# ความเป็นพิษแบบเฉียบพลันของโลหะหนักปรอท ในปลานิล (*Oreochromis niloticus*) ด้านพยาธิวิทยา

นาย เกษม รัตนภิญโญพิทักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# ACUTE MERCURY TOXICITY IN TILAPIA (*Oreochromis niloticus*), THE PATHOLOGICAL ASPECT

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Veterinary Pathology
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เกษม รัตนภิญโญพิทักษ์ : ความเป็นพิษแบบเฉียบพลันของโลหะหนักปรอทในปลานิล (*Oreochromis niloticus*) ด้านพยาธิวิทยา. (ACUTE MERCURY TOXICITY IN TILAPIA (*Oreochromis niloticus*), THE PATHOLOGICAL ASPECT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. น.สพ.ดร. อนุเทพ รังสีพิพัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.สพ.ญ.ดร. อรัญญา พลพรพิสิฐ, 78 หน้า.

ปรอทเป็นโลหะหนักที่มีความเป็นพิษสูงชนิดหนึ่งซึ่งสามารถปนเปื้อนได้ในแหล่งน้ำตามธรรมชาติ มนุษย์สามารถได้รับปรอทจากการบริโภคปลา ดังนั้นจึงสามารถใช้ปลาเป็นตัวบ่งชี้การปนเปื้อนของปรอทในสิ่งแวดล้อมได้ วัตถุประสงค์ของการศึกษาคือ เพื่อหาความเป็นพิษแบบเฉียบพลันของโลหะหนักปรอทในปลานิล (*Oreochromis niloticus*) โดยการศึกษาทางจุลพยาธิวิทยา การย้อมพิเศษด้วยวิธีออโตเมทัลโลกราฟฟี การตรวจวิเคราะห์โลหะหนักด้วยวิธี ICP-AES และการตรวจหาโปรตีนเมทัลโลไธโอนีน ด้วยเทคนิคอิมมูโนฮีสโตเคมี และการแสดงออกของจีนเมทัลโลไธโอนีน ทำการทดลองโดยการแบ่งปลานิลออกเป็น 12 กลุ่ม และกลุ่มควบคุม 1 กลุ่ม กลุ่มทดลองประกอบด้วยกลุ่มที่ได้รับสารเมอร์คิวริคคลอไรด์ ($HgCl_2$) ความเข้มข้น 0.5, 1, 2 และ 5 ไมโครกรัมต่อกรัม โดยการฉีดเข้าช่องท้อง กลุ่มที่ได้รับ $HgCl_2$ ความเข้มข้น 0.5, 1, 2 และ 5 ไมโครกรัมต่อกรัมโดยการป้อน และกลุ่มที่ได้รับโดยการสัมผัสน้ำที่มีความเข้มข้นของ $HgCl_2$ 0.5, 1, 2 และ 5 ไมโครกรัมต่อมิลลิลิตรตามลำดับ พบว่าปลาทุกตัวในกลุ่มที่ได้รับการสัมผัสน้ำที่มีความเข้มข้น 2 และ 5 ไมโครกรัมต่อมิลลิลิตรเสียชีวิตทั้งหมดภายในวันแรกของการทดลอง รอยโรคหลักทางจุลพยาธิวิทยาประกอบด้วย การเสื่อมของเซลล์เยื่อบุท่อไต การเพิ่มขึ้นของหน่วยไตใหม่ และการเกิดผลึกในท่อไต การเพิ่มจำนวนของเมลาโนแมคโครฟาจ (MMCs) ในม้าม การลดลงของไขมันที่สะสมในเซลล์ตับ การเสื่อมของเซลล์ตับ ร่วมกับการฝ่อของตับอ่อนในตับ จากการตรวจสอบด้วยวิธีออโตเมทัลโลกราฟฟี พบว่ามีปรอทสะสมอยู่ในเซลล์เยื่อบุท่อไต MMCs ในม้าม และที่ตับอ่อน ซึ่งสอดคล้องกับปริมาณปรอทที่วัดได้ โดยปริมาณและตำแหน่งที่พบการสะสมของปรอทแตกต่างกันตามวิธีและขนาดของ $HgCl_2$ ที่ได้รับ ในกลุ่มที่เลี้ยงปลาในน้ำที่มีปรอท และได้รับขนาดสูง จะพบการสะสมของปรอทในปริมาณมากกว่าการให้ด้วยวิธีอื่น และขนาดที่ได้รับต่ำกว่า การตรวจหาโปรตีนเมทัลโลไธโอนีนด้วยวิธีอิมมูโนฮีสโตเคมีพบผลบวกเฉพาะในเซลล์เยื่อบุท่อไต MMCs ในม้าม และตับอ่อนของปลาในกลุ่มที่เลี้ยงไว้ในน้ำที่มีความเข้มข้นของ $HgCl_2$ เท่ากับ 0.5 และ 1 ไมโครกรัมต่อมิลลิลิตร หลังจากวันที่ 9 และ 6 ของการทดลองตามลำดับ จากการศึกษาในครั้งนี้สรุปได้ว่า สามารถใช้รอยโรคทางจุลพยาธิวิทยา การย้อมพิเศษด้วยวิธีออโตเมทัลโลกราฟฟี และวิธีอิมมูโนฮีสโตเคมีเพื่อศึกษาความเป็นพิษแบบเฉียบพลันของโลหะหนักปรอทในปลานิลได้

ภาควิชา.....พยาธิวิทยา..... ลายมือชื่อนิสิต.....
สาขาวิชา...พยาธิชีววิทยาทางสัตวแพทย์..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
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KEYWORDS : ACUTE MERCURY TOXICITY / AUTOMETALLOGRAPHY / HISTOPATHOLOGICAL LESIONS / METALLOTHIONEIN / TILAPIA

KASEM RATTANAPINYOPITUK : ACUTE MERCURY TOXICITY IN TILAPIA (*Oreochromis niloticus*), THE PATHOLOGICAL ASPECT. THESIS ADVISOR : ASSOC. PROF. ANUDEP RUNGSIPIPAT, Ph.D., THESIS CO-ADVISOR : ASSOC.PROF. ARANYA PONPORNPISIT, Ph.D., 78 pp.

Mercury is one of most toxic heavy metals which can contaminate natural water sources. Humans can be exposed to mercury by consumption of mercury contaminated fish. Therefore, it is most appropriate model to use fish as environmental marker of mercury contamination. The purpose of this study is to investigate acute mercury toxicity in tilapia (*Oreochromis niloticus*) via histopathological, autometallography, Hg analysis by ICP-AES method and detecting metallothionein protein by immunohistochemistry techniques and metallothionein gene expression. Tilapias were divided into twelve experimental groups and one control group. The experimental groups included intraperitoneally injected with 0.5, 1, 2, 5 µg/g mercuric chloride ($HgCl_2$), oral administration with 0.5, 1, 2, 5 µg/g $HgCl_2$ , and 0.5, 1, 2, 5 µg/ml $HgCl_2$ semi-static exposure groups. All fish showed the clinical appearance of respiratory failure followed by death in 2 and 5 µg/ml $HgCl_2$ semi-exposure groups on first day (day 0) of experiment. The major histopathological lesions includes tubulonephrosis, increasing immature nephrons, and deposit of crystal in trunk kidneys, increasing melanomacrophages centers (MMCs) in spleen, and losing of fat storage, degeneration of hepatocytes, and pancreatic atrophy in hepatopancreas. Following autometallography, silver-enhanced Hg grains were visualized in renal tubular epithelium, MMCs in spleen and in pancreatic acini in accordance with high mercury levels. The amount and location of visualized grains differ from routes and doses of $HgCl_2$. The semi-exposure groups and higer concentration produced more grains than other routes and lower concentration. An expression of metallothionein (MT) protein revealed in renal tubular epithelium, MMCs in spleen, and pancreatic acini of 0.5, and 1 µg/ml semi-static exposure groups after day 9 and 6 respectively. From this present study, it concludes that pathological lesions, autometallography, expression of metallothionein protein by immunohistochemistry can be used to investigate acute mercury toxicity in tilapia.

Department : Veterinary Pathology ..... Student's Signature Kasem Rattanapinyopituk

Field of Study : Veterinary Pathobiology ... Advisor's Signature .....

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AAS | atomic absorption spectrometry |
| AFS | atomic fluorescence spectrometry |
| Ag | silver |
| $AgNO_3$ | silver nitrate |
| AMG | autometallography |
| ASV | anodic stripping voltametry |
| ATSDR | Agency for Toxic Substances and Diseases Registry |
| Bi | bismuth |
| bp | base pair (s) |
| BSA | bovine serum protein |
| β | beta |
| °C | degree Celsius (centrigrade) |
| Cd | cadmium |
| cDNA | complimentary deoxyribonucleic acid |
| cm | centrimeter |
| CV | cold vapor |
| GC | gas chromatography |
| Cu | copper |
| DO | dissolved oxygen |
| DAB | 3, 3-diaminobenzidine |
| DNA | deoxyribonucleic acid |
| $EC_{50}$ | median effective concentration |
| g | gram (s) |
| G | gravity |
| H&E | hematoxylin and eosin staining |
| $H_2O_2$ | hydrogen peroxide |
| IHC | immunohistochemistry |

| | |
|---|---|
| l | liter (s) |
| ICP-AES | Inductive coupled plasma atomic emission |
| KCN | potassium cyanide |
| $LC_{50}$ | median lethal concentration |
| Hg | mercury |
| $HgCl_2$ | mercuric chloride |
| $HgS/Se^{AMG}$ | mercury sulfide/selenide autometallography |
| ml | milliliter (s) |
| mg | milligram (s) |
| mRNA | messenger ribonucleic acid |
| MS | mass spectrometry |
| MT | metallothionein |
| µl | microliter (s) |
| µg | microgram (s) |
| µm | micrometer (s) |
| NAA | neutron activation analysis |
| Ni | nickel |
| NJDEP | New Jersey Department of Environmental Protection |
| Pb | lead |
| PBS | phosphate buffer saline |
| pH | power of hydrogen ion |
| ppm | part per million |
| % | percent |
| RFD | reference dose |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| rpm | revolution per minute |
| STREC | Scientific and Technological Research Equipment Center |
| TBE | tris borate EDTA |
| US EPA | United States Environmental Protection Agency |

| | |
|---|---|
| UV | ultraviolet |
| VMARC | Veterinary Medical Aquatic Animal Research Center |
| WHO | World Health Organization |
| Zn | Zinc |

# CHAPTER I

## INTRODUCTION

Mercury (Hg) is a heavy metal that can be contaminated in a natural water sources. It causes numerous health problems in human such as abnormalities of nervous system, kidney and immunity damages. Moreover, it is a carcinogen and mutagen (ATSDR, 1999; WHO, 2003; Zahir et al., 2005). Human is able to receive mercury from many ways, e.g. vapor inhalation, accidentally direct ingestion and especially consuming mercury contaminated fish (Oliveira Ribeiro et al., 1996; Oliveira Ribeiro et al., 1999; Limke et al., 2004; Drevnick et al., 2006). Given that human is on the top of the food chains, therefore human might be get a high level of Hg from bioaccumulation process (Raldúa et al., 2007).

Up to date, the standard method to determine heavy metal contamination is measuring level in the natural water sources. This method can identify types and measure heavy metal levels in the water but it cannot measure the level in fish that people might consume. Using natural-source-water fish as environmental biomarker is very useful worldwide and may help to assess the risk of Hg consumption of human (Oliveira Ribeiro, et al. 2002; Jewett and Duffey, 2007; Raldúa et al., 2007).

Tilapia fish is a widely distributed fresh water fish that is important in world fisheries (Eroglu et al., 2005). There are about 100 species and subspecies, such as *Orechromis niloticus*, *O. mossambicus*, and *O. aureus*, and hybrids between them. Tilapias are omnivous capable of rapid growth, being sexually mature at 6-7 months from hatch and marketable at this age (Maclean et al., 2002). Tilapia (*Oreochromis niloticus*) is one of most favorite fish in Thai cuisine. Tilapia cultures made the highest yields and total values of production more than all freshwater fish (Fishery Statistic

Analysis and Research Group, 2005). Types of tilapia cultures include pounds, ditches, paddy cums, and cage cultures. The tilapia cage cultures almost involve natural water sources therefore tilapia can directly expose toxic substances and chemicals, including Hg, that contaminate in water. Moreover, the long period of culture time (more than 6 months) increases a chance of Hg exposure in tilapia but might not cause their abnormalilties. Tilapia is the fish species that tolerant to water pollutant therefore it is popular to use in the environmental toxicological studies (Chan, 1995; Lam et al., 1998; Atli and Canli, 2003; Cheung et al., 2004; Cheung et al., 2005; Eroglu et al., 2005). Although there is numerous information of Hg toxicology in many fish species but not in tilapia that still has a few researches. Therefore, the studying of Hg toxicity in tilapia by using histopathology technique together with high specificity and sensitivity determination methods will advantage to use as biomarker and apply to appropriately diagnose heavy metal contamination in the future.

The objective of the present study is to investigate acute mercury toxicity in tilapia (*O. niloticus*) by histopathological changing, autometallography and inductive coupled plasma atomic emission spectrometry (ICPS-AES) for mercury tracing in fish tissue, and detecting of metallothionein protein by immunohistochemistry method and metallothionein gene expression.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Mercury

Mercury (Hg) is a high toxic heavy metal that can be categorized into three different categories, its compounds include elementary Hg ($Hg^0$), inorganic Hg or Hg salts, and organic Hg (WHO, 2003). In general, elementary and inorganic forms are the common forms found in nature. Elementary Hg may occur in both liquid and gaseous states. Elementary Hg is the most volatile form of mercury. It can evaporate into Hg vapor and release into the air. Hg vapor can change to other forms by passing through rain and snow which can enter to soil and water sources. Inorganic Hg occurs as salts of its divalent ($Hg^{2+}$) and monovalent cationic ($Hg^{+}$) forms. Inorganic Hg compounds, such as phenyl mercury, mercuric chloride ($HgCl_2$), mercurous chloride ($Hg_2O_2$), mercuric sulfide (HgS), have lower toxicity than organic compounds, such as methyl mercury ($CH_3Hg^{+}$), that are very volatile and able to be absorbed into the body through all portals including skin. Biotransformation of inorganic Hg to methyl mercury by aqueous microorganisms is very important as methyl mercury bioaccumulates. Inorganic Hg can change into organic form by methylation which depended on *Escherichia* sp., *Enterobacter* sp., *Streptococcus* sp., and *Bacillus* sp. Bacteria and can be absorbed by phytoplankton, or be ingested by zooplankton, other microorganisms, or fish (WHO, 2003).

## 2.2 Mercury sources

Mercury is released into environment from both natural and human sources. Natural sources, primarily in the form of elementary Hg. include volcanic activity, degassing from rocks and soil and volatilization from the ocean. Hg pollution in water sources has received a great amount of concerning since the first discovery of Hg as the cause of Minamata disease in Japan in the 1950s (Raldúa et al., 2007). Nowaday, it is found that mercury cause many diseases; Minamata disease, acrodynia (pink disease) and Mad Hatter's disease (Bernard et al., 2001). Hg contaminates to the environment is almost from human activities including industrial wastes, agricultural residues such as fertilizers, pesticides, fungicides, medical residues, such as disinfectants (Pandey et al., 2005), and wastes from communities (Table 1). The important routes of mercury intoxication are inhalation Hg vapor and ingestion of the contaminated food especially fish (Oliveira Ribeiro et al. 1996; Oliveira Ribeiro et al., 1999; Limke et al., 2004; Drevnick et al., 2006) and seafood (ATSDR, 1999). Fish and aquatic mammals used as food sources are important sources of Hg in some population (WHO, 2003). Organic Hg can deposit in fish tissue for a very long time given that Hg will catch carbon atoms in fish proteins. World health organization (WHO) determined the high level of methyl Hg daily consuming is 0.2 mg and total Hg daily consuming is 0.3 mg during 1 week (Woshiner et al., 2002).

## 2.3 Mercury toxicity in human and mammals

Acute poisoning is the major threat, but Hg is a cumulative heavy metal, subacute and chronic intoxications are also seen. Human received Hg from inhalation and ingestion (Hansen and Danscher, 1995). Accidental exposures to volatile Hg

compounds usually occur when people breathe them into the body. Hg absorption through skin also leads to a systemic poisoning. After mercury exposure, Hg circulates via blood and lymphatic system through the body. In the body, Hg combines with sulfhydril groups in the cells and depresses the enzymatic system of the cells. Hg will deposit in renal tubular epithelia and nerve cells in the brain (Zahir et al., 2005). Repeated exposure at high levels can harm the central nervous system and cause mood swings, shaky hand, difficult to walk, slurred speed, hallucinations, and loss of memory and concentration. Hg can inhibit enzymatic process in glucose metabolism which damages the nervous system, brain, kidney, and other tissues. Moreover, it is carcinogen, mutagen, and can deposit and pass through the next generation (Shimada et al., 2004). Hansen et al. (1989) had reported that they found Hg in all organs of the dogs that were fed with food contaminated with methyl mercury. The highest deposit was found in the kidneys meanwhile the gastrointestinal organs and skeletal muscle was less found. In the nervous system, it can find unusual distribution of Hg, aggregation in all area of cerebral hemispheres, brain cord, spinal cord, and nerve cells including astrocytes, microglia, and endothelial cells. Moreover, Hansen and Danscher (1995) found that mesenteric lymph nodes, liver and kidneys were the most deposit organs respectively. It can conclude that lymphatic system is the main route to distribute Hg through target organs. Parenterally administrated mercuric chloride accumulates primarily in kidneys, liver, spleen, blood, thymus, lymph nodes, bone marrow, lungs and brain of mice and rats (Loumbourdis and Danscher, 2004). WHO suggests the daily level of Hg intake could be 0.49 μg per kilogram per day meanwhile US EPA suggests a reference dose (RFD), that is the amount that could be safety consumed every day over a person's lifetime, could be 0.1 μg per day.

**Table 1** Sources of mercury (Water and Hazardous Substance Management Division. Pollution Control Department, 2002).

| Source | Example |
|---|---|
| Scientific | Thermometer, barometer, catalysts, etc. |
| Medical | Pharmaceutical, dental amalgam, antiseptics, disinfectants, etc. |
| Agricultural | Pesticides, fungicides, etc. |
| Industrial | Electrical and electronic devices, textile, paint, chlorine/caustic soda production, battery, paper manufacturing, etc. |
| Others | Mining, bomb production, waste water, etc. |

### 2.4 Mercury toxicity in fish

Acute mercury toxicity symptoms composed of respiratory abnormality; increasing respiratory movement, flaring of gill covers, loss of equilibrium, sluggishness and followed by death. 24-hour lethal concentration 50 ($LC_{50}$) of inorganic mercury ($Hg^{2+}$) in tilapia is approximately 1 part per million (ppm) (NJDEP, 2002). Table 2 also showed toxic values of mercury in fish species. If fish has been a chronic or sublethal exposure, it will impact reproduction, growth, behavior, metabolism, blood chemistry, osmoregulation and oxygen exchange in marine and freshwater fish (NJDEP, 2002).

### 2.5 Histopathology of mercury toxicity in fish and some marine mammals

Histopathology, detecting early effects in cells, tissues and organs, is now largely used in fish to evaluate the toxic effects of exposure to pollutants (Oliveira Ribeiro et al., 2002; Oliveira Ribeiro et al., 2006; Mela et al., 2007). The histopathology lesions vary from types of fish in previous experiments.

