A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Toxicity evaluation of marine algal arsenic in zebrafish model

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February, 2016

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A thesis submitted in partial fulfillment of the requirement

for the degree of Master of Science

2016. 02.

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2016.02

Date

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국문초록

제브라피쉬는 인간과 유전적 상동성과 허파를 제외하고는 간과 췌장, 지라와 흉선 등 면역계를 포함한 대부분의 기관을 가지고 있는 척추동물로 최근 실험동물 모델로써 각광받고 있다. 이들은 체외수정을 하며, 수정란을 대량으로 쉽게 확보할 수 있다. 그리고 난이 투명하여 배아의 발생과정, 혈류 흐름 및 형태적 관찰이 용이하며, 크기가 작아 공간의 제약을 받지 않는다. 또한 발생이 매우 빨라 대부분의 조직 및 장기가 하루 만에 형성된다. 이러한 장점을 이용하여 최근 독성연구에서도 동물모델로서 이용 되고 있다.

중금속은 인간의 건강을 위협하는 물질로 간주되면서 중요한 관리 대상이 되고 있다.

이중 비소는 극미량 원소 중 하나이며 다양한 형태의 화합물로 무기화합물 또는 유기화합물 형태나 비금속 상태로 지각과 생물, 환경 중에 널리 분포하는 금속 물질로서 강한 독성을 가지고 있는 주요 환경오염물질이다. 국제 암 연구기관 (International Agency for Research on Cancer)에서 비소는 인간에게 암을 일으키는 (carcinogenic) 물질, 혼합물, 노출환경 등인 'Group 1'로 평가될 만큼 유해성이 높

다. 비소를 섭취하는 것은 신경학상, 심장혈관 상의 문제뿐만 아니라 피부암, 방광암과 폐암의 원인이 될 수 있다 (ATSDR., 2007; Ming-Ho., 2005; EPA., 2011; I. G. Jeoung., 2004). 비소의 독성과 관련된 논쟁은 전 세계적인 관심의 대상이고, 몇 백만 사람들이 비소 오염에 의해 영향을 받았다.

지구 표면의 71%를 차지하고 있는 해양에는 다양한 자원을 보유하고 있으며 저온, 고온, 고압, 고염 등 다양한 극한환경을 내포하고 있다. 이러한 환경에 서식하는 해양생물자원은 세계 동물성 단백질 공급량의 약 6분의 1을 차지할 정도로 식량자원으로서 매우 중요한 부분을 차지하고 있다.

해조류(seaweed)는 전 세계적으로 바다에 서식하고 있는 수산물이다. 전 세계적으로 약 6,000종, 식용으로는 150여종이, 한국에서만 약 500종이 발견되고, 식용으로는 50여종이 이용되고 있다. 많은 종류의 해조류가 식품, 사료, 비료, 공업원료, 의약품, 그리고 심지어는 오염물질 제거용으로 이용되고 있다.

그러나 급격히 산업화가 진행되고 경제가 빠르게 성장하면서 사람들의 관심은 웰빙을 비롯한 건강과 장수에 대하여 증가하고 있다. 더불어 환경오염, 스트레스 등에 따른 건강 위해 요인도 점점 증가하고 있어서 건강한 삶에 대한 사람들의 관심과 요구가 더 증가하고 더불어 건강식품에 대한 관심도 증가하고 있다. 이

에 따라 사람들에게 건강식품으로 그리고 산업에서 식품 및 의약품 원료로서 해조류의 이용이 증가하고 있다.

