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砷暴露對魚之毒性和生化效應之比較探討以及生物指標之建立

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計畫中文摘要

水污染對人類健康及環境造成潛在的風險。當生物體暴露於特定的金屬污染物時，會造成多種不同的毒性效應，例如：立即性的死亡，或緩慢的引發癌症效應。本研究計畫之主要目標，將探討不同物種(III, V)砷暴露，對魚之毒性及其生化效應之關係。另外，對砷在不同的魚組織的濃度分布，以及其生化效應亦加以探討。另外，本計畫將建立砷暴露之生物指標。本研究計畫之結果，將有助於了解砷物種之毒性效應，以及其相關的生化反應，並藉由生物指標之建立，用來偵測水中砷污染。

關鍵詞： 砷、毒性效應、生化效應、生物指標

Abstract

Water contaminants cause a high potential risk to human health and the environment. Exposure of humans and animals to certain metal derivatives results in a variety of toxic effects ranging from immediate mortality to slow process of carcinogenesis. The primary goal of this proposal is to investigate the comparative toxicity of arsenic compounds and the corresponding biochemical effects in aquatic organisms as well as developing mechanism-based biomarkers. The toxicological responses of different redox speciation of arsenic in aquatic system will be investigated. Moreover, the biochemical effects of arsenic at different target sites of fish tilapia will be examined. Furthermore, biomarker of arsenic exposure and effect will be developed. The studies employed in this proposal will reflect the specific toxic effect of arsenic and reveal fundamental relationships between chemical structures and biological activity.

Keywords：arsenic, comparative toxicity, biochemical effects, biomarker

研究計畫之背景及目的

Overview

Water contaminants cause a high potential risk to human health and the environment. Exposure of humans and animals to certain metal derivatives results in a variety of toxic effects ranging from immediate mortality to slow process of carcinogenesis. Therefore, the environmental metal toxicity is becoming a problem of significant concerns for human and ecosystems. Increased awareness of metal contamination in aquatic ecosystems and identification of metals as carcinogens has generated public and scientific concern regarding human health and ecological risks. However, our ability to assess these risks is limited by poor understanding of (1) the relationship between metal external/internal concentrations and distribution to target sties of receptors, (2) environmental factors that control redox speciation of metals, and (3) biochemical effects of metal and mechanism of metal toxicity as well as corresponding mechanisms of cellular defense.

Protection from toxic effects of environmental water pollutants primarily involves considering the mechanism of low level toxicity and likely biological effects in organisms that live in these polluted waters. Thus, an understanding of mechanisms of toxicity at molecular level is important. Hence, it is important to understand what is being found at the molecular levels of enzymes and other biomolecules may have had a relationship to the organisms' response. Information obtained from the biochemistry or cellular physiology of toxic response will in turn suggest modes of interaction between the chemical and the organism, which may be tested.

Molecular basis for metal toxicity is generally related to certain biochemical and physiological changes in the organisms. Toxicity may result from acute and overwhelming exposure or it may be the result of a long or chronic exposure to low levels of the toxicants, and hence the mechanism of action may be very different.

Heavy metals are both ubiquitous and long-lived in the environment and play a dual role in biological systems. Some metals have no known function in cells, but have toxic effects: arsenic and cadmium are examples. Metal ions can harm cells by a variety of ways including inactivation of enzymes and by catalyzing the oxidative damage of lipids, proteins, and nucleic acids (Beysersmann, 2002). Consequently, cells often alter the expression of genes that encode proteins for the detoxification of metals when elevated concentrations are detected (Beysersmann, 2002).

Among heavy metals, arsenic has driven special attention to researchers. Arsenic has been classified as human carcinogens. Water contamination by arsenic poses a high potential risk to human health and the environment. To better assist accurate risk assessment posed by arsenic, it is important to study the biochemical effects of these metals in aquatic ecosystems.

Arsenic Exposure

Arsenic, a known human carcinogen and teratogen (Kitchin, K. T., 2001), is a widespread environmental contaminant that enters water, food, and air from both natural sources and human

activities (Hartwig, 1995). Weathering of arsenic-containing rocks, metal processing, burning of fossil fuels and using arsenic pesticides all contribute to arsenic contamination in freshwater systems. This contamination is a source of concern because of arsenic's potent toxicity. At high levels, arsenic can be lethal to many freshwater organisms. At the lower concentrations typical of most contaminated aquatic ecosystems, arsenic produces more subtle effects that can threaten the survival of aquatic life (Del Razo, et al., 2001). Relatively small amounts of arsenic also pose cancer and other health risks to humans.