**Table 2** Toxic values of mercury for fish species (NJDEP, 2002).

| Organism | $Hg^{2+}$ (µg/l) | | Methyl mercury (µg/l) | |
|---|---|---|---|---|
| | Acute ($LC_{50}$) | Chronic ($EC_{50}$) | Acute ($LC_{50}$) | Chronic ($EC_{50}$) |
| Fresh water fish | 0.03 (guppy) to 1 (tilapia) | Not available | <0.23 (minnow) to <0.26 (minnow) | 0.29 (brook trout) to 0.93 (brook trout) |
| Rainbow trout | 0.155 to 0.420 | 24 to 84 | Not available | Not available |
| Salt water fish | 0.036 (juvenile spot) to 1.678 (flounder) | 51.1 (mummichog) | Not available | Not available |

**2.5.1 Liver/ hepatopancreas**

Refers to the reports of Raldúa et al. (2007) which found that barbels (*Barbus graellsii*) showed disruption of the normal morphology and degeneration and necrosis of hepatocytes. Ceroid-lipofuscin pigments increased in liver. The pathological findings in the liver of arctic charr (*Salvelinus alpinus*) included severe necrosis and cytoplasm abnormality of hepatocytes. Lipid deposition within hepatocyte cytoplasm was remarkable reduced, heterochromatin in nuclei increased and cytoplasmic membrane became barely visible. Massive necrosis and increasing of connective tissue with infiltration of phagocytes around those lesions were also observed (Oliveira Ribeiro et al., 2002). The study in bleak (*Alburnus alburnus*) presented multifocal inflammation, such as granuloma, zonal macrovesicular steatosis with deposition of lipids in the cytoplasm and degeneration of hepatocytes (Raldúa et al., 2007). Channel catfish (*Ictalurus punctatus*) also have many histopathological changes. The hepatic parenchyma was observed a suppurative perihepatitis. The exocrine pancreatic tissue became shrunken and pancreatic cells appeared disorganized and degenerated. The

desquamation of bile duct epithelium also was found (Kendall, 1977). Liver from inorganic Hg-exposure beluga whales (*Delphinapterus leucas*) showed moderate to severe diffuse hepatocellular atrophy, multifocal talengiectasia and focal or multifocal fibrosis with bile duct proliferation. Mild periportal hepatitis was also found (Woshner et al., 2002).

### 2.5.2 Kidney

The previous study described that the hematopoietic tissue of arctic charr (*Salvelinus alpinus*) increased red blood cells, macrophages and pyknotic nuclei after exposed to inorganic mercury (Oliveira Ribeiro et al., 2002). Wolf fish (*Hoplias malabaricus*) was observed the presence of necrotic areas and atypical cells in the head of kidney. Melanomacrophage centers (MMCs) also increased the prevalence in the head of kidney (Mela et al., 2007). Histopathological findings in bowhead whales (*Balaena mysticetus*) which expose to inorganic Hg examined a generalized mild to moderate non-inflammatory periglomerular and interstitial renal fibrosis and renal lithiasis. The beluga whales were also found mild to moderate Bowman's capsular fibrosis accompanied by mild increase in mesangial matrix and renal lithiasis (Woshner et al., 2002).

### 2.5.3 Gill

The presence of exfoliative epithelium with detachment, vacuolation of epithelium cells and edema were seen in arctic charr (*Salvelinus alpinus*). Mercury exposure can induce an expansion of the vessel lumen and aneurysms (Oliveira Ribeiro et al., 2002). The morphological alteration occurred on the secondary lamella of catfish *(Trichomycterus zonatus).* The squamous epithelium and extensive epithelial

hyperplasia resulting in the formation of an intralamellar bridge was also found (Oliveira Ribeiro et al., 2000).

## 2.6 Monitoring of mercury contamination

### 2.6.1 Mercury levels in fish tissues

Hg and other heavy metal concentrations in fish have been determined in tissues (Dang et al., 1999; Woshner et al., 2002; Quirós et al., 2007; Wu et al., 2008). The analytical methods use atomic absorption spectrometry (AAS), atomic fluorescence spectrometry (AFS) or neutron activation analysis (NAA), Mass spectrometry (MS), spectrophotometry, anodic stripping voltametry (ASV). The most commonly used method is cold vapor (CV) AAS. Excellent accuracy methods have also been demonstrated with gas chromatography (GC), microwave-induced plasma atomic emission detection. Inductive coupled plasma-atomic emission spectroscopy (ICP-AES) and ICP-MS can also used to accurately determine total Hg, but less precision (ASTDR, 1999; WHO, 2003; Han et al., 2006).

### 2.6.2 Autometallography

Autometallographic (AMG) is a potent histochemical technique that aids to visualize variety of metal containing nanocrystals, such as gold (Au) and silver (Ag) nanoclusters and quantum dot of bismuth (Bi), zinc (Zn), mercury (Hg), cadmium (Cd), copper (Cu) and etc (Danscher, 1984; Danscher and Nørgaard, 1985; Danscher et al., 2000; Woshner et al., 2002; Loumbourdis and Danscher, 2004; Alvarado et al., 2006; Danscher and Stoltenberg, 2006). It has been used to determine which cell-types are involved in metal accumulation in fish and many species (Alvarado et al., 2006). Small nanocrystals can be enlarged by silver enhancement to dismensions that can be seen

under light and electron microscopes (Danscher and Stoltenberg, 2006). Basic principles are adherence of silver ions ($Ag^{+}$) to the surface of the catalytic crystal and become a part of the crystals and then reduced to silver atoms ($Ag^{0}$) by electrons released from reducing molecules (Danscher, 1984; Danscher et al., 2000; Woshner et al., 2002; Loumbourdis and Danscher, 2004; Danscher and Stoltenberg, 2006).

Hg autometallography can be used to trace mercury accumulation bound by metabolic processes as mercury sulphide/selenide clusters in tissues ($HgS/Se^{AMG}$) (Loumbourdis and Danscher, 2004). Shirabe (1978) ever used this technique to identify Hg contamination that was found in lysosomes of Minamata disease victims' celebellum. Additionally, the release of Hg from amalgam can be detected by autometallography which was found dissemination of Hg to the spinal ganglia, pancreas, lungs and pituitary gland (Danscher and Stoltenberg, 2006). Hg can also be demonstrated in lysosomes of the proximal renal tubules of rat after intraperitoneally administration of $HgCl_2$ (Nørgaard et al., 1994). A distribution of mercury in the tissue is depend on the sources, types; organic or inorganic and routes of administration (Loumbourdis and Danscher, 2004). In marine mammals, the bowhead and beluga whales were found Hg deposits in the kidney and liver among high Hg tissue concentrations especially in the renal epithelium of medullary tubules and collecting duct of their kidneys and in the hepatocellular cytoplasm and the Kupffer cells near portal triads of their liver (Woshner et al., 2002). Although there are a lot of researches study the distribution of mercury in mammals but fewer in fish and aquatic species (Alvarado et al., 2006). The previous research studying in Juvenile rainbow trout (*Sabno gairdneri*) found Hg deposit within lysosomes and extracellularly in the basal lamina of proximal tubules of kidney and within lysosomes of the hepatocytes after exposed to $HgCl_2$ for 14 days (Baatrup et al., 1986). The other studies in the mussel (*Mytilus galloprovincialis*) found that black silver deposits of Hg were localized mainly in the residual bodies and heterolysosomes of

epithelial cells of digestive tracts, and revealed in the laterofrontal and abfrontal epithelium of the gill filament after 30 and 60 day of Hg exposure (Dimitriadis et al., 2003). An additional experiment described Hg deposition as silver grains visualization in red blood cells and hepatic sinusoids, bile canaliculi and apical part of frog (*Rana ridibunda*) hepatocytes after 1-day exposure and found in endothelial and Kupffer cells of hepatocytes after 6-day exposure (Loumbourdis and Danscher, 2004).

### 2.6.3 Metallothionein

Metallothionein (MT) is a low molecular weight (6,000-7,000) and cystein-rich protein with highly affinity for divalent cations such as $Ag^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Zn^{2+}$ and $Hg^{2+}$ (Chan, 1994; Chan, 1995; Burkhardt-Holm et al., 1999; Lau et al., 2001; Atil and Canli, 2003; Eroglu and Canli, 2005; Shimada et al., 2005; Alvarado et al., 2006; Wu et al., 2008; Gao et al., 2009). MT is found to have extensive homology among amino acid sequence from diverse species from fungi, invertebrates, such as crab, and mussle, vertebrates such as fish (Chan, 1994; Cheung et al., 2004) and mammals including human (Shimada et al., 2004). Although the role of MT still unclear, it believe that MT involved in regulation of essential metals such as Zn and detoxification of non-essential metal ions such as Cd, Pb (Cheung et al., 2004) and Hg (Chan, 1994). There was a research in human that showed MT increasing in gingival amalgam tattoo patient. The researchers suggested that synthesis of MT would be expected to be upregulated initially after exposure to amalgam, important role in metal detoxification in local and systemic tissues (Lau et al., 2001). In fish, pre-exposure to sublethal levels of metal result in the induction of MT (Chan, 1994). Chan (1995) also reported that heavy metals can induce MT gene and protein in many fish species and can reduce potential toxicity of heavy metal residues therefore it can be used as biomarker for heavy metal contamination in polluted waters (Burkhardt-Holm et al., 1999 ; Cheung et al., 2004;

Cheung et al., 2005; Quirós et al., 2007). The methods to detect MT is mainly by measuring MT protein levels (Quirós et al., 2007), such as high performance liquid chromatography (HPLC), examine MT protein in tissues with immunohistochemistry technique (Shimada et al., 2005; Alvarado et al., 2006; Gao et al., 2009) and also investigate MT gene or mRNA expression (Chan, 1994; Chan, 1995; Lam et al., 1998; Cheung et al., 2004). Previous study reported that detecting MT liver, kidney, and gill mRNAs expression in $Cu^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $Ni^{2+}$, $Pb^{2+}$ and $Zn^{2+}$ exposure tilapia by reverse transcriptase polymerase chain reaction (RT-PCR) is high sensitivity and there is significantly increasing of mRNA in treated groups when compares with control group (Cheung et al., 2004). In addition, higher level of heavy metals can affect level of MT than lower levels (Chan, 1995). Alignment determination of amino acid sequences in many species of fish MT showed highly conserved and the 20 cysteine residues remain unchanged (Figure 1) (Chan, 1994). In tilapia, there are several studies of MT mRNA and gene expression. Nucleotide sequences of *O. aureus* and *O. mossambicus* have 1,452 base pairs (bp) and 1,426 bp, respectively (Figure 2) (Cheung et al., 2005).

```
MDPCECSKTGTCNCGGSCTCKNCSCTTCNK SCCPCCPSGCPKCASGCVCKGKTCDTTCCQ    (1)
----------S-------K-S--A--S-K-A---D------S---------------S---    (2)
----------S-------K-S--A--S-K- --------D-S---------------S---    (3)
---------------AT-K-T--Q----K- ----------S----------NS--SS---    (4)
------A--------AT-K-T-------K- ---F------S--------N-NS-GSS---    (5)
------A----------S-TK---KS-S- ---D------S---------------S---    (6)
```

**Figure 1** Amino acid sequences of (1) winter flounder MT (2) rainbow trout MT-A (3) rainbow trout MT-B (4) stone-loach MT (5) goldfish MT and (6) tilapia MT (Chan, 1994).

```
1       GGTTGTTCAA CATCATGATG ACTATATTAT TTTTTATTAT GGAAAAATGA GCAAGACTGA
        GGTTGTTCAA CATCATGATG ACTATATTAT TTTTTATTAT GGAAAAATGA GCAAGACTGA

61      GGAGTCACAC GATATATTAA ATGAATTATT ATTTTAGATT TGGATTTAGG ATACTTAATG
        GGAGTCACAC GATATATTAA ATGAATTATT GTTTTAGATT TGGATTTAGG ATATTTAATG

121     TGGATTTTTA CTCCAGTGAA ATGATCGCAC TATCAAAATA ATACCGGCAT AGAGTGTATT
        TGGATTTTTA CTCCAGTGAA ATGATCGCAC TATCAAAATA ATACCGGCAT AGAGTGTATT

181     AGTTTCAATT AATTAAGTAC GAGGTTAAGT TTGGAATGAA AAGAATGAAA AAATACTGCA
        AGTTTCAATT AATTAAGTAC AAGGTTAAGT TTGGAATGAA AAGAATGAAA AAATACTGCA

241     CGGAAATGTT TTCTGTGAAG AATCTTCGCA GATGCTGAAA AGTTTTTTTG TAATTTTTAA
        CGGAAATGTT TTCTGTGAAG AATCTTCGCA GATGCTGAAA AGTTTTTTTG TAATTTTTAA

301     AATAATACTG TTGGTGATAA TTCGTGCGCC CATCCACCCA ACATCCTCTC ATATTAGCCC
        AATAATACTG TTGGTGATAA TTCGTGCGCC CATCCACCCA ACATCCTCTC ATATTAGCCC

361     CAAATGGTTG TATAAATTCA CTTCAGGCCA TATAAAACTA AGACGTCCTG ATGGGAGACC
        CAAATGGTTG TATAAATTCA CTTCAGGCCA TATAAAACTA AGACGTCCTG ATGGGAGACC

421     CGCACTTCAT GTAGCCCATT CTAAATGAAT CCGTGTGTTG GTGAGCGTTT GTGACCGTGT
        CGCATGTAGC CCATTCTAAA TGAATCCGTG TGTTGGTGAG CGTTTGTGAC CTTGTGTTCA

481     GTTCAGATCT CCGTGTAGCC TGGAGATGTT CTCCTCTGTT TACATACATG AATTAATTGC
        GATCTCCGTG TAGCCTGGAG ATGTTCTCCT CTGTTTACAT ACATGAATTA ATTGCATGGT

541     ATGGTTGAAT TTCTCCAGAA GTTGACGTTC TTATATAATG TTCAGGCAGC AGGCCATAGA
        TGAATTTCTC CAGAAGTTGA CATTCTTATA TAATGTTCAC GCAGCAGGCC ATAGATCTGG

601     TCTGGGTATG AGGATCTCTC TGTTCCCGCT GCTGAAAGGT TGTCGTGTGC ACAACCTCTT
        GTATGAGGAT CTCTCTGTTC CCGCTGCTGA AAGGTTGTCG TGTGCACAAC CTCTTCGTAA

661     CGTAATCATT AACCACGTGC TTGCGTGTAG CGTGACAGGC GTGTATTGCA CTCGGCTCTG
        TCATTAACCA CGTGCTTGCG TGTAGCGTGA CAGGCGTGTT TTGCACTCGG CTCTGTTGTT

721     TTGTTCTCAG GACGCTATAA AAGAGCCACT CCTACACCGT CATTCACAAC ATTCATTCAA
        CTCAGGACGC TATAAAAGAG CCACTCCTAC ACCATCATTC ACAACATTCA TTCAAGTCCC

781     GTCCCGAAGA GACAAGAGCA ACGCCAGCAT CACTCGGAAC AAACGAGCCA TCAACTGCAA
        GAAGAGACAA GAGCAACGCC AGCATCACTC TGAACAAACG AGCCATCAAC TGAAAAATGG

841     AATGGACCCC TGCGAGTGCG CCAAGAGTGA GTGTTCCTGC CGCTGTTTAA CAAGGCTATT
        ACCCCTGCGA GTGCGCCAAG AGTGAGTGTT CCTGCCGCTG TTTAACAAGG CTATTTAATA

901     TAATACGCTG CTTGTTACCA GCGGACCGCA TGAAACTTAA CTGTCTTTTT TATGTTCAGC
        CGCTGCTTGT TACCAGCGGA CCGCATGAAA CTTAACTGTC TTTTTTATGT TCAGCTGGAA

961     TGGAACCTGC AACTGCGGAG GATCCTGCAC GTGCACAAAG TGCTCCTGCA AGAGCTGCAA
        CCTGCAACTG CGGAGGATCC TGCACGTGCA CAAAGTGCTC CTGCAAGAGC TGCAAGAAGA

1021    GAAGAGTAAG TCAAACACCT CACAGCACAG CAAACAATCT ATCGACCTGT TAATGTTACG
        GTAAGTCAAA CACCTCACAG CACAGCAAAC AATCTATCGA CCTGTTAATG TTACGCTCCT

1081    CTCCTGTTTA TCTTCCCAAG TAACATGCAT AGTGTGACGA TGGTCTCTTA TAGTCATGTC
        GTTTATCTTC CCCAGTAACA TGCATAGTGT GACGATGGTC TCTTATAGTC ATGCCCCATT

1141    CCATTTCTTC AAAATTGCAC ATTTGATGCC TTTTTTTTTT TAATGCTGCG CAGGTGGCTC
        TCTTCAAAAT TGCGCATTTG ATGCCTTTTT TAATGCTGCG CAGGTGGCTC TAATTGCTTT

1201    TAATTGCTTT AATGACTTAA GTTCTTGTTT TCTTGTGTTC TGTCACTAAT GATGTCTGTC
        AATGACTTAA GTTCTTGTTT TCTTGTGTTC TGTCACTGCA GGCTGCTGCG ACTGCTGCCC

1261    ACTGCAGGCT GCTGCGACTG CTGCCCATCC GGCTGCAGCA AATGCGCCTC TGGCTGCGTG
        ATCCGGCTGC AGCAAATGCG CCTCTGGCTG CGTGTGCAAA GGAAAGACAT GCGACACCAG

1321    TGCAAAGGAA AGACATGCGA CACCAGCTGC TGCCAGTGAG GAGTCTGCAG CATCAGCTCT
        CTGCTGCCAG TGAGGAGTCT GCAGCATCAG CTCTCTGCTG CAATTATGGA GTCATTTATT

1381    CTGCTGCAAT TATGGAGTCA TTTATTTGCC ACTAATCATG AATTTGCACA TGTCCAGAAA
        TGCCACTAAT CATAAATTTG CACATGTCCA GAAATGATAA CGAATG

1441    TGATAACGAA TG
```

**Figure 2** Nucleotide sequences of *O.aureus* (upper line) and *O.mossambicus* (lower line) MT gene (Cheung et al., 2005).

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Fish

Tilapias (*Oreochromis niloticus*) (9-12 cm; 25-50 g) were obtained from Aquaculture Research Laboratory, Faculty of Veterinary Science, Chulalongkorn University, Nakhon Pathom Campus. The fish were maintained and the experiment was conducted at Veterinary Medical Aquatic Animal Research Center, Faculty of Veterinary Science, Chulalongkorn University Bangkok. All fish were fed twice daily with commercially pellet food throughout the experimental period. Fifty percents of the water in the aquaria was changed and the sediment was suction out every two days.

## 3.2 Aquaria preparation

The 62.5-liter glass aquaria filled with 50 liter aerated carbon filter water was prepared. Water temperature, pH and dissolved oxygen (DO) were measured before the experiment was conducted.

## 3.3 Mercury exposure

Tilapias were divided into thirteen experimental groups, in each group, twenty five tilapias were placed in 62.5-liter glass aquaria filled with 50 liter aerated carbon filter water. Four of the groups were intraperitoneally injected with 0.5 ml of 0.5, 1, 2, and 5 µg/g (ppm) mercuric chloride ($HgCl_2$, Sigma-Aldrich, USA), respectively. Other four groups were orally administration with 0.5 ml of 0.5, 1, 2, and 5 µg/g (ppm) and the fish

in the rest of the aquaria were semi-exposed with 0.5, 1, 2, and 5 μg/ml (ppm) $HgCl_2$ respectively. One aquarium was kept as a negative control groups.

### 3.4 Tissue sampling

The samples were collected after hypothermic euthanasia every three days for fifteen days. The specimens are included the kidney, liver, spleen, gill, intestine, brain, and muscle tissues. The fish then were dissected and examined all lesions. Fish organs included the gill, kidney, hepatopancreas, spleen, gastrointestinal organ, brain and skeletal muscle were collected. All samples were divided into 3 parts, one were fixed in 10% buffered formalin and subjected to histopathological, autometallography and immunohistochemistry examination and one was freeze in -80°C for measuring total Hg level using ICP-AES method and another one for detecting metallothionein gene expression by reverse transcriptase polymerase chain reaction (RT-PCR).

### 3.5 Histological examination, criteria and scores

Samples were fixed in 10% buffered formalin and routine histologic processed, embedded in paraffin wax, sectioned at 4 μm thickness and stained by hematoxylin and eosin (HE). The sections were observed under light microscope for noticing histopathological change of all organs and scored by lesion scores.

#### 3.5.1 Kidney (Trunk)

Kidney lesion was evaluated by an appearance of hydropic degeneration of tubular epithelium with intracytoplasmic hyaline droplets. Level of kidney damage was divided

into three according to the following scores (Oliveria Ribeiro et al., 2002; Peebua et al., 2006).

Score 0: normal kidney

Score +1: mild hydropic degeneration of tubular epithelium

Score +2: moderate hydropic degeneration of tubular epithelium with hyaline droplets

Score +3: severe hydropic degeneration of tubular epithelium with diffuse hyaline droplets

### 3.5.2 Liver (Hepatopancreas)

Hepatopancreatic lesion was evaluated by an appearance of fat storage and pancreatic acinar atrophy (Oliveria Ribeiro et al., 2002). Lesion score was given as the following.