해조류에는 육상식물에 비하여 많은 비소가 함유되어 있으며 높은 비소함량에도 불구하고 대부분이 독성이 거의 없다고 알려진 유기비소인 Arsenobetaine 형태로 존재하기 때문에 안전하다고 한다(Abernathy CO., 2003). 그러나 해조류 중의 비소함량에 대한 기준 및 규격은 아직까지 정해지지 않고 있는 실정이다. 우선, 해조류에 존재하는 유기비소 화합물을 비롯한 총 6개의 유기비소화합물, 2개의 유기비소 화합물을 제브라피쉬 embryos에 0.25, 0.5, 그리고 1ppm의 농도로 처리하였으며 생존율, 심박수, 부종크기 및 형태학적 관찰 등을 통해 유기비소 화합물보다 무기비소 화합물의 독성이 센 것을 확인하였다. 성체에서도 이와 같은 실험을 진행하였으며 5, 10, 그리고 20ppm 농도의 각 화합물에 노출된 간 조직을 조직학적 및Real-time PCR과 western blot을 통해 apoptosis pathway의 관련 인자 및 단백질을 확인하였다. 그 결과, 모든 농도에서 유기비소 화합물보다 무기비소화합물로 인한 독성으로 간 조직 병변 및 apoptosis 관련 인자 및 단백질의 발현을 통해 유기비소 화합물 보다 무기비소 화합물로 인한 독성이 심한 것을 확인하였다.

특히, 해조류에 포함된 유기비소 화합물(AsB)를 처리한 제브라피쉬에서는 유의적인 차이가 없음을 확인하였다. 따라서 본 연구에서는 영양가가 풍부하고 생리활성작용 및 건강기능성을 가지고 있는 자연식품 및 기능성식품의 원료로서 이용이 증가하고 있는 제주 연안에 서식하고 있는 감태, 미역, 그리고 톳으로부터의 비소를 추출하여 비소추출물에 대한 독성평가를 동물모델인 제브라피쉬를 통해 우리가 섭취하는 해조류의 안전성을 확인하였다. 해조류의 비소추출물을 embryos에서 100, 500, 그리고 1000ppm의 농도로 처리하였으며 생존율, 심박수, 부종 크기 및 형태학적 관찰 등을 통해 1000ppm에서 처리하지 않은 그룹과 비교하여 유의적으로 차이가 있음을 확인하였다. 성체에서도 이와 같은 실험을 진행하였으며200, 400, 그리고 800ppm의 농도로 처리하였으며800ppm에서 각 해조류의 추출물에 노출된 간 조직을 조직학적 및 Real-time PCR을 통해apoptosis pathway의 관련 인자를 확인하였다. 그 결과, 각 해조류의 비소추출물에 노출된 성체는 모든 농도에서 다 생존하였으나 800ppm의 농도로 처리된 그룹의 간 조직 및 apoptosis 관련 인자를 확인한 결과 처리하지 않은 그룹에 비해 유의적으로 차이가 있음을 확인하였다.

추출 후 해조류 비소의 건조중량에 비교하여 100ppm농도의 감태, 미역, 그리

고 톳의 비소 추출물의 비소함량은 각 4.9, 3.5, 그리고 5.4 mg/kg 함량으로 계산되었다. 비소함량이 최대인 톳 5.4 mg/kg을 기준으로 했을 때 연 평균 0.1 g의 톳을 섭취하는 한국인에게서 1일을 기준으로 섭취하였을 때 약 0.3 mg의 양으로 이는 1일 잠정섭취허용량의 약 228배임을 확인하였다.

이 모든 결과를 종합해 볼 때 OECD guide line에 따른 독성평가에서 유기비소가 무기비소에 비해 독성이 없음을 확인하였으며 비소 1일 잠정섭취허용량은 2 μg/kg이다. 60kg의 몸무게를 가진 성인의 비소 1일 잠정섭취허용량은 120 μg/kg으로, 연구에 사용된 시료 중 비소함량이 최대인 톳 100ppm (5.4mg/kg)을 기준으로 했을 때, 22.2 g/day의 톳을 섭취한 양에 해당된다. 따라서, 한국인에게서 22.2 g/day 이상의 톳을 지속적으로 먹지 않는 이상 톳의 섭취에 따른 독성 가능성은 미비하여 안전한 수준으로 판단되어진다.

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Fig. 1-1. Measurement of survival rates after treating with arsenic compounds. (A) 0.25ppm arsenic compounds, (B) 0.5ppm arsenic compounds, and (C) 1ppm arsenic compounds. The zebrafish were treated with various of concentrations of arsenic compounds. And then we measured survivorship of zebrafish for 7dpf. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

Fig. 1-2. Measurement of toxicity of the arsenic compounds by the heartbeat rate. (A) 0.25ppm arsenic compounds, (B) 0.5ppm arsenic compounds, and (C) 1ppm arsenic compounds. The heartbeat rate of both atrium and ventricle were counted at 48 hpf by under the microscope for 1 min. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

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Fig. 1-4. Measurement of toxicity of the organic and inorganic arsenic compounds by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 24 hpf.