Arsenic was ranked first on the Agency for Toxic Substances and Disease Registry / Environmental Protection Agency "Top 20 Hazardous Substances Priority List" in 2001. Arsenic also tops the U.S. Environmental Protection Agency's list of chemicals of concern at toxic waste sites. Exposure to arsenic in drinking water represents a significant health problem for people around the world. In 1997, the World Health Organization recommended that arsenic in drinking water be recognized as a major public health issue that should be addressed on an emergency basis.

In nature, As is found as oxides or sulfur compounds and it is mainly distributed throughout the environment by water. Among the chemicals species of As present in the environment, inorganic arsenic (iAs) is generally considered the most hazardous (Moore et al., 1997; Yamauchi and Flwler, 1994). The characteristics of As salts' reactivity and toxicity depend on their oxidative states, the trivalent form being the most reactive (Del Razo et al., 2001).

In humans, exposure to arsenic has been linked to increased risk of cancer, heart disease, diabetes and reproductive disorders (Garcia-Vargas and Cebrian, 1996). However, most studies linking arsenic with human disease have involved people exposed to very high levels - in the workplace, for example, or in parts of Taiwan, Pakistan and other areas of the world where levels of arsenic in drinking water are unusually elevated. In Bangladesh, India, an estimated 70 million people have been slowly poisoned by well water tainted by arsenic from natural sources deep within the ground (Garcia-Vargas and Cebrian, 1996).

The toxicity of As compounds highly depends on the oxidation state and chemical composition of the arsenical (Moore et al., 1997; Yamauchi and Flwler, 1994). As^{III} appears to be the most toxic regardless of being inorganic or organic form. As^{III} toxicity could be carried out either directly, by attacking –SH groups, or indirectly, through the generation of reactive oxygen species (ROS) (Chen et al., 1998). The toxicity of iAs^{V} appears to be mediated through its ability to substitute phosphate groups, affecting enzymes that depend on this group for its activity.

Arsenic is an important xenobiotic in aquatic ecosystems. Because fish are an important food resource and a major ecosystem component, it is important to assess the biochemical effects of arsenic in fish, determine acceptable levels of arsenic in the aquatic environment, and develop new methods for detecting deleterious effects of arsenic at an early stage.

Stress Proteins induced by arsenic

The stress response is a phenomenon of adaptation of organisms. The induction of stress

protein synthesis is highly tissue specific and it is related to damage induced by stress to specific proteins and protein complexes (Ritossa, 1996). This response is also metal specific and it is related factors such as mode of metal uptake, distribution and accumulation among tissues, subcellular distribution within tissues, and secondary generation of molecular stressors (Honda et al., 1992; Bauman et al., 1993). Target-tissue-specific alterations in protein synthesis may represent "biochemical fingerprint" or toxicological signature" of exposure and toxicity, respectively. However, knowledge about how cells adapt to stress at the molecular or cellular levels is limited.

Exposure to arsenicals either *in vitro* or *in vivo* in a variety of model systems has been shown to cause the induction of a number of the major stress protein families such as metallothionein (MT) and heat shock proteins (HSP) (Maitani et al., 1987; Albores et al., 1992). The study of the HSP genes has revealed that all of them play similar roles and contain highly conserved homologous sequences among very different organisms ranging from bacteria to humans (Del Razo et al., 2001). In most of the cases, the induction of stress proteins depends on the capacity of the arsenical to reach the target, its valence, and the type of exposure, arsenite being the biggest inducer of most HSP in several organs and systems. HSP induction is a rapid dose-dependent response (1-8 h) to the acute exposure to arsenite (Kitchin et al., 1999; Wijeweera et al., 2001). Thus, the stress response appears to be a viable biomarker to monitor the sub-lethal toxicity resulting from exposure to arsenic.

HSPs as biomarkers of arsenic exposure / effect

In environmental mixture, the often-unpredictable antagonistic, synergistic or additive effects between pollutants can occur that make complete chemical analysis complicated. Chemical analyses of complex mixtures are expensive and time consuming and they can not reflect or predict the ultimate bioavailability of a chemical. In this respect, lethality tests have long been used to supplement chemical analyses. However, in majoring acute toxicity at high doses, these tests have little or no predictive value for chronic exposure at lower dose levels making that lethality is too coarse a measure for toxicity (Basu et al., 2002). In contrast, changes at biochemical level are usually the first detectable responses to environmental pollution. This approach examines the effect at the site of the interaction of chemicals with the different macromolecules in the cell. When the target molecule is part of the defense, repair or detoxification mechanism of the cell, it becomes a direct and specific marker of exposure and effect (biomarker). Therefore, biomarkers may be used in an early warning system to predict biological effects for a wide range of environmental contaminants such as heavy metals (Basu et al., 2002).