Fat storage

Score 0: No fat storage in hepatocytes

Score +1: fat storage in hepatocytes less than 25%

Score +2: fat storage in hepatocytes about 50-75 %

Score +3: fat storage in hepatocytes more than 75%

Pancreatic acinar atrophy

Score 0: normal pancreatic acini

Score +1: mild pancreatic acinar atrophy

Score +2: moderate pancreatic acinar atrophy

Score +3: severe diffuse pancreatic acinar atrophy

### 3.5.3 Spleen

Splenic lesion was evaluated by an increasing of MMCs in white pulp. (Mela et al., 2007) The score was given as the following.

Score 0: no MMCs

Score +1: mild increase MMCs

Score +2: moderate increase MMCs

Score +3: severe increase MMCs

### 3.5.4 Gill

Gill affected was evaluated by gill inflammation (Oliveira Ribeiro et al., 2002). The score was given as the following.

Score 0: normal gill

Score +1: mild gill inflammation

Score +2: moderate gill inflammation

Score +3: severe gill inflammation

### 3.5.5 Gastrointestinal organs

Gastrointestinal lesion was evaluated by gastroenteritis, increasing of mucous glands or goblet cells. The score was given as the following.

Score 0: normal gastrointestinal tract

Score +1: mild gastroenteritis/ mild increasing of mucous glands

Score +2: moderate gastroenteritis/ moderate increasing of mucous glands

Score +3: severe gastroenteritis/ severe increasing of mucous glands

### 3.5.6 Brain

Brain lesion was evaluated by encephalitis, degeneration and/or necrosis of brain tissue. The score was given as the following.

Score 0: normal brain

Score +1: mild encephalitis/ degeneration/ necrosis

Score +2: moderate encephalitis/ degeneration/ necrosis

Score +3: severe encephalitis/ degeneration/ necrosis

### 3.5.7 Muscle

Muscle lesion was evaluated by myositis, muscle degeneration and/or necrosis. The score was given as the following.

Score 0: normal muscle

Score +1: mild myositis/ muscle degeneration

Score +2: moderate myositis/ muscle degeneration

Score +3: severe myositis/ muscle degeneration

## 3.6 Autometallography

Autometallography was performed for detecting intracellular mercury deposition. Sectioned slides were deparaffinized in xylene for 30 minutes. Other metal residue elimination was performed by placing the slides in 1% KCN for 2 hours, then washed in tap water for 1 hour and distilled water for 5 minutes. Silver amplification was performed by placing the slides in physical developer (50% Arabic gum, 50% citrate buffer, 5.6% hydroquinone, and 17% $AgNO_3$) for 1 hour in automatic shaker at 26°C. Then, the sections were washed in distilled water 5 minutes at 60°C for 3 times and washed in tap water for 1 hour, then the sections were applied to silver residue elimination method with 10% sodium thiosulfate and Farmer's solution. (20% sodium thiosulfate and 7.5% potassium ferric cyanide) Then, washed in tap water for 5 minutes and counterstained with Mayer's hematoxylin. The sections were observed under light microscope to identify cell types and locations of silver grains depositon. The positive score is depended on distribution and intensity of silver grains in each cell by mild (+1), moderate (+2), and

severe (+3) compared to positive control. Positive control was a lung section of mercury-vapor-inhaled mouse (kindly provide from Prof. Dr. Akinori Shimada).

### 3.7 Immunohistochemistry

Immunohistochemistry staining was performed for detection metallothionein protein by using monoclonal mouse anti-horse metallothionein (MT; E9, Dako®, Denmark) as primary antibody with an EnVision™ detection system (Dako®, Denmark). Briefly, sectioned slides were deparaffinized in xylene for 30 minutes. Antigen retrieval method was done by autoclaved at 121°C for 20 minutes and microwave technique (medium degree, 700 watt) for 5 minutes in citrate buffered pH 6.0, blocked endogenouse peroxidase reaction with 3% $H_2O_2$ in methanol for 30 minutes and slides were washed in PBS 5 minutes for 3 times. Then, sections were blocked with 1% BSA for 30 minutes at 37°C and washed in PBS 5 minutes for 3 times. 1:50 diluted monoclonal mouse anti-horse metallothionein were applied onto section and incubated at 4°C overnight. After washing section slides with PBS 5 minutes for 3 times, biotinylated anti-mouse IgG antibody and EnVision polymer (Dako REAL™ EnVision™ detection system, Dako®, Denmark) was used as secondary antibody and sections were incubated at room temperature for 45 minutes. After washing with PBS 5 minutes for 3 times, sections were stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 3 minutes and counterstained with Mayer's hematoxylin. The sections were observed under light microscope to identify cell types of metallothionein protein in the positive cells. Positive control was a section of canine mammary adenocarcinoma showed an intense cytoplamic expression of metallothionein gene product.

### 3.8 Inductive coupled plasma atomic emission spectrometry (ICP-AES)

The frozen samples were grinded, lyophilized, weighed the sample and recorded. The dry samples were digested with 10 ml of nitric acid: hypochloric acid (10:1) overnight. The digested samples were evaporated at 60°C until remaining 1 ml of solution. After that, the solutions were diluted to 10 ml and subjected to measure Hg levels with inductive coupled plasma atomic emission spectrometer (Perkin Elmer model PLASMA-100) at Scientific and Technological Research Equipment Center Chulalongkorn University (STREC). The mercury was reported in ppm per dry weight (ppm/g).

### 3.9 Metallothionein gene expression by RT-PCR

#### 3.9.1 RNA extraction

50-100 mg of frozen tissues was homogenized with grinder in 1 ml of TRIZOL reagent; RNA extraction buffer (Invitrogen®, USA) then mixed 200 µl of chloroform to the mixture and maintained for 3 minutes at room temperature. The mixture was then centrifuged at 14,000 G for 10 minutes at 4°C. The colorless supernatant was transferred to a sterile eppendrof. RNA was then precipitated by the addition of 500 µl of 2-propanol. The mixture was kept at room temperature for 5 minutes before centrifugation at 12,000 G for 10 minutes at 4°C. The supernatant was discarded and RNA pellets were washed with 75% ethanol followed by centrifugation at 7,000 G for 45 minutes at 4°C. The pellet containing total RNA was air-dried for 3-5 minutes and dissolved in RNAase-free distilled water.

### 3.9.2 Semi-quantitative RT-PCR

Reverse transcription was using total RNA extraction from the hepatopancreas, trunk of kidney, spleen, intestine and muscle. The reaction was performed in the final volume of 0.5 µl at 48°C for 45 minutes using kit condition (AccessQuick™ RT-PCR, Promega, USA). Primers were designed from metallothionein sequence of tilapia (*Oreochromis mossambicus*) reported in GenBank (AY257202) (Cheung et al., 2004). Forward primers and reverse primers were TMTB (5'-GAGGATCCTGCTCGTGCACT-3') and TMTC (5'-CAGCAAATGCGCCTCCGGCTG-3'), respectively. β-actin was used as constitutional expression control. β-actin primers were designed from β-actin sequences of GenBank (AY116536) (Hwang et al., 2003). Forward primer and reverse primer were 5'-GCCCCACCTAGCGTAAATA-3' and 5'-AAAGGTGGACAGGAGGCCA-3', respectively (Table 3). For PCR condition, 3 µl of samples were supplemented with the addition of 0.5 µl of forward primer, 0.5 µl of reverse primer, 8 µl of nuclease-free water and 12.5 µl of dNTP mix (AccessQuick™ Master Mix, Promega, USA).

The PCR reaction for metallothionein gene was performed as follow (modified from Cheung et al., 2004).

| | | | |
|---|---|---|---|
| Initial denaturation step: | | 94°C, 2 minutes | for 1 cycle |
| Denaturing step | : | 94°C, 40 seconds | for 30 cycles |
| Annealing step | : | 58°C, 40 seconds | |
| Extension step | : | 72°C, 40 seconds | |

Then, the samples were held at 72°C for 10 minutes and cooled down to 15°C

The PCR products from each sample were applied to 1.5% agarose gel electrophoresis. Gel was stained with ethidium bromide. DNA bands were visualized and documented under UV light. The positive band size was in 127 kb.

```
1     ATG GAT CCG TGC GAA TGC GCC AAG ACT GGA ACC TGC AAC TGC
      Met Asp Pro Cys Glu Cys Ala Lys Thr Gly Thr Cys Asn Cys

43    GGA GGA TCC TGC TCG TGC ACT AAG TGC TCC TGC AAG AGC TGC
      Gly Gly Ser Cys Ser Cys Thr Lys Cys Ser Cys Lys Ser Cys

85    AAG AAG AGC TGC TGC GAC TGC TGC CCA TCC GGC TGC AGC AAA
      Lys Lys Ser Cys Cys Asp Cys Cys Pro Ser Gly Cys Ser Lys

127   TGC GCC TCC GGC TGC GTG TGC AAA GGA AAG ACA TGC GAC ACC
      Cys Ala Ser Gly Cys Val Cys Lys Gly Lys Thr Cys Asp Thr

169   AGC TGC TGC CAG TGAggagtctgcagcatcagctctctgctgcaattctgga
      Ser Cys Cys Gln End

221   gtctttatttgccactaatcatgaatttgcacatgtccagaaatgataacggatga

277   ttttgtacttgtgtttgaaataaacatgtttgttgacgcta
```

**Figure 3** Nucleotide and amino acids sequences of Tilapia cDNA (*O. mossambic*us) (Chan, 1994).

**Table 3** Primers for RT-PCR.

| Primer name | sequences (5'-3') | Nucleotide position |
|---|---|---|
| TMTB | GAGGATCCTGCTCGTGCACT | 44-63 |
| TMTC | CAGCAAATGCGCCTCCGGCTG | 120-140 |
| tActins | GCCCCACCTGAGCGTAAATA | 1,072-1,091 |
| tActinas | AAAGGTGGACAGGAGGCCA | 1,118-1,136 |

### 3.10 Statistical analysis

Statistical analysis for the histopathology, autometallography, immunohistochemistry and metallothionein gene expression by RT-PCR were performed using descriptive statistic analysis. Total Hg levels in fish tissues from ICP-AES were analyzed using one-way ANOVA and followed by Newman-Keuls multiple comparison test ($p<0.05$).

# CHAPTER IV

# RESULTS

## 4.1 Tilapia weight and length

An average weight of tilapias was 34.41±8.22 g. and length was 11.52±1.17 cm.

## 4.2 Temperature, pH and dissolved oxygen (DO) of water

An average water temperature was 27.14±0.39 $^{o}$C, pH was 7.30±0.11 and DO was 9.56±0.60 mg/l.

## 4.3 Clinical signs and gross lesions

Tilapias of 2 and 5 ppm semi-static exposure groups showed remarkable signs such as swimming to the surface of the water, increasing respiration by moving gill opercula in high frequency, splash swimming and following by death one to two hours after exposure. In intraperitoneal groups, fish decreased moving activity and staying on aquarium bottom after treatments in the first days of experiment. Two fish of 2 and 5 ppm i.p. groups showed swelling of abdomen after day 3 of intraperitoneal injection of $HgCl_2$ (Figure 4).

No remarkable lesion different among groups and days was observed (Figure 6). Fish were injected with 2 and 5 ppm i.p. that had swelling abdomen revealed accumulation of purulent exudates in abdomen and abdominal organs adhesion (Figure 5).

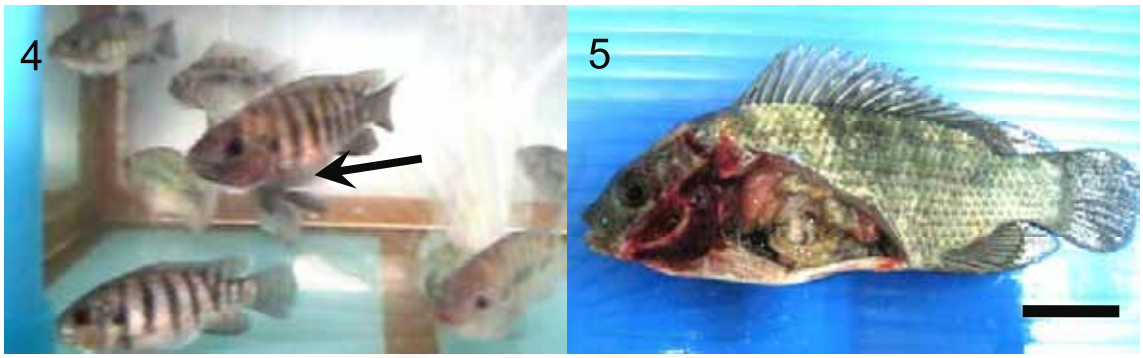


**Figure 4** Swelling of abdomen was observed in 2 ppm $HgCl_2$ intraperitoneally injection group (arrow).

**Figure 5** Postmortem examination of 2 ppm $HgCl_2$ intraperitoneally administration tilapia showed swelling abdomen by accumulation of purulent exudates in abdomen and adhesion of abdominal organs, bar = 2.5 cm.

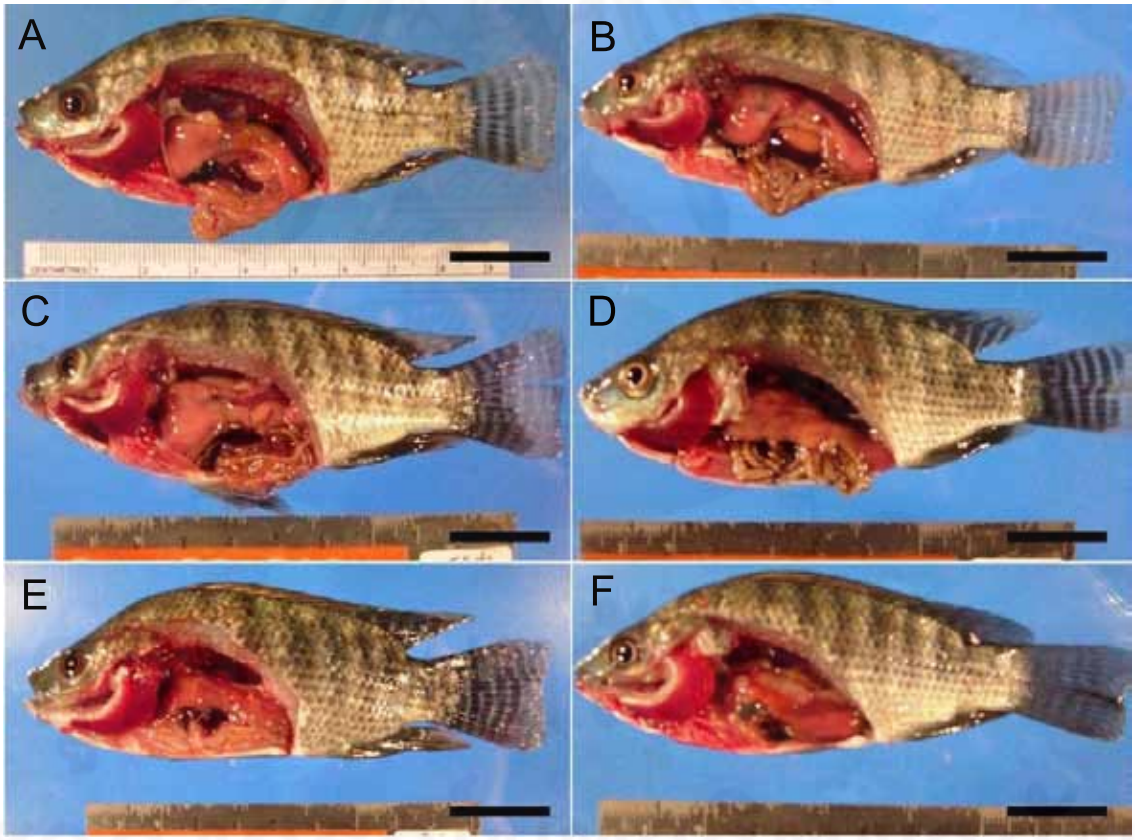


**Figure 6** Postmortem examination of tilapias in all groups revealed no remarkable lesions; A. Control, B. 2 ppm $HgCl_2$ i.p. tilapia, C. 5 ppm $HgCl_2$, D. 0.5 ppm $HgCl_2$ bath, E. 1 ppm $HgCl_2$ bath and F. 5 ppm $HgCl_2$ oral administration tilapias on day 15 of experiment, bar = 2 cm.

## 4.4 Histopathological lesions

### 4.4.1 Kidney (trunk)

Degeneration and necrosis of tubular epithelium were observed in all experimental groups (Figure 8). Severe tubulonephrosis were found in semi-static exposure groups and lesion scores were the highest when compared with control and other experimental groups (score +3). On the first day (day 0), lesion scores of 0.5, 1, 2, and 5 ppm semi-static exposure groups were +2, +3, +3 and +2 respectively. On day 3 and 6 of 0.5 and 1 ppm semi-static exposure groups, the lesion scores decreased to +2 and +2 respectively and were the lowest in day 12 and 15. On day 12 and 15, the lesion scores were +1 and +1 in 0.5 and 1 ppm semi-static exposure groups respectively.

Hyaline droplets deposited in degenerated tubular epithelium were also found in all semi-static exposure groups (Figure 9). In i.p. administration groups, mild to moderate degrees of tubular degeneration were found. Lesion scores of 0.5, 1, 2 and 5 ppm i.p. groups were +1, +1, +1 and +2 respectively. After day 3, the lesion scores were +2, +2, +2 and +3 in 0.5, 1, 2, and 5 ppm i.p. groups respectively. On day 15, the lesion scores were +1, +1, +2 and +2 respectively. The lowest lesion scores were found in oral groups. On day 0 of experiment, the tubular degeneration was mild degree. The lesion scores were +1, +2, +2 and +2 in 0.5, 1, 2 and 5 ppm oral groups respectively. After day 3, the lesion scores decreased to +1, +1, 0 and +1 respectively.

Immature nephrons, regeneration of tubular epithelium, were also found in experimental groups (Figure 10 and 11). The lesion scores are shown in table 4. The highest scores were showed on day 12 of 5 ppm i.p. group (score +3). In all semi-static exposure groups, the lesion scores of immature nephrons were +1 in day 0, 3, 6, 9 and 15.

Pleomorphic crystals also deposited in tubular lumen in experimental groups (Figure 12). Severe tubular crystals (score +3) were found in semi-static exposure groups from day 0 of 1, 2 and 5 ppm doses and moderate pleomorphic tubular crystal were found from day 0 of 0.5 ppm dose (score +2).

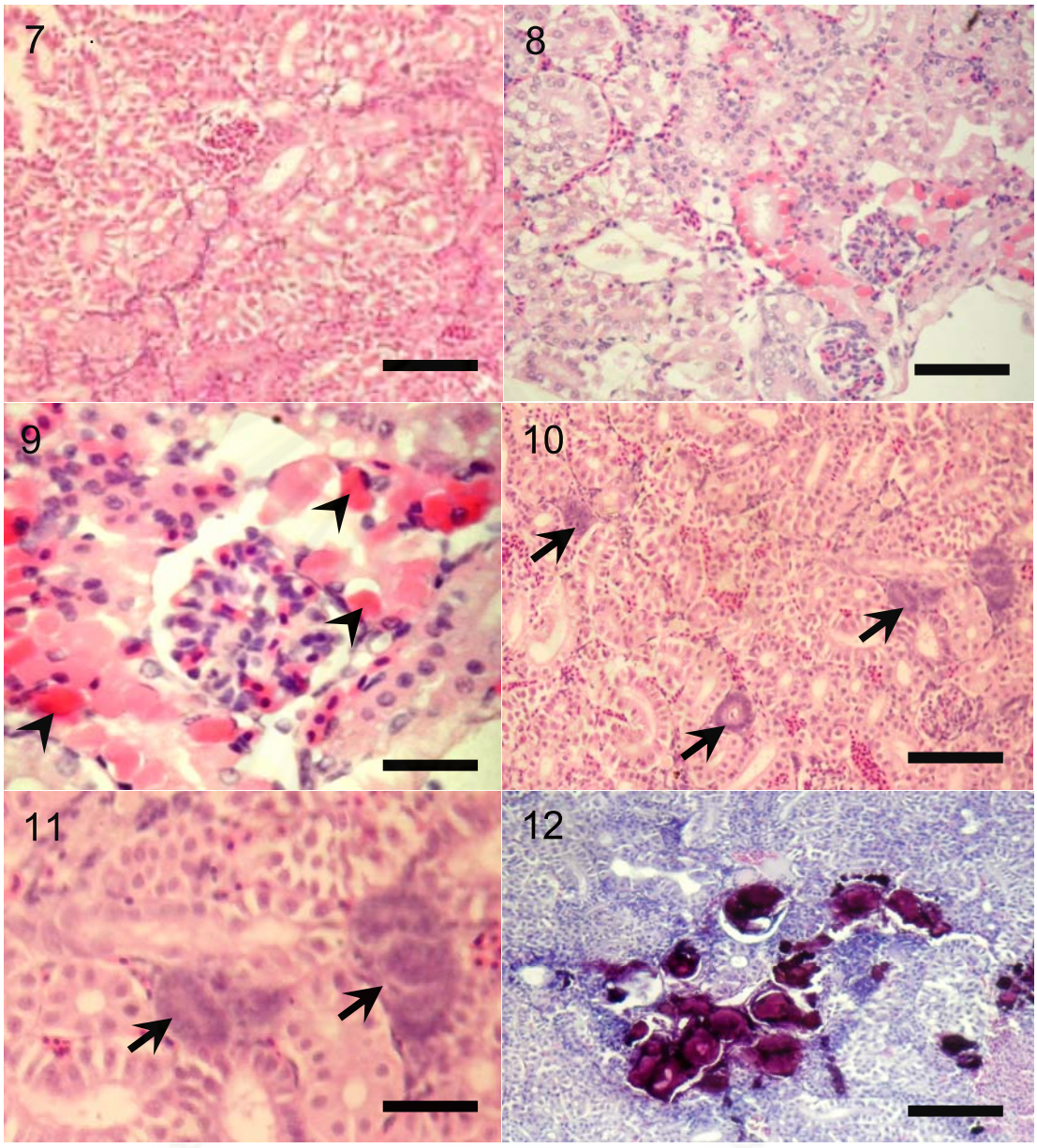


**Figure 7** Trunk kidney of control tilapia on day 15 (H&E staining, bar = 150 μm).

**Figure 8** Severe diffuse tubulonephrosis (score +3) with hyaline granules were revealed in trunk of kidney of 2 ppm semi-static exposure tilapia (H&E staining, bar = 150 μm).

