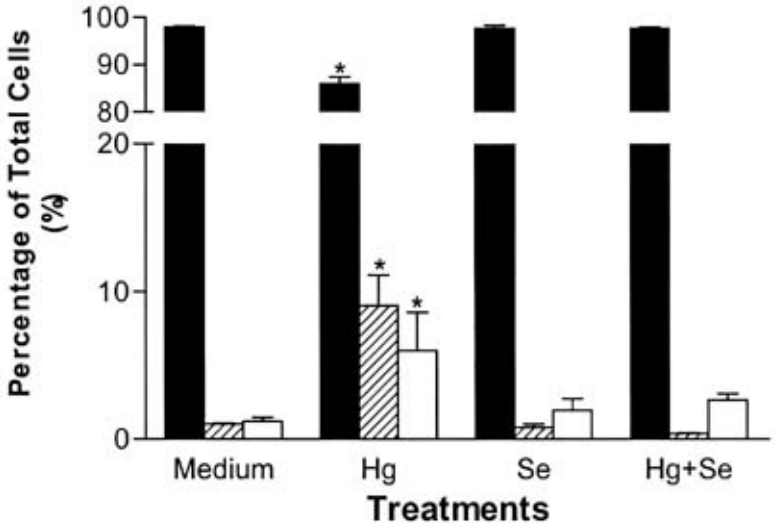


Fig. 4.5 Types of cell injury following 72 hour exposure to different treatments. Medium = medium only (control). Hg = $HgCl_2$ 20 μM. Se = Na_2SeO_3 80 μM. Hg + Se = $HgCl_2$ 20 μM plus Na_2SeO_3 80 μM. ■ = Normal cells. ▨ = Apoptotic cells. □ = Oncotic, necrotic, and dead cells. Statistical significance is shown as ✶ ($P<0.05$) when compared to matched parameters in medium only (control) group.

Fig. 4.5


Percentage of Total Cells (%)
100
90
80
20
10
0
*
*
*
Medium
Hg
Se
Hg+Se
Treatments

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PART V

CONCLUTIONS

Mercuric chloride ($HgCl_2$) altered the ultrastructures of Atlantic spotted dolphin renal cells (Sp1K cells). Cell proliferation of Sp1K cells and Rhesus monkey renal cells (MK2 cells) were both decreased by $HgCl_2$. Although $HgCl_2$ caused changes in the cell cycle, the $HgCl_2$-induced decrease in cell proliferation may mainly result from the induction of apoptosis rather than from the changes in cell cycle status. The resistance to the toxicity of $HgCl_2$ was higher in Sp1K cells than in MK2 cells.

Sodium seleniate (Na_2SeO_3) protected Sp1K cells from $HgCl_2$-induced decrease in the cell proliferation and $HgCl_2$ -induced increase in apoptosis. From the data of different administration order, the protection may involved in formation mercury-selenium complex and competition of binding sites.

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EDUCATION

Master of Veterinary Medicine Sciences, expected August 2000
Graduate student (M.S.) in marine mammal toxicology in the Department of Biomedical Sciences and Pathobiology at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM), Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA
Thesis topic: Mercuric chloride toxicity on cultured Atlantic Spotted renal cells and the role of selenium in protection.
Advisor: Carl J. Pfeiffer, Ph.D.

Bachelor of Veterinary Medicine, June 1996
National Taiwan University, Taipei, Taiwan

SCHOLARSHIPS AND AWARDS

Student Travel Award, Society for Marine Mammalogy, 1999
Commencement speaker, National Taiwan University, 1996
President's Award by National Taiwan University, 1993. Awarded to top 5% students in each academic class.
Liu's Scholarship by Liu's Memorial Foundation, 1993. For distinct academic progression.
Ministry of Education Scholarship, 1992. Awarded by Taiwan government for the study of Sun Yat-Sen Thought.

GRANTS

$2175 for Master's thesis study, Fall 1999. Awarded by the Research Focus Unit for Environmental Medicine and Toxicology, VMRCVM.
$2380 for Master's thesis study, Spring 1999. Awarded by the Department of Biomedical Sciences and Pathobiology, VMRCVM.

PROFESSIONAL EXPERIENCES

Graduate Research Assistant, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, VA. August 1999-present.

Developed and printed photographs taken under transmission electron microscope and assisted in other electron microscope related work.

Part-time Veterinary Clinician, NTU Veterinary Hospital, Taipei, Taiwan. November 1996-June 1998.

Treated cats and dogs in the Veterinary Hospital, including emergency and rehabilitation service.