It has been suggested that HSPs can be used as biomarkers of environmental pollution (Basu et al., 2002). Three aspects of HSP have been characterized in order to be used as biomarkers of pollution: (1) they are part of the cellular protective response; (2) their synthesis is likely to be induced by a large number of chemicals; and (3) they are highly conserved in all organisms from bacteria to plants and man (Bierkens, 2000). Among HSPs family, studies on

the induction of HSP70 by environmental stressors have been demonstrated in various organisms and the regulation of hsp70 gene expression occurs mainly at the transcriptional level (Basu et al., 2002).

Since hsp70 genes are highly conserved among organisms (Morimoto, 1994), we hypothesized that hsp70 gene expression will be induced in response to arsenic exposure in fish tilapia (*Oreochromis mossambicus*). Therefore, We propose that expression of heat shock protein 70 is sensitive to levels of arsenic well below those that kill organisms outright. We will measure protein and messenger RNA in the fish and use this measurement to provide a reliable molecular indicator of arsenic exposure. We expect that the stress protein response can be used as a useful endpoint, or biomarker, for detecting chemical stress in ecosystems.

The primary goal of this proposal is to investigate the comparative toxicity of arsenic compounds and the corresponding biochemical effects in aquatic organisms as well as developing mechanism-based biomarkers. In the original proposal, we proposed four specific aims to accomplish the research goal with 3 years period. However, the NSC only supported one year funding. Therefore, we have adjusted our focus on some experiments. We use arsenic as our focus of study and fish tilapia (*Oreochromis mossambicus*) as experimental organism. The toxicological responses of arsenic in aquatic system will be investigated. Additionally, the biochemical effects of metals at different target sites of aquatic organisms will be examined. Furthermore, mechanism-based biomarkers will be developed. The studies employed in this proposal will reflect the specific toxic effect of arsenic and reveal fundamental relationships between chemical structures and biological activity. The information generated from this proposal enables mechanisms of arsenic toxicity to be elucidated at the molecular and cellular level and the development of an early warning system for the exposure to arsenic.

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研究方法及進行步驟

Animals

Juvenile tilapia (*Oreochromis mossambicus*) was acclimated for 2 weeks prior to the experiments. 50 animals were kept individually in glass aquaria (one fish per aquarium) with constant aerated freshwater (25 l) at water temperatures of 22-25 ^{o}C. Temperature was recorded and water quality determined for DO_2, pH in the beginning of experimental period.

Metal exposure

After acclimatization, fish was weighted, maintained individually in glass aquaria that were randomly allocated to control and different concentrations of arsenic (iAs^{III})(10 isolated fish for each group).

Subchronic toxicity test

Subchronic toxicity tests are performed to determine the growth rate parameter during different sub-lethal level of metal exposure. During the experimental period, all groups will be fed daily (3% of body weight - BW) and the unfed diet remains is collected and dried to constant weight. The water is changed once a week to maintain As concentrations. Fish were exposed to a range of arsenic(iAs^{III})c: 0, 1, 2, 4 ppm for 4 weeks. After this period, they are removed at

the same time from each aquarium, anaesthetized in benzocaine before killing by transecting the spinal cord. The fish are put on water- dissolved benzocaine (80 mg/l) and are anesthetized in seconds. No side effects were related with this procedure. Liver, muscle, gills, and kidney are removed, weighed, frozen in liquid nitrogen and stored at -80^{o}C for further metal analyses.

Arsenic analyses

The inorganic arsenic compound is measured using atomic absorption spectrometer.

RNA isolation

Frozen tissues are thawed and homogenized in TRIzol Reagent (GibcoBRL) using a glass-Teflon homogenizer according to the manufacturer's instructions. Briefly, after 5 min room temperature incubation (20°C), chloroform is added for phase separation. The upper aqueous phase is collected, and the RNA is precipitated by mixing with isopropyl alcohol. The RNA pellet is washed once with 75% ethanol, and the RNA pellet is air-dried and finally re-dissolved in RNase-free water.