**Figure 9** Hyaline droplets were found in degenerative renal tubular epithelium of 2 ppm semi-static exposure tilapia (arrow heads) (HE staining, bar = 35 μm).

**Figure 10 and 11** Trunk kidney of 2 ppm i.p. tilapia showed increasing of immature nephrons (arrows) (HE staining, bar; figure 10 = 300 μm and figure 11 = 80 μm).

**Figure 12** Pleomorphic crystals appeared in renal tubular lumen of 0.5 ppm $HgCl_2$ i.p. tilapia (HE staining, bar = 400 μm).

#### 4.4.2 Hepatopancreas

The fat storage in hepatocytes scores of control group was all +3 (Figure 13). Fat deposition scores of hepatocytes are shown in table 4. Hepatocytes of experimental groups were enlarged and had large nuclei with prominent nucleoli. In i.p. groups, severe fat losing was found in day 6 of 2 ppm dose and after day 3 of 5 ppm dose (Figure 14). Severe losing of fat storage can be found in semi-static exposure group after day 3 in 0.5 ppm dose. Hyaline droplets were also found on day 12 of 1 ppm semi-static exposure group (Figure 16).

Pancreatic atrophy was found in all experimental groups. Pancreatic acini were decreased in number and appeared as remnant around hepatic blood vessels (Figure 15). Moderate to severe pancreatic atrophy (score +2 and +3) was noted in i.p. and semi-static exposure groups from first day of experiment.

#### 4.4.3 Spleen

There was an increasing the numbers of MMCs in splenic parenchyma in experimental groups compared with control group (Figure 17). Lymphoid depletion also found in some groups.

#### 4.4.4 Gastrointestinal organs

Diffuse goblet cell proliferation and mononuclear cells infiltration in intestinal lamina propria and submucosa layer were noted in oral and semi-static exposure groups (Figure 18). The lesion scores were shown in table 4. There was also found epithelial necrosis of gastric mucosa with mononuclear cells infiltration in 2 oral ppm treated fish on day 3 (Figure 19).

#### 4.4.5 Other organs (gills, brain and skeletal muscle)

There were no remarkable lesions on the gills, brain and skeletal muscle different between control group and all experimental groups.

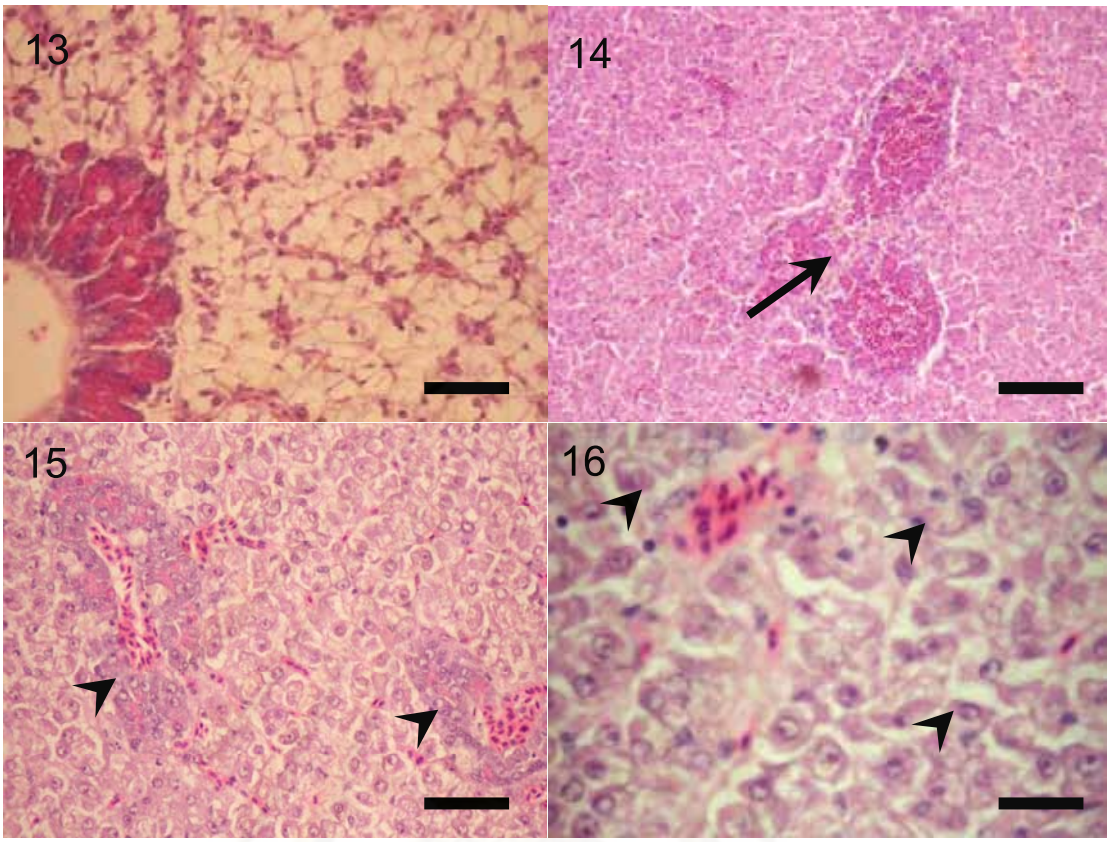


**Figure 13** Hepatopancreas of control tilapia on day 15 (fat storage score +3) (H&E staining, bar = 30 μm).

**Figure 14** Liver of tilapia showed severe losing of fat in hepatocytes (fat storage score +1) 3 days after 5 ppm $HgCl_2$ intraperitoneally injection. Moderate pancreatic acinar atrophy also appeared with MMCs infiltration (arrow) (H&E staining, bar = 125 μm).

**Figure 15** Severe losing of fat storage (fat storage score +1) was observed in 1 ppm $HgCl_2$ semi-static exposure group on day 15 and severe pancreatic atrophy also observed (arrow heads) (H&E staining, bar = 60 μm).

**Figure 16** In 1 ppm $HgCl_2$ semi-static exposure group on day 12, hepatocytes had large nuclei with prominent nucleoli with intracytoplasmic hyaline droplets (arrow heads) (H&E staining, bar = 15 μm).

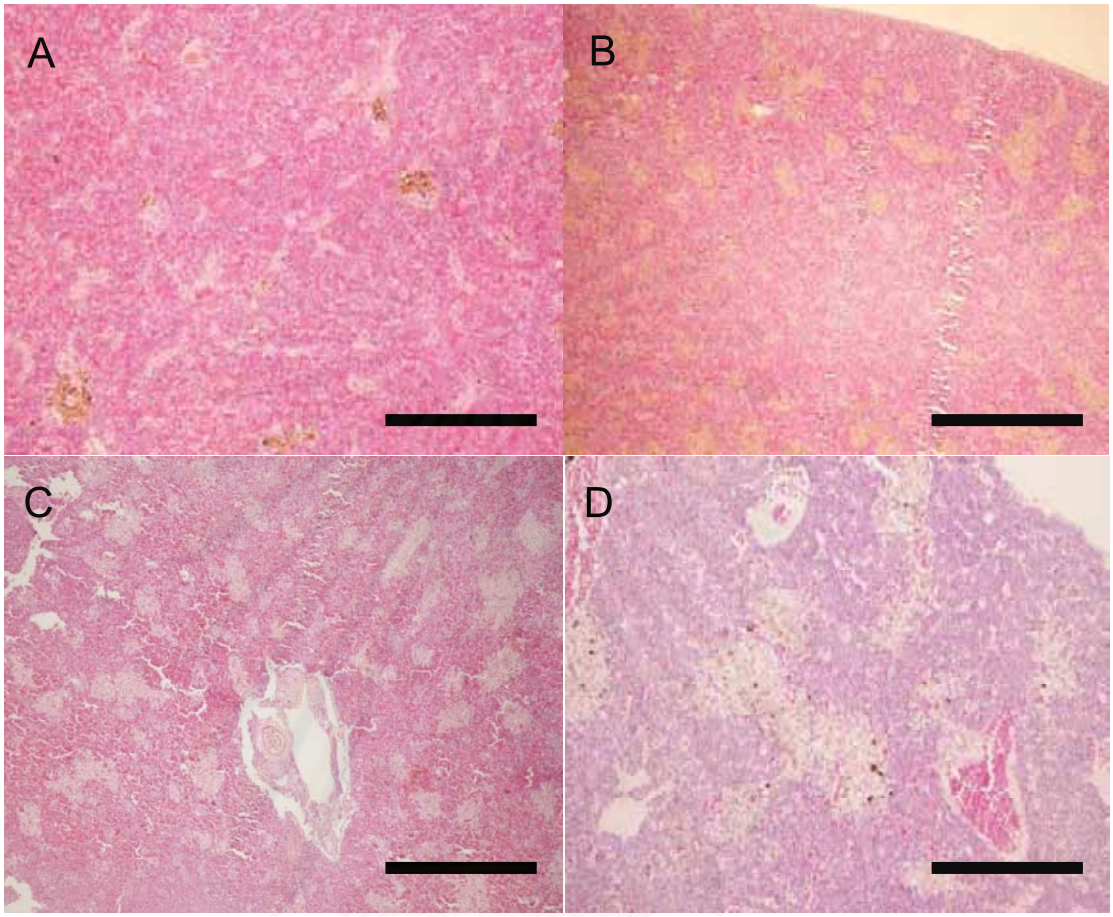


**Figure 17** Tilapia spleens showed increasing of MMCs in experimental group compared with control; Control (A), 0.5 ppm $HgCl_2$ semi-static exposure group on day 15 (score +3) (B), 1 ppm $HgCl_2$ semi-static exposure group on day 12 (C) and day 15 (D) (score+3) (H&E staining, bar; A = 450 μm, B = 450 μm, C = 450 μm, and D = 250 μm).

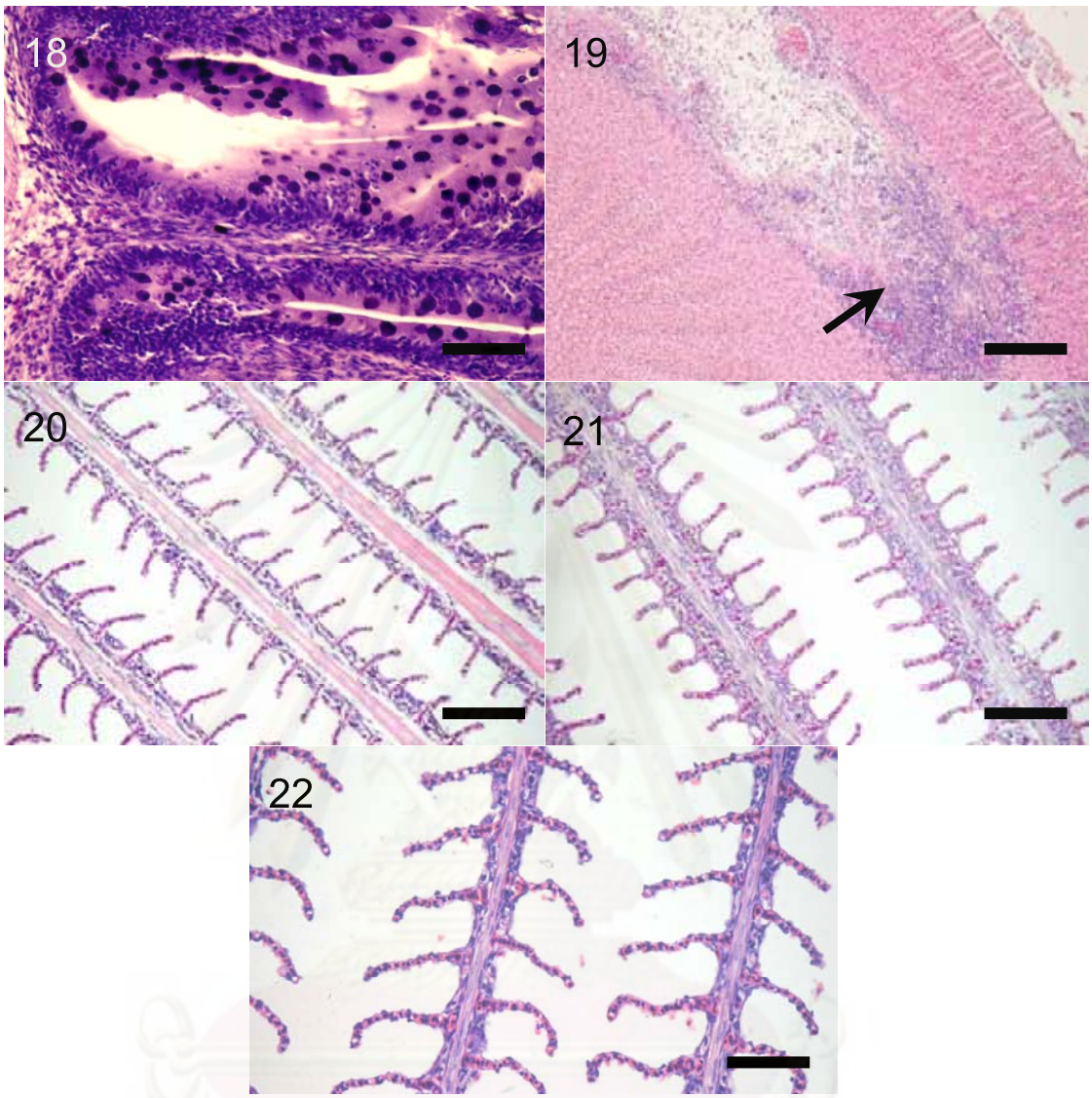


**Figure 18** Goblet cells proliferation in intestinal epithelium was observed in 1 ppm semi-static exposure groups on day 6 (H&E staining, bar = 80 μm).

**Figure 19** Gastric epithelium was necrosis with mononuclear cells infiltration in 2 ppm $HgCl_2$ oral administration tilapia on day 3 (arrow) (H&E staining, bar = 300 μm).

**Figure 20** Secondary gill lamellae of control tilapia on day 15 (H&E staining, bar = 150 μm).

**Figure 21** Secondary gill lamellae of 1 ppm semi-static exposure group on day 15 (H&E staining, bar = 160 μm).

**Figure 22** Secondary gill lamellae of 5 ppm semi-static exposure group on day 15 (H&E staining, bar = 100 μm).

**Table 4** Histopathological scores of control group.

| Group | Dose | Day | Histopathological lesion score | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Trunk of kidney | | | Hepatopancreas | | Spleen | Gastrointestinal organs | | Other organs |
| | | | Degeneration/ necrosis of tubular epithelium | Immature nephron | Crystal in tubules | Fat storage | Pancreatic acinar atrophy | MMCs | Infiltration of mononuclear cells | Goblet cells proiferation | (gill, brain, muscle) |
| Control | 0 | 0 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 3 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 6 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 9 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 12 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 15 | 0 | 0 | 0 | +3 | 0 | +1 | 0 | 0 | NRL |

NRL: no remarkable lesion, +1: mild, +2: moderate, +3: severe

**Table 5** Histopathological scores of i.p. groups.

| Group | Dose | Day | Histopathological lesion score | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Trunk of kidney | | | Hepatopancreas | | Spleen | Gastrointestinal organs | | Other organs |
| | | | Degeneration/ necrosis of tubular epithelium | Immature nephron | Crystal in tubules | Fat storage | Pancreatic acinar atrophy | MMCs | Infiltration of mononuclear cells | Goblet cells proiferation | (gill, brain, muscle) |
| i.p. | 0.5 µg/g | 0 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 3 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 6 | 0 | 0 | +1 | +1 | 0 | +3 | 0 | 0 | NRL |
| | | 9 | +2 | 0 | +3 | +2 | 0 | +3 | 0 | 0 | NRL |
| | | 12 | +2 | 0 | +1 | +2 | 0 | +3 | 0 | 0 | NRL |
| | | 15 | +2 | 0 | +3 | +2 | +1 | +3 | 0 | 0 | NRL |
| | 1 µg/g | 0 | +1 | 0 | 0 | +3 | +1 | 0 | 0 | 0 | NRL |
| | | 3 | 0 | 0 | 0 | +3 | +1 | 0 | 0 | 0 | NRL |
| | | 6 | +2 | +2 | +1 | +2 | +1 | +1 | 0 | 0 | NRL |
| | | 9 | +1 | 0 | +2 | +2 | +2 | +3 | 0 | 0 | NRL |
| | | 12 | +1 | 0 | +3 | +2 | +1 | +2 | 0 | 0 | NRL |
| | | 15 | +1 | +1 | +3 | +2 | +2 | +2 | 0 | 0 | NRL |

NRL: no remarkable lesion, +1: mild, +2: moderate, +3: severe

**Table 5** Histopathological scores of i.p. groups (continue).

| Group | Dose | Day | Histopathological lesion score | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Trunk of kidney | | | Hepatopancreas | | Spleen | Gastrointestinal organs | | Other organs |
| | | | Degeneration/ necrosis of tubular epithelium | Immature nephron | Crystal in tubules | Fat storage | Pancreatic acinar atrophy | MMCs | Infiltration of mononuclear cells | Goblet cells proiferation | (gill, brain, muscle) |
| i.p. | 2 µg/g | 0 | +1 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 3 | +1 | 0 | 0 | +2 | +2 | +1 | 0 | 0 | NRL |
| | | 6 | +2 | +2 | +1 | +2 | +2 | +1 | 0 | 0 | NRL |
| | | 9 | +2 | +2 | +2 | +1 | +2 | +2 | 0 | 0 | NRL |
| | | 12 | +2 | 0 | +3 | +2 | +1 | +2 | 0 | 0 | NRL |
| | | 15 | +2 | +1 | +3 | +2 | +2 | +2 | 0 | 0 | NRL |
| | 5 µg/g | 0 | +2 | 0 | 0 | +2 | +1 | 0 | 0 | 0 | NRL |
| | | 3 | +3 | 0 | 0 | +1 | +2 | +1 | 0 | 0 | NRL |
| | | 6 | +2 | 0 | 0 | +2 | +2 | +2 | 0 | 0 | NRL |
| | | 9 | +2 | +1 | +1 | +2 | +2 | +2 | 0 | 0 | NRL |
| | | 12 | +2 | +3 | +3 | +2 | +3 | +2 | 0 | 0 | NRL |
| | | 15 | +2 | +1 | +1 | +1 | +2 | +3 | 0 | 0 | NRL |