Veterinary Assistant, Pao-An Animal Hospital, Taipei, Taiwan, 1994-1995

Assisted in surgery, blood and fecal testing, animal nursing. Performed neutering and ovariohysterectomy of stray dogs at stray animal centers.

TEACHING EXPERIENCES

Speaker, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, VA. February 2000

-Lectured "Introduction to Manatee Biology and Medicine" in "Marine Mammal Biomedicine" course.

Instructor, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, VA. June 1999-present

-Instructed a veterinary student in cell culture technique, experimental design, and data analysis as part of a summer fellowship program.

-Instructed a technician in cell culture technique.

Graduate Teaching Assistant, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, VA, January 1998- June 1998

-Graded quizzes and assignments

OTHER EXPERIENCES

Editor and Consultant, Eastern Publication Company, Ltd., Taipei, Taiwan, 1993-1994.

Editor and Consultant for *The Life of Birds*, one of the Sciences Cartoon Encyclopedia, a serious of children's books and winner of the *Golden Tripod Award* for outstanding publications; Providing information on birds biology, behavior and bird-watching; ensuring accuracy of illustrations and text.

PROFESSIONAL MEMBERSHIPS

Society for Marine Mammalogy, member, 1999-present.

International Association for Aquatic Animal Medicine (IAAAM), membership, 1999-present.

Taipei Veterinary Medical Association, Taipei, Taiwan, member, 1996-1998.

AFFILIATIONS

Chinese Student Association, Virginia Tech.

Acting President, January 1999- August 1999.

Vice president, June- December 1998.

Member, January 1998- present.

Taiwan Cetacean Stranding Network, Taiwan, Volunteer, October 1996-present
Performed necropsies on stranded and by-caught cetaceans, provided medical advice, translated parts of *Marine Mammals Ashore* and other books from English to Chinese, assisted in organizing conferences and overseas workshops.

China Youth Corps, Taiwan, Volunteer, February 1996- August 1996.

Association for Public Studies, NTU.
Director of the Executive Board, September 1994- September 1995.
Chairman of Orientation Committee, and Farewell Committee, February 1994- September 1994.
Chairman of Farewell Committee, September 1993- February 1994.
Member, February 1992-June 1996.

Taiwan Museum, Taiwan, Volunteer Guide, June 1992- September 1992.
Volunteer guide for the animal section.

Parliamentary Procedure Study Camp, NTU, Counselor, February 1992.

PUBLICATIONS

Protective effects of selenium against mercury toxicity in cultured Altantic spotted dolphin (*Stenella plagiodon*) renal cells. **A. Wang**, D. Barber, and C.J. Pfeiffer (2000) Arch. Environ. Contam. Toxicol. (submitted)

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ABSTRACTS

Lingual ultrastructure of the long-finned pilot whale. D. Pfeiffer, **A. Wang**, and C.J. Pfeiffer. The 8th annual Atlantic Coastal Dolphin Conference, Wilmington, North Carolina, 24-26 March, 2000.

Effects of mercuric chloride on Atlantic Spotted Dolphin(*Stenella frontalis*) renal cells. **A. Wang**, L. Sharova, K. Carlson, and C.J. Pfeiffer. The 13th Biennial Conference on the Biology of Marine Mammals, Maui, Hawaii, 28 November - 3 December, 1999.

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OTHER PUBLICATIONS

Tigers and lions. Benesse Challenge magazine, Taipei, Fukutake Publication Co., LTD. The magazine is for 1st to 3rd grade children, includes a tape cassette.

CONTINUING EDUCATION

Training the Future Professoriate Workshop. Research and Graduate Studies Blacksburg, VA. April 29, 2000.

One day workshop for preparing an academic career. Topics included balancing teaching, research and service duties and writing proposals.

1999 North Regional Stranding Conference. National Aquarium in Baltimore, Baltimore, MD. May 20-23, 1999.

30th Annual Conference of International Association for Aquatic Animal Medicine. Boston, MS, May 2-5. 1999

1999 Atlantic Coastal Dolphin Conference. Virginia Marine Science Museum, Virginia Beach, VA. March 19-21, 1999.

1998 Northeast Regional Stranding Conference. Virginia Marine Science Museum, Virginia Beach, VA. March 27-29, 1998.

Presentation Skill Workshop. Center of Excellence Undergraduate Teaching, Blacksburg, VA. March 11, 1998

Continuing Education in Veterinary Medicine. University of California at Davis, Davis, CA. August 8 – September 2, 1994.

120 hours of lecture and laboratory in the short course on small animal topics.

FOREIGN TRAVEL EXPERIENCE

Lived in Taiwan and United States.
Traveled in Egypt, Greek, Indonesia, Italian, Singapore, Thailand, and Canada.