Semi-quantitative RT-PCR

RT-PCR is performed using Ready-To-Go^{TM} RT-PCR beads (Amersham Pharmacia Biotech). Briefly, total RNA is diluted to 1 µg/µl in RNase-free water, mixed with 0.5 µg of pd $(T)_{12-18}$ and 47 µl of RNase-free water to a final volume of 49 µl in a reaction tube containing RT-PCR bead. The reaction is incubated at 42°C for 30 min, followed by 95°C for 5 min to inactivate the reverse transcriptase and completely denature the template. Forward / reverse primers to the amplification of HSP70 will be designed from the literatures. One of the gene specific primer sets (5' and 3'primers for HSP70) is added to give a final volume of 50 µl. The number of PCR cycle is varied to determine the optimal number that would allow detection of the appropriate messages, while still keeping amplification for these genes in the log phase. Five µl aliquots of the PCR reaction are collected after 18, 20, 22, 24, 26, 28 and 30 cycles to determine the linear range of the reaction, in two independent experiments. Control amplifications are done either without RT or without RNA. As a control, a DNA fragment of the constitutively expressed L18 ribosomal protein gene is amplified using the L18 primers. Following PCR amplification, the reaction products are run at 100 V on a 1% agarose gel with 0.5 µg/ml ethidium bromide. The HSP70 and L18 reactions are analyzed in the same slot on a 2% agarose gel. The total band volumes of amplified products are calculated by gel-documentation software (Kodark). All glass- and plastic ware are treated with diethyl pyrocarbonate and autoclaved.

Statistical analysis

All data are presented as mean ± standard deviation. The levels of HSP70 mRNA in the different tissues are analyzed by ANOVA to test if different heavy metal exposure causes significant quantitative changes in the gene expressions. The level of significance is set at p value < 0.05.

結果與討論

We conducted subchronic toxicity test for tilapia fish exposed to a range of sub-lethal level of arsenic exposure. Juvenile tilapia (*Oreochromis mossambicus*) was acclimated for 2 weeks prior to the experiments. Fish were kept in glass aquaria with constant aerated freshwater (25 L), under 12:12 light-dark cycle, at water temperatures of 22-25 ^{o}C. Temperature was recorded and water quality determined for DO_2, pH in the beginning of experimental period. Table 1 and Table 2 show the measurement of fish weight and size during arsenic exposure period.

Subchronic toxicity tests were performed to determine internal body burden and hsp70 gene expression for fish exposed to a range of sub-lethal level of arsenic exposure. During the experimental period, all groups were fed daily (3% of body weight - BW) and the unfed diet remains were collected and dried to constant weight. The water was changed once a week to maintain arsenic concentrations. Fish were exposed to a range of arsenic: 0, 1, 2, 4 ppm for 4 weeks. For each dose of metal, 10 fish were exposed. Control and each test concentration were conducted in duplicate. After this period, fish were removed at the same time from each aquarium, anaesthetized in benzocaine before killing by transecting the spinal cord. The fish were put on water- dissolved benzocaine (80 mg/l) and were anesthetized in seconds. No side effects were related with this procedure. Liver, muscle, gills, intestine, blood, and kidney were removed, weighed, immediately frozen in liquid nitrogen and stored at -80^{o}C for further analyses. Figure 1 shows where tissue samples were removed from tilapia fish.

The measurement of arsenic concentration in fish tissue is in the process of being determined by using atomic absorption spectrometer.

Table 1. Measurement of the weight of fish samples

Exposure period	Arsenic exposure concentration (ppm)						
	0	1		2		4	
NO.	0	1	1R	2	2R	4	4R
Week1	20.8	17.4	22.3	32.9	49.2	30.8	36.5
Week2	32.6	17.4	22.1	13.5	20.6	18.7	35.7
Week3	-	21.2	25.4	25.6	16.1	15.4	24.4
Week4	-	28.2	19.5	22.0	21.2	18.4	14.3

(unit：g)

Table 2. Measurement of the size (length) of fish samples

Exposure period	Arsenic exposure concentration (ppm)						
	0	1		2		4	
NO.	0	1	1R	2	2R	4	4R
Week1	10.2	10.2	12.3	14.0	14.5	12.5	14.2
Week2	13.1	10.3	11.2	9.8	11.3	10.5	13.5
Week3	-	11.0	11.3	11.7	10.0	10.0	11.7
Week4	-	12.5	11.1	11.2	11.0	10.2	9.7

(unit：cm)