NRL: no remarkable lesion, +1: mild, +2: moderate, +3: severe

**Table 6** Histopathological scores of oral groups.

| Group | Dose | Day | Histopathological lesion score | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Trunk of kidney | | | Hepatopancreas | | Spleen | Gastrointestinal organs | | Other organs |
| | | | Degeneration/ necrosis of tubular epithelium | Immature nephron | Crystal in tubules | Fat storage | Pancreatic acinar atrophy | MMCs | Infiltration of mononuclear cells | Goblet cells proiferation | (gill, brain, muscle) |
| Oral | 0.5 µg/g | 0 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 3 | +1 | 0 | 0 | +2 | +2 | 0 | 0 | +3 | NRL |
| | | 6 | +1 | 0 | +1 | +2 | +2 | +3 | 0 | +3 | NRL |
| | | 9 | +1 | 0 | +1 | +2 | +2 | +2 | 0 | +3 | NRL |
| | | 12 | 0 | 0 | 0 | +2 | +1 | +2 | 0 | +2 | NRL |
| | | 15 | +1 | 0 | +2 | +2 | +2 | +2 | 0 | +3 | NRL |
| | 1 µg/g | 0 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 3 | +2 | 0 | +1 | +2 | +2 | +2 | 0 | +3 | NRL |
| | | 6 | +1 | 0 | 0 | +2 | +1 | +2 | 0 | +2 | NRL |
| | | 9 | +1 | 0 | 0 | +2 | +1 | +1 | 0 | +3 | NRL |
| | | 12 | 0 | 0 | 0 | +2 | +1 | +2 | 0 | 0 | NRL |
| | | 15 | +1 | 0 | +1 | +2 | +1 | +3 | 0 | 0 | NRL |

NRL: no remarkable lesion, +1: mild, +2: moderate, +3: severe

**Table 6** Histopathological scores of oral groups (continue).

| Group | Dose | Day | Histopathological lesion score | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Trunk of kidney | | | Hepatopancreas | | Spleen | Gastrointestinal organs | | Other organs |
| | | | Degeneration/ necrosis of tubular epithelium | Immature nephron | Crystal in tubules | Fat storage | Pancreatic acinar atrophy | MMCs | Infiltration of mononuclear cells | Goblet cells proiferation | (gill, brain, muscle) |
| Oral | 2 µg/g | 0 | +1 | 0 | +3 | +1 | +3 | 0 | 0 | 0 | NRL |
| | | 3 | 0 | 0 | 0 | +2 | +1 | 0 | +2 | +2 | NRL |
| | | 6 | 0 | 0 | +1 | +2 | +1 | +2 | 0 | 0 | NRL |
| | | 9 | 0 | 0 | 0 | +3 | 0 | +2 | 0 | 0 | NRL |
| | | 12 | 0 | 0 | 0 | +3 | 0 | +2 | 0 | 0 | NRL |
| | | 15 | 0 | 0 | 0 | +2 | +1 | +2 | 0 | 0 | NRL |
| | 5 µg/g | 0 | 0 | 0 | 0 | +3 | 0 | 0 | 0 | 0 | NRL |
| | | 3 | 0 | 0 | 0 | +2 | +1 | +1 | 0 | 0 | NRL |
| | | 6 | +1 | 0 | +1 | +3 | +1 | +2 | 0 | 0 | NRL |
| | | 9 | +1 | 0 | +1 | +3 | +1 | +2 | +1 | +2 | NRL |
| | | 12 | +1 | 0 | 0 | +2 | +1 | +2 | 0 | +2 | NRL |
| | | 15 | +2 | 0 | +3 | +2 | +1 | +3 | 0 | 0 | NRL |

NRL: no remarkable lesion, +1: mild, +2: moderate, +3: severe

**Table 7** Histopathological scores of semi-static exposure groups.

| Group | Dose | Day | Histopathological lesion score | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Trunk of kidney | | | Hepatopancreas | | Spleen | Gastrointestinal organs | | Other organs |
| | | | Degeneration/ necrosis of tubular epithelium | Immature nephron | Crystal in tubules | Fat storage | Pancreatic acinar atrophy | MMCs | Infiltration of mononuclear cells | Goblet cells proiferation | (gill, brain, muscle) |
| Semi-static exposure | 0.5 µg/ml | 0 | +3 | +1 | +2 | +2 | +1 | +1 | 0 | +1 | NRL |
| | | 3 | +2 | +1 | +2 | +1 | +2 | +3 | 0 | +1 | NRL |
| | | 6 | +2 | +1 | +1 | +2 | +1 | +3 | +2 | 0 | NRL |
| | | 9 | +2 | +1 | +1 | +1 | +2 | +3 | +2 | 0 | NRL |
| | | 12 | +1 | 0 | +2 | +2 | +2 | +3 | +2 | 0 | NRL |
| | | 15 | +2 | +1 | +1 | +2 | +2 | +3 | +2 | 0 | NRL |
| | 1 µg/ml | 0 | +3 | 0 | +3 | +3 | +2 | +2 | 0 | 0 | NRL |
| | | 3 | +2 | 0 | +2 | +2 | +2 | +2 | 0 | 0 | NRL |
| | | 6 | +2 | +1 | +2 | +1 | +2 | +3 | 0 | 0 | NRL |
| | | 9 | +1 | 0 | +2 | +1 | +2 | +3 | 0 | 0 | NRL |
| | | 12 | +2 | 0 | +2 | +1 | +2 | +3 | 0 | 0 | NRL |
| | | 15 | +1 | 0 | +2 | +1 | +3 | +3 | 0 | 0 | NRL |

NRL: no remarkable lesion, +1: mild, +2: moderate, +3: severe

**Table 7** Histopathological scores of semi-static exposure groups (continue).

| Group | Dose | Day | Histopathological lesion score | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Trunk of kidney | | | Hepatopancreas | | Spleen | Gastrointestinal organs | | Other organs |
| | | | Degeneration/ necrosis of tubular epithelium | Immature nephron | Crystal in tubules | Fat storage | Pancreatic acinar atrophy | MMCs | Infiltration of mononuclear cells | Goblet cells proiferation | (gill, brain, muscle) |
| Semi-static exposure | 2 µg/ml | 0 | +3 | 0 | +3 | +3 | +2 | +1 | 0 | 0 | NRL |
| | | 3 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 6 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 9 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 12 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 15 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | 5 µg/ml | 0 | +3 | NA | +3 | +3 | +2 | +1 | 0 | 0 | NRL |
| | | 3 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 6 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 9 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 12 | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| | | 15 | NA | NA | NA | NA | NA | NA | NA | NA | NA |

NRL: no remarkable lesion, +1: mild, +2: moderate, +3: severe

### 4.5 Autometallographic tracing of Hg accumulation

Hg deposition revealed as yellow-brown to black silver grains in cytoplasm of cells. The positive degrees and location of positive cells were different between organs and days after exposure.

#### 4.5.1 Kidney (trunk)

The silver grains were found in tubular epithelium. Degrees of deposition were high in i.p and semi-static exposure groups. The autometallography scores were showed in table 6. Severe deposition of silver grains (score +3) was found in 5 ppm i.p. and 0.5 and 1 ppm semi-static exposure groups after day 3 of experiments (Figure 24, 25 and 26).

The grains were also found in MMCs that aggregated in renal parenchyma. All fish of control group was not found the accumulation of silver grains in the tubular epithelium and MMCs (score 0) (Figure 23).

#### 4.5.2 Hepatopancreas

The silver grains were found in MMCs that located in atrophic pancreatic acini (Figure 27). Degrees of deposition were showed in table 5, compared to negative staining in control group.

#### 4.5.3 Spleen

MMCs were found yellow to black silver grains in all experiment groups (Figure 28). MMCs were found after day 3 of experiment, compared to negative staining in control group (Table 8).

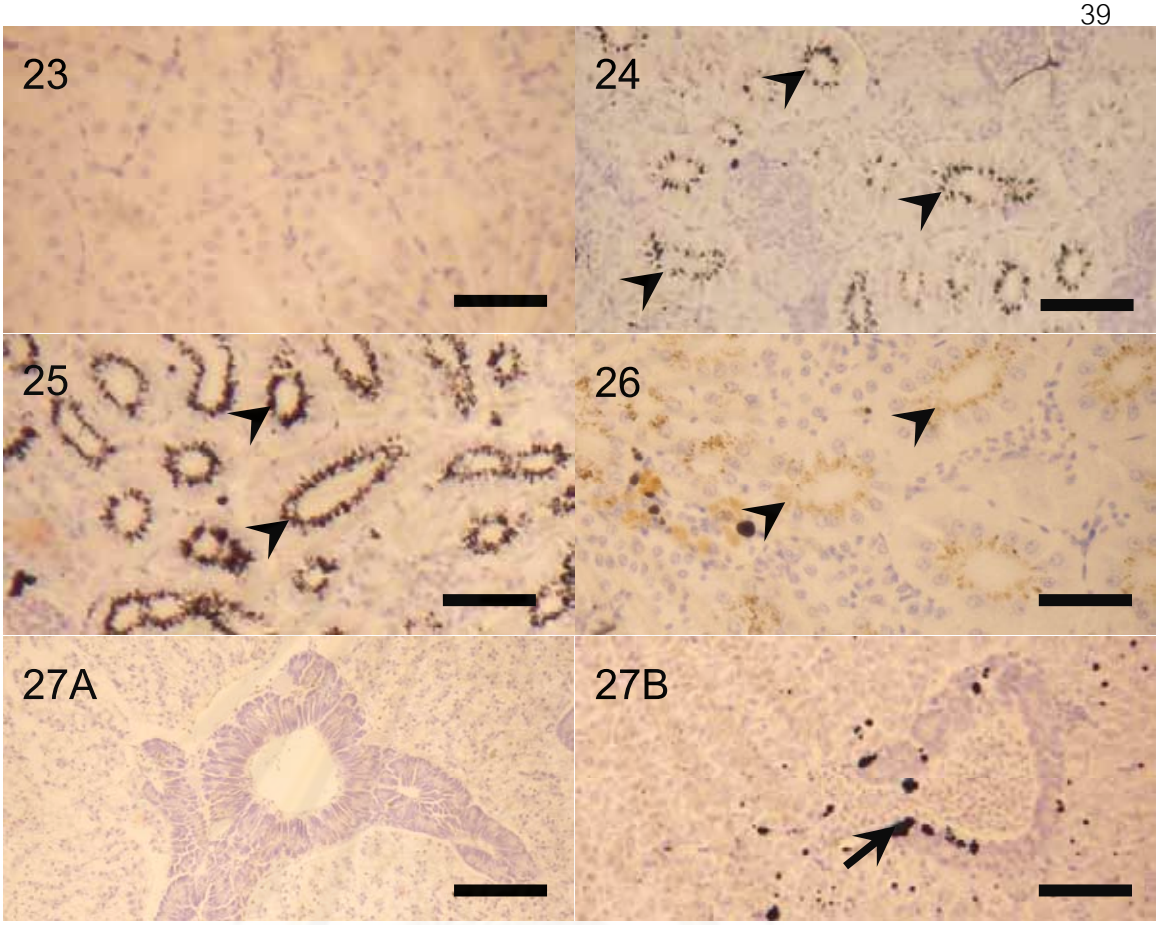


**Figure 23** Trunk kidney of control tilapia was negative staining (AMG staining, bar = 50 µm).

**Figure 24** Silver grains were produced as yellow to brown and black grains (score +2) in renal tubular epithelium of trunk kidney of 2 ppm $HgCl_2$ i.p. injected tilapia on day 15 (arrow heads) (AMG staining, bar = 150 µm).

**Figure 25** Intense silver grain was produced as black grains (score +3) in renal tubular epithelium of 1 ppm $HgCl_2$ semi-static exposure tilapia on day 15 (arrow heads) (AMG staining, bar = 150 µm).

**Figure 26** Silver grains were produced as yellow to brown grains (score +2) in renal tubular epithelium of 0.5 ppm $HgCl_2$ semi-static exposure tilapia on day 15 (arrow heads) (AMG, bar = 45 µm).

**Figure 27** A. Silver grains were observed in MMCs at pancreatic acini of 0.5 ppm $HgCl_2$ semi-static exposure tilapia on day 3 (arrow). B. No grain produced in hepatopancreas of control group (AMG staining, bar; A = 300 µm and B = 150 µm).

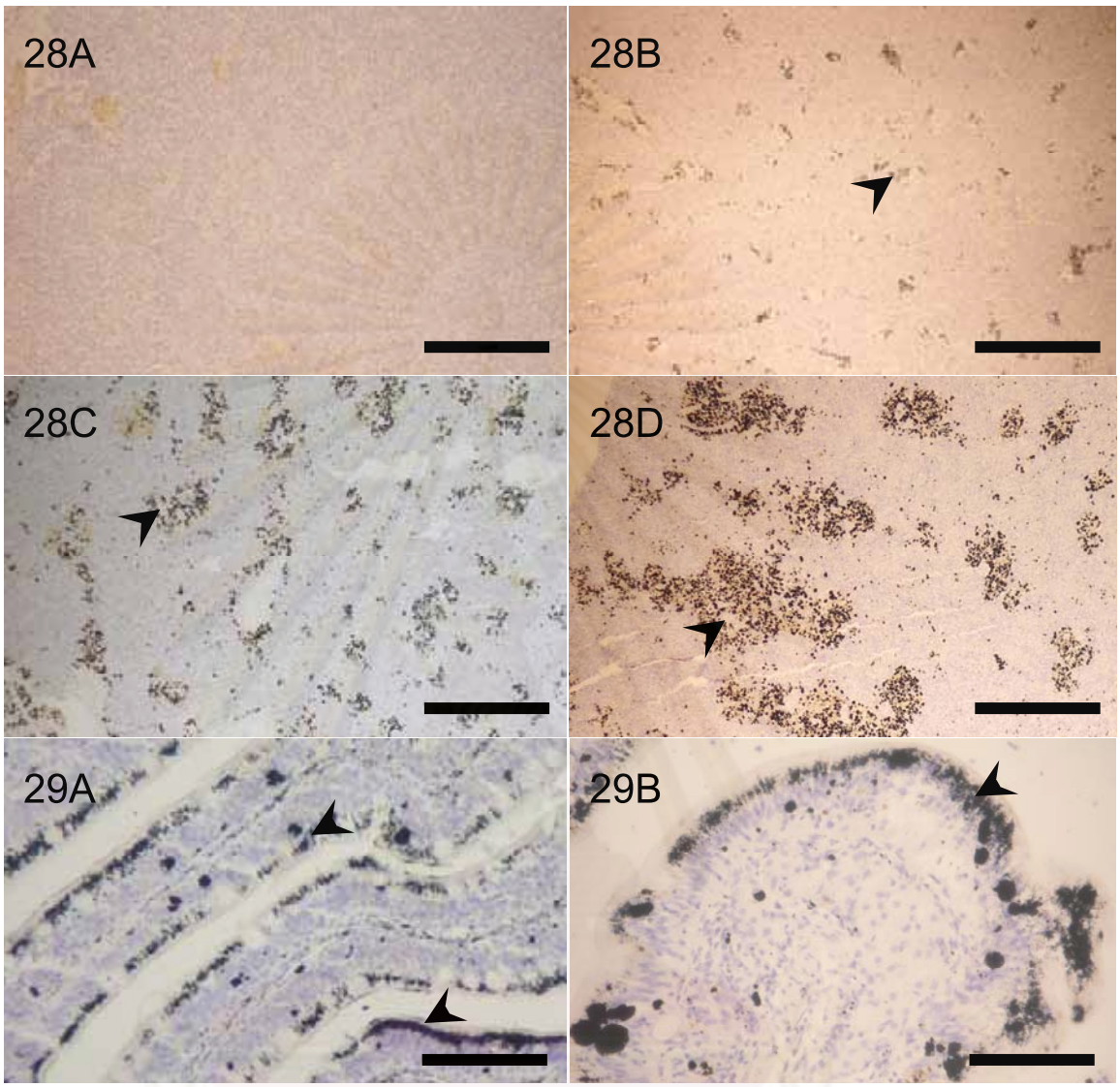


**Figure 28** Tilapia spleens performed autometallography. A. No silver grain revealed in control spleen, B. 1 ppm $HgCl_2$ i.p. tilapia was found silver grains in increasing MMCs on day 3 (score +1), C. 0.5 ppm $HgCl_2$ semi-static exposure tilapia on day 15 (score +3), and D. 1 ppm $HgCl_2$ semi-static exposure tilapia on day 15 (score+3) (AMG staining, bar; A = 450 µm, B = 550 µm, C = 450 µm, and D = 450 µm).

**Figure 29** A. and B. Intestinal mucosa of 1 ppm $HgCl_2$ semi-static exposure tilapia on day 15. Silver grains (score +3) were observed in epithelial cells (arrow heads) (AMG staining, bar; A = 120 µm and B = 80 µm).

#### 4.5.4 Gastrointestinal organs

There was silver grains deposit in epithelial cells of intestinal mucosa of oral and semi-static exposure treated groups. In oral groups, AMG positive was found only in 5 ppm dose on day 3 and 6 of experiment. The silver grains were found after day 3 of 0.5 and 1 semi-static exposure groups (Table 8 and figure 29).

#### 4.5.5 Other organs (brain and skeletal muscle)

No silver grain was found in every cell types in gill, brain tissues and skeletal muscle cells in control and all experimental groups (Table 8).

### 4.6 An expression of metallothionein protein by IHC

Positive control is canine mammary adenocarcinoma showed intense brown cytoplasmic staining (Figure 30). An expression of MT protein could be detected in 0.5 and 1 ppm semi-static exposure groups. In 0.5 ppm semi-static exposure group, there was an expression of MT immunostaining in cytoplasm of renal tubular epithelium after day 9 of experiment. The scores were +3, +3 and +3 on day 9, 12 and 15 respectively (Table 8 and figure 31). Spleen was also shown expression of MT protein in MMCs on day 15 (score +2) (Figure 32).

Trunk kidney of 1 ppm semi-static exposure groups revealed the expression of MT protein after day 6 of experiment. The scores were +1, +3, +3 and +3 on day 6, 9, 12 and 15 respectively. Spleen had the expression of MT protein on day 15 as same as 0.5 ppm semi-static exposure group but the score was +3 (Table 8 and figure 31).

The pancreatic acini of 0.5 and 1 ppm semi-static exposure groups also had the expression of MT protein on day 15. The scores were +2 and +3 in 0.5 ppm and 1 ppm doses, respectively (Table 8 and figure 33).

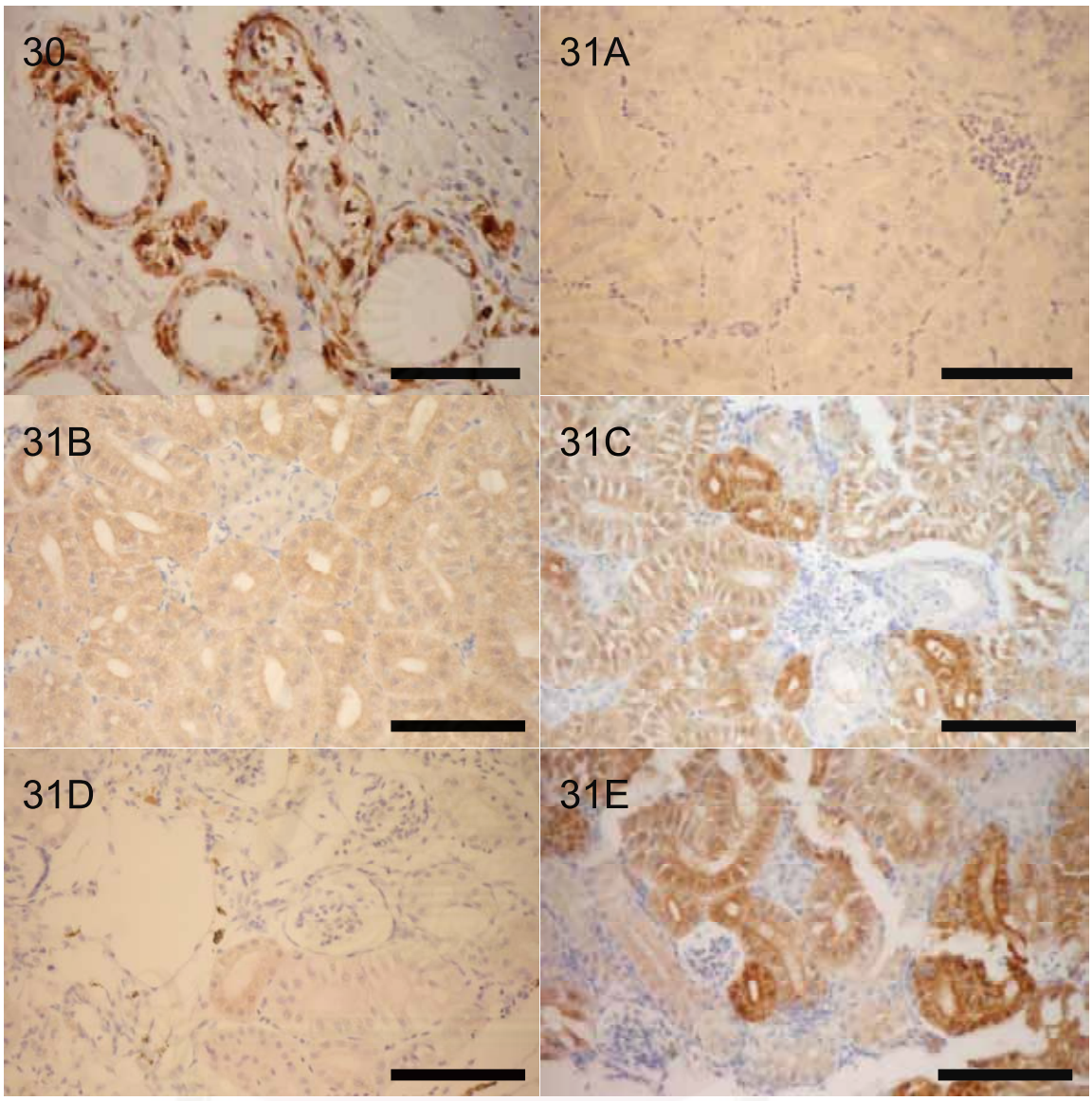


**Figure 30** Canine mammary adenocarcinoma revealed an expression of MT protein (IHC, DAB, counter stained with Mayer's hematoxylin, bar = 100 µm).

**Figure 31** A. Control group revealed no expression of MT protein in trunk of kidneys. B. Renal tubular epithelium revealed an expression of MT protein in 0.5 ppm $HgCl_2$ semi-static exposure tilapia on day 9 (score +2), B. 0.5 ppm $HgCl_2$ Semi-static exposure tilapia on day 15 (score +3), C. 1ppm $HgCl_2$ semi-static exposure tilapia on day 6 (score +1), and D. 1 ppm $HgCl_2$ semi-static exposure tilapia on day 15 (score +2) (IHC, DAB, counter stained with Mayer's hematoxylin, bar = 80 µm).

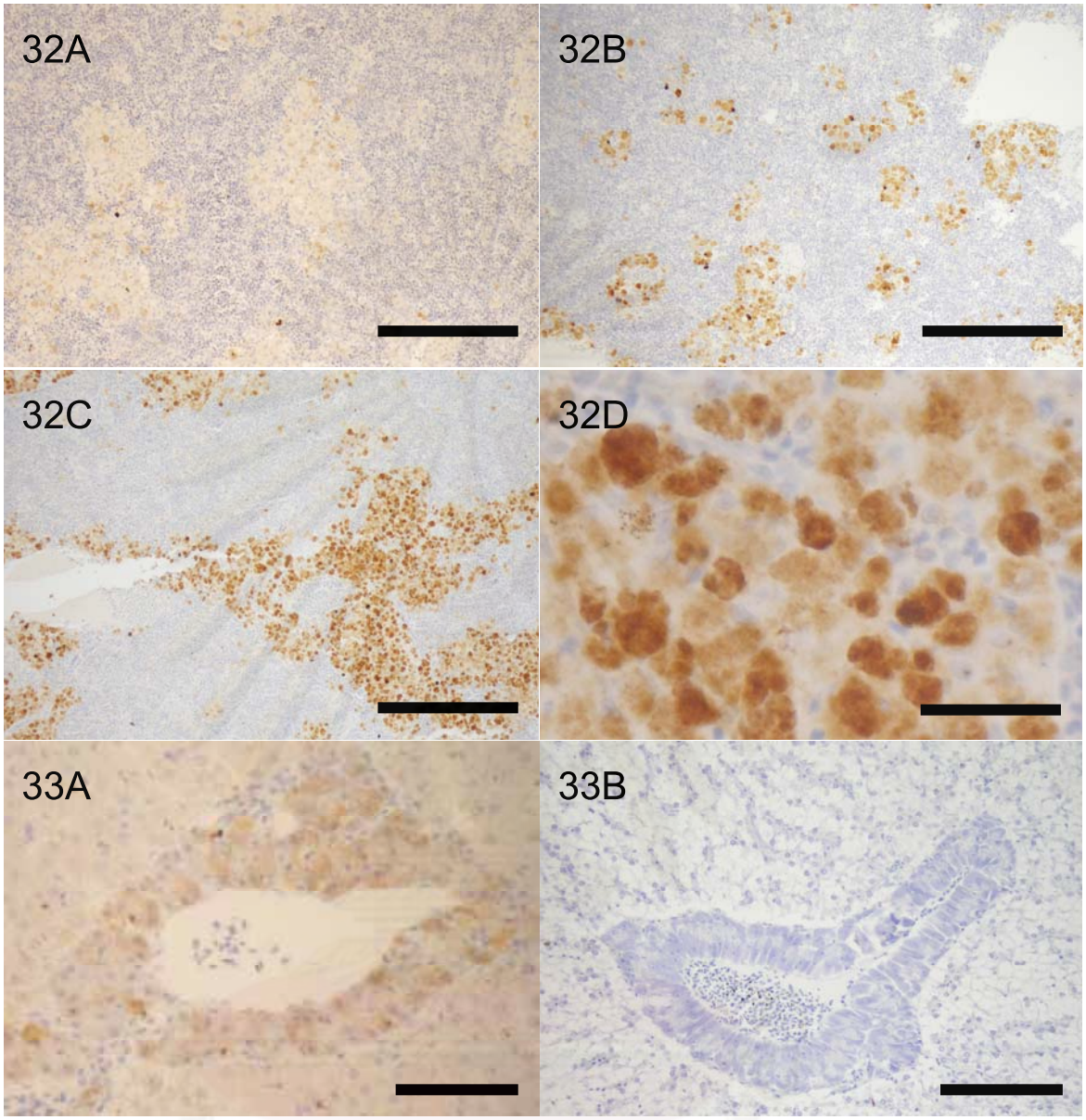


**Figure 32** A. 1 ppm $HgCl_2$ semi-static exposure tilapia on day 12 revealed no expression of MT protein. B. 0.5 ppm $HgCl_2$ semi-static exposure tilapia on day 15 revealed an expression in MMCs (score +2), C. and D. 1 ppm $HgCl_2$ Semi-static exposure tilapia on day 15 (score +3) (IHC, DAB, counter stained with Mayer's hematoxylin, bar; A, B, and C = 270 µm, and D = 40 µm).

**Figure 33** A. 1 ppm $HgCl_2$ semi-static exposure tilapia on day 15 revealed an expression of MT (score +1), and B. Control revealed no expression of MT (IHC, DAB, counter stained with Mayer's hematoxylin, bar; A = 60 µm, and B = 125 µm).

**Table 8** Autometallography and immunohistochemistry scores in experimental tilapias.

| Group | Dose | Day | AMG score | | | | IHC score | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Renal tubular epithelium | MMCs at pancreatic acini | MMCs at slpeen | Epithelium of GI mucosa | Renal tubular epithelium | Pancreatic acini | MMCs at spleen |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| i.p. | 0.5 µg/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 9 | +2 | 0 | +2 | 0 | 0 | 0 | 0 |
| | | 12 | +2 | +1 | +2 | 0 | 0 | 0 | 0 |
| | | 15 | +2 | 0 | +2 | 0 | 0 | 0 | 0 |
| | 1 µg/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | +1 | 0 | 0 | 0 | 0 | 0 |
| | | 6 | +1 | +1 | +1 | 0 | 0 | 0 | 0 |
| | | 9 | +2 | +1 | +2 | 0 | 0 | 0 | 0 |
| | | 12 | +2 | +1 | +2 | 0 | 0 | 0 | 0 |
| | | 15 | +1 | +1 | +1 | 0 | 0 | 0 | 0 |

NA = not examined, +1: mild, +2: moderate, +3: strong

**Table 8** Autometallography and immunohistochemistry scores in experimental tilapias (continue).

| Group | Dose | Day | AMG score | | | | IHC score | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Renal tubular epithelium | MMCs at pancreatic acini | MMCs at slpeen | Epithelium of GI mucosa | Renal tubular epithelium | Pancreatic acini | MMCs at spleen |
| i.p. | 2 µg/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | +1 | 0 | +1 | 0 | 0 | 0 | 0 |
| | | 6 | +1 | +1 | +2 | 0 | 0 | 0 | 0 |
| | | 9 | +1 | +1 | +2 | 0 | 0 | 0 | 0 |
| | | 12 | +2 | +1 | +1 | 0 | 0 | 0 | 0 |
| | | 15 | +2 | +1 | +1 | 0 | 0 | 0 | 0 |
| | 5 µg/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | +3 | +2 | +3 | 0 | 0 | 0 | 0 |
| | | 6 | +2 | +1 | +2 | 0 | 0 | 0 | 0 |
| | | 9 | +2 | +1 | +2 | 0 | 0 | 0 | 0 |
| | | 12 | +2 | +1 | +1 | 0 | 0 | 0 | 0 |
| | | 15 | +2 | +1 | +1 | 0 | 0 | 0 | 0 |
| Oral | 0.5 µg/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | +1 | +1 | 0 | 0 | 0 | 0 |
| | | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

NA = not examined, +1: mild, +2: moderate, +3: strong

**Table 8** Autometallography and immunohistochemistry scores in experimental tilapias (continue).

| Group | Dose | Day | AMG score | | | | IHC score | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Renal tubular epithelium | MMCs at pancreatic acini | MMCs at slpeen | Epithelium of GI mucosa | Renal tubular epithelium | Pancreatic acini | MMCs at spleen |
| Oral | 1 µg/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | +1 | 0 | 0 | 0 | 0 |
| | | 6 | 0 | 0 | +1 | 0 | 0 | 0 | 0 |
| | | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 µg/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | +1 | 0 | 0 | 0 | 0 |
| | | 6 | 0 | 0 | +1 | 0 | 0 | 0 | 0 |
| | | 9 | 0 | 0 | +1 | 0 | 0 | 0 | 0 |
| | | 12 | 0 | 0 | +1 | 0 | 0 | 0 | 0 |
| | | 15 | 0 | 0 | +1 | 0 | 0 | 0 | 0 |
| | 5 µg/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | +2 | 0 | +1 | +2 | 0 | 0 | 0 |
| | | 6 | 0 | 0 | +1 | +3 | 0 | 0 | 0 |
| | | 9 | +1 | 0 | +2 | 0 | 0 | 0 | 0 |
| | | 12 | 0 | 0 | +2 | 0 | 0 | 0 | 0 |
| | | 15 | 0 | 0 | +1 | 0 | 0 | 0 | 0 |

NA = not examined, +1: mild, +2: moderate, +3: strong

**Table 8** Autometallography and immunohistochemistry scores in experimental tilapias (continue).

| Group | Dose | Day | AMG score | | | | IHC score | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Renal tubular epithelium | MMCs at pancreatic acini | MMCs at slpeen | Epithelium of GI mucosa | Renal tubular epithelium | Pancreatic acini | MMCs at spleen |
| Bath | 0.5 µg/ml | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | +1 | +3 | +1 | +2 | 0 | 0 | 0 |
| | | 6 | +2 | +2 | +1 | +3 | 0 | 0 | 0 |
| | | 9 | +2 | +1 | +2 | +3 | +2 | 0 | 0 |
| | | 12 | +2 | +1 | +2 | +3 | +3 | 0 | 0 |
| | | 15 | +2 | +1 | +3 | +2 | +3 | 0 | +2 |
| | 1 µg/ml | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | +1 | +1 | +1 | +2 | 0 | 0 | 0 |
| | | 6 | +2 | +1 | +1 | +2 | +1 | 0 | 0 |
| | | 9 | +3 | +2 | +2 | +3 | +3 | 0 | 0 |
| | | 12 | +3 | +1 | +3 | +2 | +3 | +1 | 0 |
| | | 15 | +3 | +1 | +3 | +2 | +3 | +1 | +3 |
| | 2 µg/ml | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | NA | NA | NA | NA | NA | NA | NA |
| | | 6 | NA | NA | NA | NA | NA | NA | NA |
| | | 9 | NA | NA | NA | NA | NA | NA | NA |
| | | 12 | NA | NA | NA | NA | NA | NA | NA |
| | | 15 | NA | NA | NA | NA | NA | NA | NA |

NA = not examined, +1: mild, +2: moderate, +3: strong

**Table 8** Autometallography and immunohistochemistry scores in experimental tilapias (continue).

| Group | Dose | Day | AMG score | | | | IHC score | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Renal tubular epithelium | MMCs at pancreatic acini | MMCs at spleen | Epithelium of GI mucosa | Renal tubular epithelium | Pancreatic acini | MMCs at spleen |
| Bath | 5 µg/ml | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | NA | NA | NA | NA | NA | NA | NA |
| | | 6 | NA | NA | NA | NA | NA | NA | NA |
| | | 9 | NA | NA | NA | NA | NA | NA | NA |
| | | 12 | NA | NA | NA | NA | NA | NA | NA |
| | | 15 | NA | NA | NA | NA | NA | NA | NA |

NA = not examined, +1: mild, +2: moderate, +3: strong

### 4.7 ICP-AES for measurement total mercury in tissues

A cut-off value of total Hg level in control group in 3 organs was less than 0.2 ppm/g dry weight (Figure 34). Hg levels measured by ICP-AES were differently between routes and doses (Table 9). Total Hg levels in visceral organs in abdomen were high when compared within groups. It was significantly different between visceral organs in abdomen and other organs (gills and muscle) in 0.5 ppm $HgCl_2$ i.p. groups on day 3 ($p<0.05$) but Hg levels were not significantly different between organs on day 9 and day 15. In this group, total Hg levels of visceral organs had significant statistically different among day 3, day 9, and day 15 in different routes of exposure (0.5 ppm i.p., 2 ppm i.p., 5 ppm i.p, 0.5 ppm oral, 2 ppm oral, and 5 ppm oral) (Figure 35A, 35C, 35D, 36A, 36C and 36D). 2 ppm oral group showed statistic significantly different differ from other doses in oral groups. It had significantly different between visceral organs in abdomen and other organs on day 3, day 9, and day 15. ($p<0.05$) When compared between the same organ, in this group revealed significantly different in visceral organs in abdomen on every days. The total Hg concentrations were 6.266±0.460, 4.223±0.115 and 1.384±0.131 ppm/g dry weight respectively. Total Hg concentration in gills on day 9 was also significantly differing from day 3 and day 15 ($p<0.05$). In addition, visceral organs in abdomen in 2 ppm oral group were also significantly different from other organs on every day ($p<0.05$) (Figure 36C). In 1 ppm semi-static exposure tilapia group, it had the highest level compared with other groups (Table 9). Visceral organs in abdomen Hg concentration showed significantly different from other organs on every day ($p<0.05$) and the different day also showed significantly different (Figure 37B).

**Table 9** Total Hg in experimental tilapias.

| Group | Dose | Day | Organ | | |
|---|---|---|---|---|---|
| | | | Gill (ppm/g dry weight) | Visceral organs in abdomen (ppm/g dry weight) | Muscle (ppm/g dry weight) |
| Control | 0 | 3 | 0.163 ± 0.014 | 0.132 ± 0.023 | 0.152 ± 0.018 |
| | | 9 | 0.110 ± 0.005 | 0.149 ± 0.007 | 0.180 ± 0.032 |
| | | 15 | 0.101 ± 0.019 | 0.133 ± 0.003 | 0.173 ± 0.051 |
| i.p. | 0.5 µg/g | 3 | 1.157 ± 0.439 | 5.709 ± 1.609 | 0.424 ± 0.241 |
| | | 9 | 0.888 ± 0.124 | 1.689 ± 0.786 | 0.981 ± 0.575 |
| | | 15 | 0.687 ± 0.191 | 1.857 ± 1.417 | 0.515 ± 0.219 |
| | 1 µg/g | 3 | 2.212 ± 0.142 | 14.757 ± 7.114 | 1.870 ± 1.142 |
| | | 9 | 1.467 ± 1.039 | 9.965 ± 1.359 | 1.588 ± 0.657 |
| | | 15 | 0.895 ± 0.030 | 3.119 ± 2.787 | 0.233 ± 0.097 |
| | 2 µg/g | 3 | 5.884 ± 5.484 | 19.701 ± 2.100 | 1.047 ± 0.364 |
| | | 9 | 4.152 ± 1.845 | 8.239 ± 3.261 | 3.163 ± 0.430 |
| | | 15 | 3.783 ± 1.577 | 9.518 ± 4.014 | 2.584 ±0.970 |
| | 5 µg/g | 3 | 4.144 ± 0.686 | 31.629 ± 11.392 | 4.659 ± 1.302 |
| | | 9 | 2.257 ± 0.974 | 13.070 ± 1.151 | 3.195 ± 1.590 |
| | | 15 | 0.989 ± 0.339 | 4.786 ± 1.403 | 1.378 ± 0.335 |
| Oral | 0.5 µg/g | 3 | 0.395 ± 0.040 | 0.910 ± 0.143 | 0.389 ± 0.037 |
| | | 9 | 0.237 ± 0.071 | 0.330 ± 0.139 | 0.284 ± 0.087 |
| | | 15 | 0.365 ± 0.108 | 0.411 ± 0.016 | 0.160 ± 0.081 |
| | 1 µg/g | 3 | 0.484 ± 0.030 | 0.727 ± 0.186 | 0.099 ± 0.040 |
| | | 9 | 0.365 ± 0.146 | 0.496 ± 0.115 | 0.178 ± 0.057 |
| | | 15 | 0.421 ± 0.007 | 0.606 ± 0.212 | 0.337 ± 0.043 |
| | 2 µg/g | 3 | 1.337 ± 0.472 | 6.266 ± 0.460 | 1.465 ± 0.272 |
| | | 9 | 3.292 ± 0.314 | 4.223 ± 0.658 | 1.419 ± 0.662 |
| | | 15 | 0.778 ± 0.136 | 1.384 ± 0.131 | 0.685 ± 0.201 |
| | 5 µg/g | 3 | 3.422 ± 0.306 | 9.263 ± 4.737 | 0.761 ± 0.299 |
| | | 9 | 1.337 ± 0.472 | 3.936 ± 0.446 | 0.385 ± 0.015 |
| | | 15 | 1.438 ± 0.286 | 3.015 ± 0.515 | 0.552 ± 0.170 |

**Table 9** Total Hg in experimental tilapias (continue).

| Group | Dose | Day | Organ | | |
|---|---|---|---|---|---|
| | | | Gill (ppm/g dry weight) | Visceral organs in abdomen (ppm/g dry weight) | Muscle (ppm/g dry weight) |
| Semi-static exposure | 0.5 µg/ml | 3 | 15.847 ± 2.884 | 33.279 ± 13.171 | 2.473 ± 0.646 |
| | | 9 | 36.861 ± 4.785 | 51.859 ± 11.140 | 10.616 ± 0.923 |
| | | 15 | 55.778 ± 2.113 | 42.654 ± 26.111 | 18.235 ± 5.642 |
| | 1 µg/ml | 3 | 20.210 ± 2.583 | 45.747 ± 25.849 | 3.208 ± 1.330 |
| | | 9 | 33.568 ± 9.335 | 183.274 ± 7.208 | 10.041 ± 3.847 |
| | | 15 | 44.972 ± 2.847 | 139.507 ± 8.611 | 15.917 ± 8.627 |
| | 2 µg/ml | 3 | NA | NA | NA |
| | | 9 | NA | NA | NA |
| | | 15 | NA | NA | NA |
| | 5 µg/ml | 3 | NA | NA | NA |
| | | 9 | NA | NA | NA |
| | | 15 | NA | NA | NA |