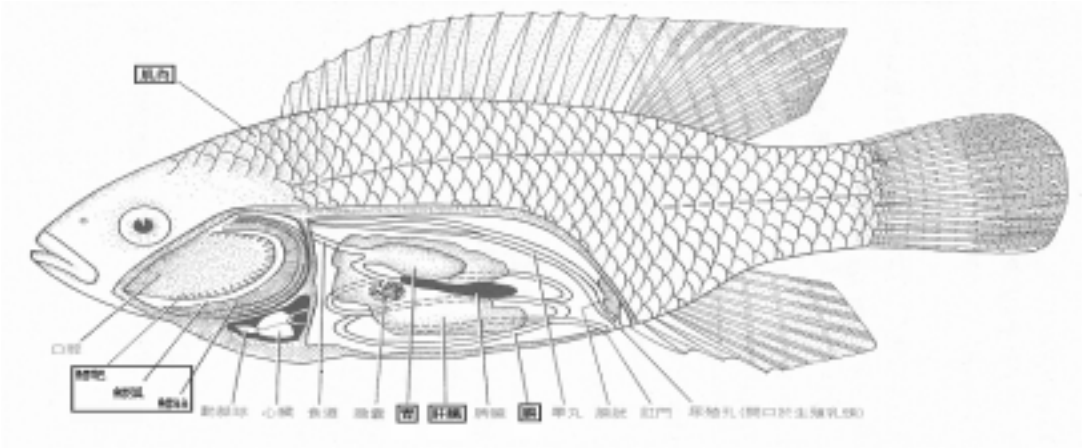

Figure 1. This figure shows where the tissues samples were removed from tilapia fish in current research.

We isolated RNAs from arsenic-exposed fish tissues. Frozen fish tissues (liver, muscle, gills, intestine, blood, and kidney) were thawed and homogenized in TRIzol Reagent (GibcoBRL) using a glass-Teflon homogenizer according to the manufacturer's instructions. Briefly, after 5 min room temperature incubation, chloroform was added for phase separation. The upper

aqueous phase which contains RNA was collected, and the RNA was precipitated by mixing with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol, and the RNA pellet was air-dried and finally re-dissolved in RNase-free water.

The amount and yield of isolated RNAs from various fish tissues were quantified by measuring A_{260} and ratio of A_{260} / A_{280} was used to indicate the quality of RNAs. In addition, the integrity and quality of RNAs were further examined by electrophoresis of RNAs through Agarose Gels Containing Formaldehyde. The representative RNA gel was shown in Figure 2.

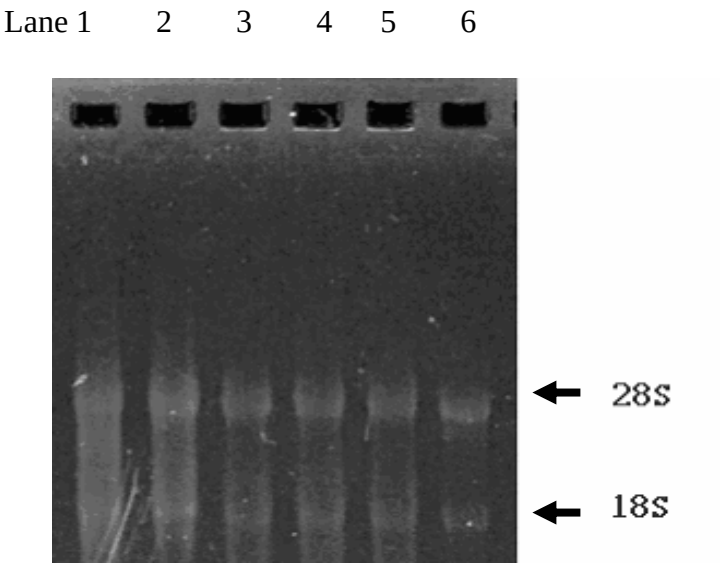

Figure 2. Representitive RNAs gel. Purified RNAs from liver, muscle, gills, intestine, blood, and kidney were located in lane 1 ~6, respectively.

In semi-quantitative RT-PCR experiment, we were unable to detect the hsp70 transcript. There are several possibilities to account for such issue. One is that the exposure concentration of As(III) is not high enough to induce the hsp70 gene expression. Currently we are exposing fish with higher concentration of As(III). Quality of RNA may represent the other important factor affecting the detection of hsp70 transcript in RT-PCR experiment. This is can be overcome by isolation of RNA with careful caution to avoid RNase activity.