NA: not examined

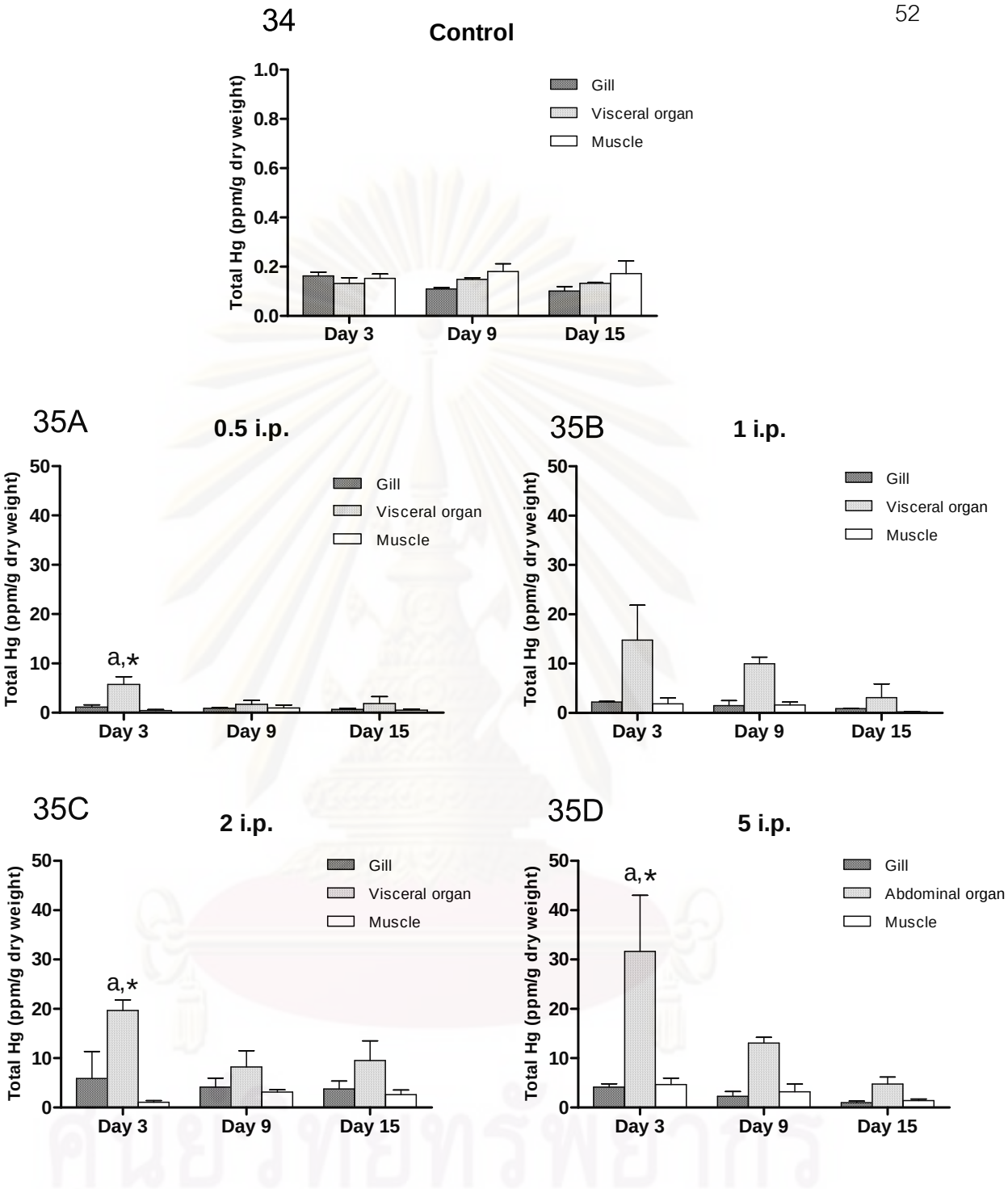


**Figure 34** Total Hg levels from ICP-AES of Control group.

**Figure 35A-D** Total Hg levels from ICP-AES of i.p. groups. The different alphabets shows when statistic significantly different in the same organ between days. ($p$<0.05) and the stars (*) showed when statistic significantly different between organs in the same day. ($p$<0.05) Statistic analysis was using one-way ANOVA and followed by Newman-Keuls multiple comparison test.

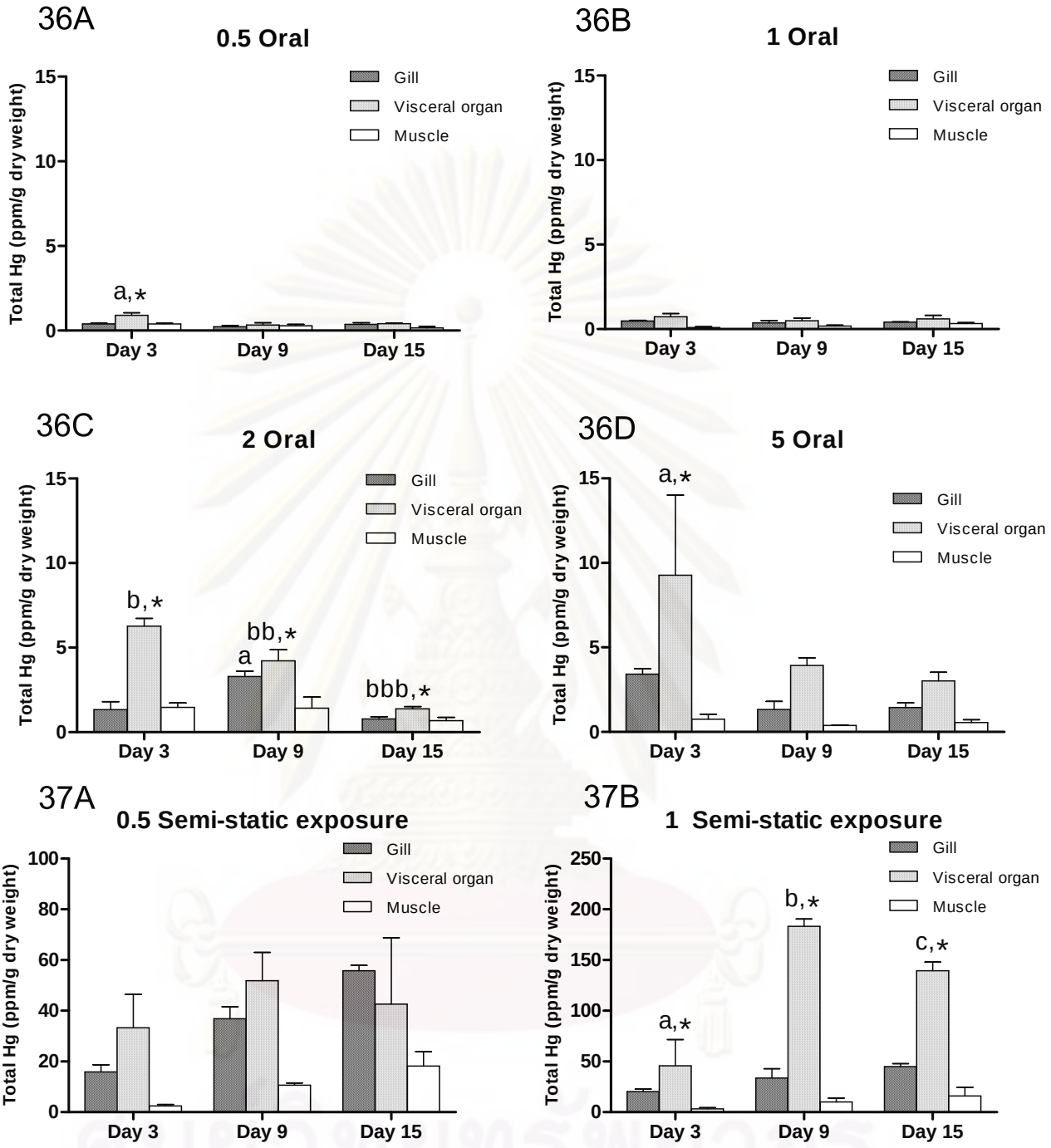


**Figure 36A-D** Total Hg levels from ICP-AES of oral group.

**Figure 37A-B** Total Hg levels from ICP-AES of semi-static exposure groups. The different alphabets shows when statistic significantly different in the same organ between days. ($p<0.05$) and the stars (*) showed when statistic significantly different between organs in the same day. ($p<0.05$) Statistic analysis was using one-way ANOVA and followed by Newman-Keuls multiple comparison test.

**4.8 Reverse transcriptase polymerase chain reaction for detecting metallothionein gene expression**

All groups of experiment includes control group showed many unspecific bands by using 2 pairs of primers, forward and reverse primers of tilapia (*O. mossambica*) MT gene (TMTB and TMTC) and forward and reverse primers of tilapia β-actin gene (Figure 38 and 39). The PCR products were chosen for nucleotide sequencing (150 and 300 bp PCR products). The nucleotide sequences were compared with *O. mossambica* MT nucleotide sequence in GenBank (AY257202) (Cheung et al., 2004). The results showed no similarity between PCR products and nucleotide sequence in GenBank (Table 10).

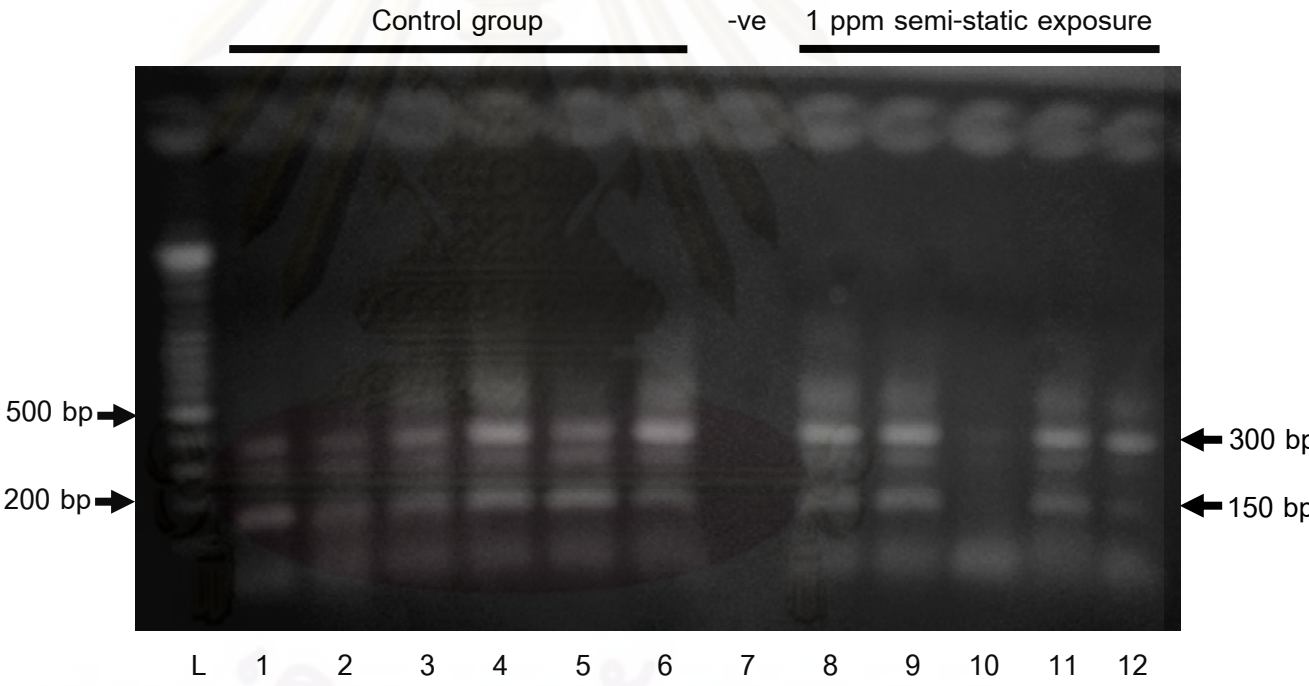


**Figure 38** RT-PCR products when using 2 pairs of primers (tilapia (*O. mossambicus*) MT; TMTB and TMTC) and tilapia β-actin. The samples were kidney tissues of control and 1 ppm $HgCl_2$ semi-static exposure group. Lane 1, 2, 3, 4, 5 and 6: control, lane 7: negative control, lane 8, 9, 10, 11 and 12: 1 ppm $HgCl_2$ semi-static exposure group and L: 100 bp marker.

When using only forwards and reverse primers of tilapia (TMTB and TMTC), the result revealed all negative in every groups (Figure 37).

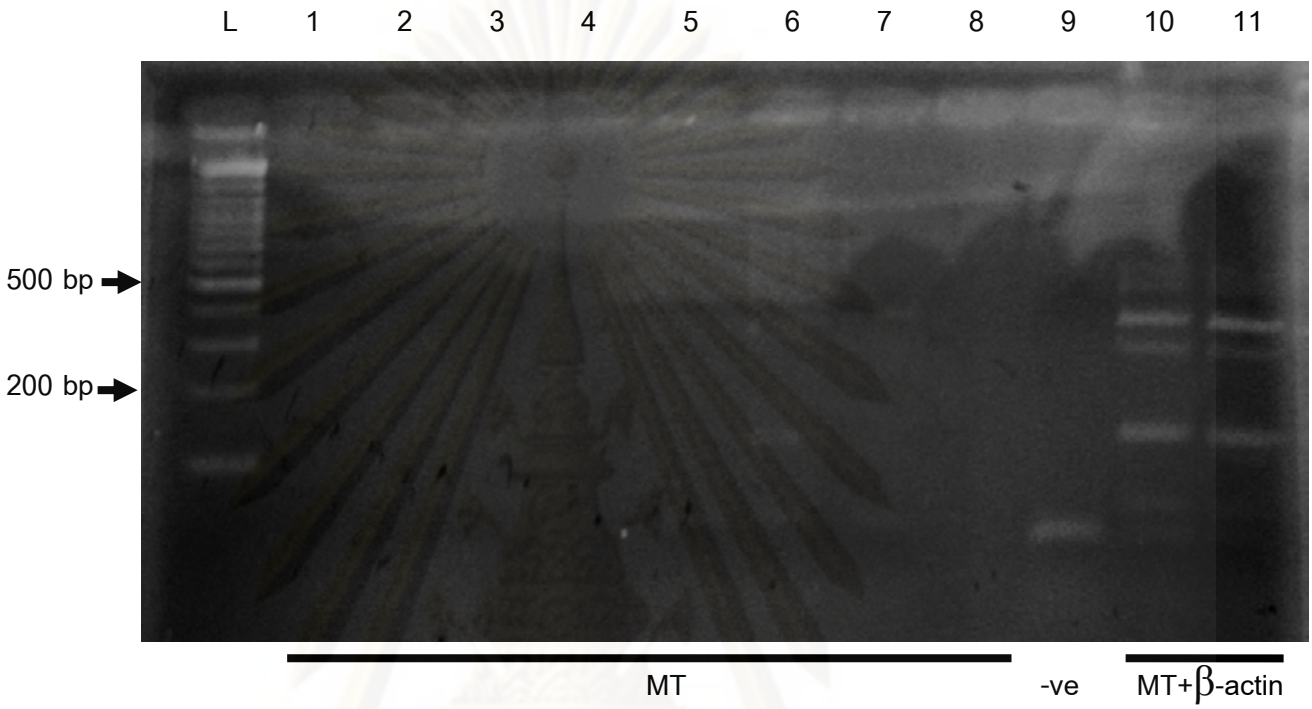


**Figure 37** RT-PCR products. Lane 1, 2, 3, 4, 5, 6, and 7 were using forward and reverse primers of tilapia (*O. mossambica*) MT; TMTB and TMTC. Lane 10 and 11 were using 2 pairs of primers MT and tilapia β-actin. The samples were kidney tissues of control (lane 1, 2, 3 and 4) and 1 ppm $HgCl_2$ semi-static exposure group (lane 5, 6, 7 and 8). The results were all negative when using only MT primers. L: 100 bp marker, lane 9: negative control.

**Table 10** The sequences of nucleotides of RT-PCR products from the current experiment.

| Sequence of nucleotides | Length (bp) |
|---|---|
| 5'-CCACCAAGATCTGCACCCGCGGCGGCTCCACCCGGGCTCGCGCCCTAGGCT TCCGTGCTCACCGCGGCGGCCCTCCTACTCGTCGCGGCGTAGCCCTCGCGGCT CCTATTGCCGGCGACGGCCGGGTATGGGCCCGACGCTCCAGCGCCATCCATTTT CAGGGCTAGTTGATTCGGCAGGTGAGTTGTTACACACTCCTTAGCGGATTCCAACT TCCATGGCCTCCTGTCCACCTTTA -3' | 238 |

**Table 11** Comparison of histopathological lesions, autometallography and expression of metallothionine protein between experimental groups.

| Group | Histopathological lesions | Autometallography | Expression of metallothionine protein by Immunohistochemistry |
|---|---|---|---|
| Control | - No histopathogical lesion | - Negative AMG | - Negative IHC |
| i.p. | - Mild to severe tubulonephrosis, immature nephrons and deposition of crystal in renal tubules.<br>- Mild to severe loss of fat deposition in hepatocytes, and mild to severe pancreatic acinar atrophy.<br>- Marked increased MMCs. | - Mild to severe localized of silver grains in tubular epithelium, MMCs of spleen and pancreatic acini. | - Negative IHC |
| Oral | - Mild to moderate tubulonephrosis and deposition of crystal in renal tubules.<br>- Mild to severe loss of fat deposition in hepatocytes, and mild to moderate pancreatic acinar atrophy.<br>- Marked increased MMCs.<br>- Mild to severe infiltration of mononuclear cells and goblet cells in GI epithelium. | - Mild to moderate localized of silver grains in tubular epithelium, and MMCs of spleen.<br>- Mild to moderate localized of silver grains in GI epithelium. | - Negative IHC |

**Table 11** Comparison of histopathological lesions, autometallography and expression of metallothionine protein between experimental groups (continue).

| Group | Histopathological lesions | Autometallography | Expression of metallothionine protein by Immunohistochemistry |
|---|---|---|---|
| Bath | - Severe tubulonephrosis, mild immature nephrons and deposition of crystal in renal tubules.<br>- Moderate to severe loss of fat deposition in hepatocytes, and mild to severe pancreatic acinar atrophy.<br>- Marked increased MMCs.<br>- Mild to severe infiltration of mononuclear cells and goblet cells in GI epithelium | - Mild to severe localized of silver grains in tubular epithelium, MMCs of spleen and pancreatic acini.<br>- Mild to severe localized of silver grains in GI epithelium. | - Positive IHC in renal tubular epithelium after day 6, MMCs in spleen and pancreatic acini after day 15 |

# CHAPTER V

# DISCUSSION AND CONCLUSION

## Discussion

Many studies have documented that mercury can be toxic to fish. In this study, all tilapia were dead in 2 and 5 ppm (µg/l) $HgCl_2$ semi-static exposure groups after 2 hours on the first day of experiment. It was accordance with 24-hour mean lethal concentration ($LC_{50}$) of $Hg^{2+}$ in tilapia, which is about 1 µg/ml (NJDEP, 2002). The clinical appearances of high doses $Hg^{2+}$ toxicity may affect respiratory system so fish showed obvious clinical sign of respiratory failure, such as swimming to the surface of the water and increasing respiration by moving gill opercula. Hypoxemia has been reported as the main toxicological effect in fish exposed to inorganic Hg in water. Inorganic Hg alters in the respiratory function of gills caused decreasing their gas exchange capability (Raldúa et al., 2007).

Various tissues of tilapia were affected by inorganic Hg. Acute $Hg^{2+}$ mercury toxicity mainly affects on kidney, hepatopancreas and spleen. The main pathological lesions of acute mercury toxicity in tilapia were found in renal tubular epithelial cells susceptible to heavy metal exposure, such as Hg like in mammals (Hansen and Danscher, 1995). In this study, the kidney appeared to be primary target tissue for $Hg^{2+}$. The present study showed severe tubulonephrosis; degeneration and necrosis of tubular epithelium with accumulation of hyaline droplets and crystal in tubular lumen. The effect of Hg can be found after first day of experiment in high dose with semi-static exposure route. The previous study in channel catfish (*Ictalurus punctatus*) were noted that severe tubular and glomerular alteration at 24-hour after exposing to Hg. Because the renal tubular epithelium has its major function to excrete of divalent ions, including $Hg^{2+}$, so renal tubular epithelium can be damaged easily by those toxic substances especially nephrotoxins (Kendall, 1975).

The pathological lesions in hepatopancreas included losing of fat storage in hepatocytes when fish exposed to inorganic mercury suggests that this process involved an increasing of metabolism response to toxic substances as we seen by enlargement of nuclear size and prominent nucleoli of degenerative hepatocytes. Oliveira Ribeiro et al. (2002) also observed losing of fat in arctic charr (*Salvelinus alpinus*) liver. They described that change may be from oxidative stress and an accumulation of lipid peroxidation products. Howerver, Drevnick et al. (2008) hypothesized decreasing in fat reserves within hepatocytes in northern pike (*Esox lucius*) may happen from undergoing starvation deplete fat reserves in the liver for energy consumption.

In this study found that obvious pathological lesions of liver differed from the previous studies in some points. The previous study in arctic charr associated with MeHg exposure showed severe necrosis of hepatocytes (Oliveira Ribeiro et al., 2002). Necrosis may be a consequence from inhibiting of enzymatic process, cell membrane disturbances, and alteration of protein and carbohydrate synthesis (Mela et al., 2007). In Hg-exposed beaks, there was a presence of multifocal granuloma, macrovesicular steatosis and nuclear pyknosis of hepatocytes. Proliferation of bile ducts and the epithelial cells were also observed in bleaks but not found in the present study (Raldúa et al., 2007).

Pancreatic atrophy also appeared after exposed to Hg. It was similar liver pathology described by Kendall (1977), he reported that exocrine pancreatic tissue in the catfish (*I. punctatus)* hepatopancreas demonstrated the earliest histopathologic change.

In experimental groups, there was marked increasing of MMCs in splenic parenchyma. MMCs were increased both number and size of cells. Such increasing was fluctuated according to time of exposure. Increasing of MMCs in spleen and other organs may involve an increase phagocytic activity due to tissue damage by $Hg^{2+}$. Macrophages may belong to the first line of protection of Hg intoxification (Loumbourdis and Dancher, 2004). MMCs contained melanin that is well-known as its ability to

neutralize free radicals and cations for inactivating them and protecting from tissue damages (Robert et al., 1978). They may also involve removing debris during inflammation (Mela et al., 2007) and tissue repairing process (Agius, 1985). Therefore, the highly increasing of MMCs in fish spleen can use as a potent biomarker for Hg pollutant (Mela et al., 2007).

Autometallography development, silver-enchanced Hg grains are visualized as yellow-brown to black granules in renal tubular epithelium, MMCs in spleen, MMCs in pancreatic acinai and intestinal epithelium of experimental tilapias. From autometallographic tracing of mercury, the trunk of the kidney and spleen of tilapia were major organs for mercury accumulation. Hg also involved in MMCs aggregation in the destructive tissues such as in pancreatic acini and trunk kidneys. The strong positive intensity scores were observed in high doses. The highest scores showed in semi-static exposure groups followed by i.p. group, and the least were in the oral groups in kidney and MMCs of spleen. The intestinal mucosa also found postitive with AMG method but only on semi-static exposure and oral groups. It may describe that Hg can distribute to various organs different from route of exposure. i.p. routes could not find silver grains in intestinal mucosa but it showed stronger positive in renal epithelial cells and MMCs of spleen more than oral route. It suggested that $Hg^{2+}$ may absorb into circulation and directly distribute into target organs; kidney and spleen, like in mammals (Zahir et al., 2005). When fish received $Hg^{2+}$ by oral route, $Hg^{2+}$ may absorb via intestinal epithelial cells and deposit in mucosal layers before distribution to the circular system and circulate to target organs. The semi-static exposure groups were repeated exposed to $Hg^{2+}$ all the time and they can receive $Hg^{2+}$ from many organs; gill and oral route so it was not surprising that revealed strong positive in every organs when compared among treatment groups. In the current study, there was no AMG grains deposit in gill tissues unlike the previous study of Alverado et al. (2006) that found silver grains accumulated in mucocytes, filament epithelium, and the secondary lamellae in Cu exposed turbot (*Scophthalmus maximus*). It may describe that heavy metal types can show different AMG pattern (Danscher and Stoltenberg, 2006).

Therefore, it may conclude that Hg AMG pattern was dependent on types of heavy metals, sources of Hg, route of administration, dose and time of exposure (Danscher and Stoltenberg, 2006).

An expression of MT protein detected by IHC method, the semi-static exposure group showed positive results in renal epithelial cells, pancreatic acini and MMCs in splenic parenchyma. The presence of MT in tilapia tissue suggested that MT gene involved in the process of heavy metal dextoxification. MT protein can be detected after day 6 of experiment in 1 ppm $HgCl_2$ semi-static exposure group and after day 9 of 0.5 ppm $HgCl_2$ semi-static exposure group. Other experimental groups and control group showed negative results in all tissues. It suggested that MT protein expression in tilapia is depended on dose, time of exposure and route of administration. MT was found after day 6 of experiment in 1 ppm $HgCl_2$ group. It may be because there were not enough MT levels for detecting by IHC method. There were expression of MT protein in renal tubular cells and pancreatic acini as same as in this experiment tilapias but there were also positive in hepatocytes of mandarin fish.

Chan (1995) described that hepatic levels of MT in fish are dose-dependently increased after injection of Cd or exposure to Cu and Zn. The previous study, significant induction of MT mRNA levels in the liver and gills of heavy metal-exposed-tilapia was observed at week 3 (Lam et al., 1998). An expression of MT protein had been reported in kidney and liver are similar to the study in mandarin fish (*Siniperca chuatsi*).

The expression in MMCs in spleen using DAB system may be confusing with normal MMCs tissue because normal MMCs contained yellow-brown pigments; melanin pigments (Agius, 1985). The previous reported also describe that It was hardly detectable MT in the spleen (Gao et al., 2009), Therefore, this study demonstrated an expression on MMCs by compared with control and other groups. In positive IHC groups; 0.5 and 1 ppm $HgCl_2$ semi-static exposure groups, there were obviously different intensity of reaction products in MMCs so the result suggested that may be positive from MT more than from melanin pigments.

The previous report suggested the expression of MT protein in some cell-type of gills of tilapia (*O. mossambica*) after exposure to Cu that is different from current study, no positive result in gill tissues. In addition, there are reported of immunohistochemical detection in gill of brown trout (*Salmotrutta fario* L.), rainbow trout (*Oncorrhynchus mykiss* Richardson) and turbot (*Scophthalmus maximus*). There was MT protein expression in chloride cells of gill filaments after exposed to the sewage in brown trout and rainbow trout (Burkhardt-Holm et al., 1999). In turbot, there were positive MT immunohistochemistry in mucocytes after exposureto Cd, Cu and Zn (Alvarado et al., 2006). From this current study, it may suggested that renal tubular cells, pancreatic acini and MMCs in spleen were involved in MT production after exposed to Hg.

Total Hg levels measured by ICP-AES in present study showed that visceral organs in abdomen were mainly accumulation locations. One single dose of $Hg^{2+}$ revealed higher level in day 3 and decreased respectively until day 15 of experiment but differed from semi-static exposure group that repeatedly exposed to $Hg^{2+}$ every 3 days, Hg level revealed increased until day 15 of experiment. It may suggest that if fish exposes to Hg all time, as well as Hg toxicity in their environment culture, will have high concentration of Hg in tissues and it was accordance to severity of histopathological lesions and AMG pattern in $HgCl_2$ semi-static exposure groups. In group one single dose exposure, the Hg level decreased may be from elimination of those organs although some of them may accumulate in internal organs, such as trunk kidney and spleen. From the results of ICP-AES, muscle showed no significantly different between days in the same experimental group. It may describe that muscle was not the important organs for Hg accumulation in this study. It's because the form of Hg was different from the natural accumulation that inorganic Hg may change to organic Hg by biomethylation process before fish contaminated. In nature, organic form is the most important form that accumulates in muscle. (WHO, 2003) However, the total mercury concentration from ICP-AES analysis may be not accurate when tissue contained high concentration because the detection limit was between 20 ppb (20 µg/l) to 2 ppm (2 mg/l) (Han et al., 2006).

The previous reports MT gene mRNA expression has often been use as a sensitive and efficient biomarker for heavy metal exposure in various aquatic species (Gao et al., 2009). In the present study, the examining metallothionein gene expression from Hg-exposure-tilapia by RT-PCR used a method modified from Cheung et al. (2004) The primers were designed for RT-PCR reaction from MT cDNA of *O. mossamiba*. The internal control used forward and reverse primers of β-actin gene, house keeping gene for tilapia species, designed from GenBank (AY116536) (Hwang et al., 2003). The aspect positive bands were 127 bp for MT gene product and 90 bp for β-actin gene product. The results of RT-PCR showed many unspecific bands when using two pairs of primers and the results were all negative in control and IHC for MT positive groups when using only MT forward and reverse primers. Two unspecific bands were chosen for nucleotide sequencing (300 and 150 bp). The nucleotide sequences reveals no similarity between MT nucleotides that was suggested by Cheung et al. (2004). It may suggest that there are no similarity between *O. mossambica* and *O. niloticus* although there are highly conserved in MT among fish species (Chan, 1994). In tilapia, there are several studies of MT mRNA and gene expression. Nucleotide sequences *O. aureus* and *O. mossambicus* have been reported (Cheung et al., 2005) but the data in *O. niloticus* is still unclear. Thus, it is important to investigate the specific MT nucleotide of tilapia (*O. niloticus*) for approving specific RT-PCR method to detect MT gene expression in this species.

From all information in the previous study, there were no remarkable changes in histopathological lesions and the results of AMG and IHC for MT were negative in brain, heart, and muscle tissue. It may describe that those organs were not involved in acute mercury toxicity in tilapia.

## Conclusion

Mercury is also known as one of highly toxic heavy metal that contaminated in environment. Bioaccumulation process of mercury through the

food chain is primary cause for much of the concern with this metal. Tilapia (*O. niloticus*) is chosen as an experimental fish in Thailand because it is the most commonly found in local fish cultures. Semi-static exposure may be a suitable route for studying acute heavy metal toxicology and toxicopathology, such as mercury toxicity because it can produce severe and remarkable histopathological lesions.

According to this study, the main pathological lesions of mercury toxicity in tilapia were observed in kidneys, spleen, and hepatopancreas. Autometallography possibly use to study the toxicological profiles of heavy metal, such as mercury. This method is specific for mercury detection in tissue and can easily perform in ordinary pathology laboratory. Hg autometallographic pattern is dependent on type of heavy metals, sources of Hg, route of administration, dose and time of exposure.

Metallothionein is a protein that involved in heavy metal exposure in various animals, includes fish species. Therefore, detecting metallothionein is also specific on investigate heavy metal contamination in environment by using fish as a biomarker. Metallothionein can be investigate by immunohistochemistry technique and also investigate metallothionein gene or mRNA expression. Specific primers for *O. niloticus* cDNA may be necessary for specific RT-PCR reaction.

From this present study, it concludes that we can use pathological lesions, autometallography, an expression of metallothionine protein by immunohistochemistry to investigate acute mercury toxicity in tilapia.

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# APPENDICES

# APPENDICES

## Appendix A

Table 1A Total mercury concentration of gills

| Group | Day | Total Hg concentration (ppm/g dry weight) | | | |
|---|---|---|---|---|---|
| Control | 3 | 0.190 | 0.140 | 0.160 | |
| | 9 | 0.12 | 0.11 | 0.100 | |
| | 15 | 0.064 | 0.12 | 0.120 | |
| 0.5 i.p. | 3 | 2.000 | 0.947 | 0.524 | |
| | 9 | 0.730 | 1.133 | 0.800 | |
| | 15 | 0.320 | 0.778 | 0.962 | |
| 1 i.p. | 3 | 1.940 | 2.417 | 2.278 | |
| | 9 | 0.710 | 3.522 | 0.169 | |
| | 15 | 0.950 | 0.846 | 0.889 | |
| 2 i.p. | 3 | 0.400 | 11.368 | | |
| | 9 | 7.474 | 1.100 | 3.882 | |
| | 15 | 0.950 | 4.000 | 6.400 | |
| 5 i.p. | 3 | 2.090 | 4.695 | 4.912 | 4.879 |
| | 9 | 3.227 | 0.308 | 3.235 | |
| | 15 | 0.530 | 0.789 | 1.650 | |
| 0.5 oral | 3 | 0.437 | 0.316 | 0.433 | |
| | 9 | 0.305 | 0.311 | 0.095 | |
| | 15 | 0.151 | 0.444 | 0.500 | |
| 1 oral | 3 | 0.509 | 0.523 | 0.394 | |
| | 9 | 0.118 | 0.694 | 0.520 | |
| | 15 | 0.428 | 0.407 | | |
| 2 oral | 3 | 2.220 | 1.186 | 0.605 | |
| | 9 | 3.441 | 2.688 | 3.746 | |
| | 15 | 0.699 | 0.592 | 1.043 | |
| 5 oral | 3 | 2.840 | 3.552 | 3.875 | |
| | 9 | 2.220 | 1.186 | 0.605 | |
| | 15 | 1.915 | 1.475 | 0.925 | |
| 0.5 semi-static exposure | 3 | 10.295 | 17.266 | 19.980 | |
| | 9 | 38.545 | 27.861 | 44.178 | |
| | 15 | 53.664 | 57.891 | | |
| 1 semi-static exposure | 3 | 16.984 | 27.917 | 18.289 | 17.649 |
| | 9 | 51.412 | 19.889 | 29.402 | |
| | 15 | 42.125 | 47.819 | | |

Table 1B Total mercury concentration of visceral organ in abdomen

| Group | Day | Total Hg concentration (ppm/g dry weight) | | | |
|---|---|---|---|---|---|
| Control | 3 | 0.086 | 0.150 | 0.160 | |
| | 9 | 0.136 | 0.150 | 0.160 | |
| | 15 | 0.130 | | 0.130 | |
| 0.5 i.p. | 3 | 7.318 | 4.100 | | |
| | 9 | 0.690 | 1.136 | 3.240 | |
| | 15 | 0.440 | 3.273 | | |
| 1 i.p. | 3 | 1.490 | 16.938 | 25.842 | |
| | 9 | 8.133 | 12.619 | 9.143 | |
| | 15 | 0.300 | 0.364 | 8.692 | |
| 2 i.p. | 3 | 22.272 | 15.540 | 21.291 | |
| | 9 | 1.833 | 12.500 | 10.385 | |
| | 15 | 2.920 | 16.778 | 8.857 | |
| 5 i.p. | 3 | 2.400 | 57.720 | 30.242 | 36.154 |
| | 9 | 15.057 | 11.071 | 13.083 | |
| | 15 | 3.330 | 3.438 | 7.591 | |
| 0.5 oral | 3 | 0.981 | 0.633 | 1.116 | |
| | 9 | 0.088 | 0.333 | 0.568 | |
| | 15 | 0.444 | 0.395 | 0.395 | |
| 1 oral | 3 | 0.357 | 0.937 | 0.888 | |
| | 9 | 0.282 | 0.386 | 0.820 | |
| | 15 | 0.403 | 1.029 | 0.386 | |
| 2 oral | 3 | 7.054 | 5.461 | 6.283 | |
| | 9 | 4.995 | 4.760 | 2.914 | |
| | 15 | 1.643 | 1.221 | 1.289 | |
| 5 oral | 3 | 14.000 | 4.526 | | |
| | 9 | 4.612 | 3.094 | 4.103 | |
| | 15 | 2.902 | 3.959 | 2.185 | |
| 0.5 semi-static exposure | 3 | 4.683 | 67.821 | 35.465 | 25.148 |
| | 9 | 73.984 | 43.082 | 38.512 | |
| | 15 | 16.543 | 68.765 | | |
| 1 semi-static exposure | 3 | 14.590 | 25.597 | 121.274 | 97.053 |
| | 9 | 26.000 | 185.002 | 194.805 | 170.015 |
| | 15 | 148.118 | 130.895 | | |

Table 1C Total mercury concentration of muscle tissues

| Group | Day | Total Hg concentration (ppm/g dry weight) | | | |
|---|---|---|---|---|---|
| Control | 3 | 0.110 | 0.170 | 0.140 | 0.190 |
| | 9 | 0.090 | 0.190 | 0.240 | 0.200 |
| | 15 | 0.040 | 0.290 | 0.180 | 0.180 |
| 0.5 i.p. | 3 | 0.190 | 0.906 | 0.175 | |
| | 9 | 0.280 | 0.542 | 2.120 | |
| | 15 | 0.280 | 0.952 | 0.314 | |
| 1 i.p. | 3 | 0.860 | 4.150 | 0.600 | |
| | 9 | 0.340 | 2.571 | 1.852 | |
| | 15 | 0.330 | 0.136 | | |
| 2 i.p. | 3 | 0.370 | 1.154 | 1.618 | |
| | 9 | 2.576 | 2.914 | 4.000 | |
| | 15 | 0.750 | 4.050 | 2.952 | |
| 5 i.p. | 3 | 0.950 | 5.778 | 4.958 | 6.950 |
| | 9 | 0.381 | 5.885 | 3.318 | |
| | 15 | 0.720 | 1.596 | 1.818 | |
| 0.5 oral | 3 | 0.421 | 0.431 | 0.315 | |
| | 9 | 0.353 | 0.111 | 0.389 | |
| | 15 | 0.312 | 0.035 | 0.133 | |
| 1 oral | 3 | 0.083 | 0.175 | 0.038 | |
| | 9 | 0.271 | 0.074 | 0.190 | |
| | 15 | 0.400 | 0.355 | 0.256 | |
| 2 oral | 3 | 1.824 | 1.639 | 0.932 | |
| | 9 | 0.777 | 0.737 | 2.742 | |
| | 15 | 1.077 | 0.411 | 0.568 | |
| 5 oral | 3 | 0.696 | 0.280 | 1.308 | |
| | 9 | 0.416 | 0.368 | 0.372 | |
| | 15 | 0.256 | 0.555 | 0.845 | |
| 0.5 semi-static exposure | 3 | 0.657 | 2.435 | 2.973 | 3.683 |
| | 9 | 8.908 | 12.078 | 10.862 | |
| | 15 | 1.385 | 24.207 | 24.949 | 22.399 |
| 1 semi-static exposure | 3 | 0.733 | 3.601 | 5.291 | |
| | 9 | 2.505 | 12.467 | 15.152 | |
| | 15 | 2.505 | 32.024 | 13.222 | |

## Appendix B

### Preparing of 1,000 ppm $HgCl_2$ (stock $HgCl_2$)

0.675 g $HgCl_2$ (FW=271.50) added distilled water to yield 500 ml

### Reagent for autometallography

A. 1% potassium cyanide (5 g of potassium cyanide add with 500 ml of distilled water)
B. Physical developer
    a. 60 ml of 50% gum Arabic (30 g gum Arabic from acacia tree add with 30 ml of a distilled water and incubate in 60°C overnight)
    b. 10 ml of citrate buffer (2.55 g citric acid anhydrous and 2.375 g tri-sodium citrate dehydrate add with 10 ml of a distilled water)
    c. 30 ml of 5.6% hydroquinone (1.68 g of hydroquinone add with 30 ml of distilled water)
    d. 0.5 ml of 17% silver nitrate (0.34 g of silver nitrate add with 2 ml of distilled water)
C. 5% sodium thiosulfate (25 g of sodium thiosulfate add with 500 ml of distilled water)
D. Farmer's solution
    a. 0.6 ml of 7.5% potassium ferric cyanide (0.75 g of potassium ferric cyanide add with 10 ml of distilled water)
    b. 2.4 ml of 2% sodium thiosulfate (2 g of sodium thiosulfate add with 100 ml of distilled water)
    c. Add with 120 ml of distilled water

**Reagent of immunohistochemistry**

A. 0.1 M phosphate buffer saline (stock PBS)
    a. 40 g sodium chloride
    b. 1 g potassium chloride
    c. 5.75 g di-sodium hydrogen orthophosphate
    d. 1 g potassium dihydrogen phosphate
    e. Add distilled water to yield 500 ml
B. Citrate buffer pH 6.0
    a. citric acid
C. Peroxidase blocking solution
    a. 750 µl of 30% hydrogen peroxide
    b. Add 75 ml of methanol
D. 1% BSA
    a. 1 g BSA
    b. 100 ml of working PBS (1X)

**Reagent of ICP-AES samples**

10 : 1 sulfuric acid : hypochroric acid

A. 900 ml of sulfuric acid
B. 100 ml of hypochloric acid

**Reagent for RT-PCR**

A. TBE (17 g TBE buffer powder (Amresco®, USA) add with 1 l of distilled water)
B. 1.5% agarose gel for electrophoresis
    a. 1.5 g agarose gel
    b. 100 ml of TBE

# BIOGRAPHY

Mr. Kasem Rattanapinyopituk was born on August 26, 1982 in Ratchaburi, Bangkok, Thailand. He graduated with Doctor of Veterinary Medicine (D.V.M., second class honor) in academic year 2006 from Faculty of Veterinary Science, Chulalongkorn University. He continued studying a master degree in Veterinary Pathobiology program of Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University on academic year 2007.