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Fig. 1-23. Western blot analysis of Cleaved Caspase 3, Cleaved Caspse 9, and Caspase 8 in the liver tissues of adult zebrafish with arsenic compounds of 20ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

Fig. 2-1. Measurement of survival rates after treating with the algae arsenic. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The zebrafish were treated with various of concentrations of arsenic extracts. And then we measured survivorship of zebrafish for 7dpf. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

Fig. 2-2. Measurement of toxicity of the algae arsenic by the heartbeat rate. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The heartbeat rate of both atrium and ventricle were counted at 48 hpf by under the microscope for 1 min. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

Fig. 2-3. Measurement of toxicity of the algae arsenic by the yolk sac edema size. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The yolk sac edema size was measured at 48 hpf using the microscope. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

Fig.2-4. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 24 hpf

Fig.2-5. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 48 hpf.

Fig.2-6. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 72 hpf.

Fig.2-7. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 96 hpf.

Fig.2-8. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 120 hpf.

Fig.2-9. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 144 hpf.

Fig. 2-10. Measurement of toxicity of algae arsenic by the cell death. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The cell death was measured by image analysis using fluorescence microscope and levels using Image J. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values

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Fig. 2-11. Measurement of toxicity of the algae arsenic compounds by the ROS production. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The ROS production was measured by image analysis using fluorescence microscope and levels using Image J. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

Fig. 2-12. Expression of p53 (A), Bax (B), Bcl-2 (C), Caspase-3 (D), Caspase-9 (E), and Caspase-8 (F) mRNA species in the embryos treated with algae arsenic of 100 and 500ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

Fig.2-13. Measurement of survival rates after treating with algae arsenic. (A) 200ppm algae arsenic, (B) 400ppm algae arsenic, and (C) 800ppm algae arsenic. The zebrafish were treated with vatious of concentrations of arsenic extracts. And then we measured survivorship of zebrafish for 7dpf. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

Fig. 2-14. Measurement of toxicity of algae arsenic by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and

malformation in the adult zebrafish at 7 days post-exposure after treated arsenic extracts with various concentrations.

Fig. 2-15. The bill duct of zebrafish at 7 days post-exposure after treated ECAE, UPAE, and HFAE with various concentrations.

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Fig. 2-17. Expression of p53 (A), Bax (B), Bcl-2 (C), Caspase-3 (D), Caspase-9 (E), and Caspase-8 (F) mRNA species in the liver tissues of adult zebrafish treated with algae arsenic of 200, 400, and 800ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

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Introduction

Heavy metals are significant environmental pollutants, and their toxicity are found naturally in the earth. Also, toxicity of heavy metals has been recognized as serious pollutants of the aquatic environment with harmful effects on the associate organisms (Akintade O.A., 2013). Also, toxic heavy metals become concentrated as a result of human caused activities.

Arsenic is an environmental pollutant and can occur from natural sources such as rocks and sediments and also as a result of anthropogenic activities such as coal burning, copper smelting and the processing of mineral ores (Choi, H.P., 2011). It exists in inorganic and organic forms and in different oxidation sates (-3, 0, +3, +5). Levels of arsenic in the aquatic environment are higher than in most areas of land as it is fairly water-soluble and may be washed out of arsenic-bearing rocks. In particular, seaweed is known to contain high concentrations of arsenic in comparison to terrestrial plants due to the ability of marine plants to concentrate the arsenic they derive from sea water (Zhao, Y.F., 2014).

In recent years, many people have increased interest in the health care considered important for safety of the drug. Therefore, it is expected to become effective therapies together with a natural product to apply such societal expectations. Seaweeds are known to compose a variety of bioactive substances (polysaccharides, pigments, minerals, peptides, and polyphenols) with valuable pharmaceutical and biomedical potentials (Kim. E.A., 2013). Consumption of fish and seafood is known to have beneficial health effects and it is recommended that the general population in some countries, especially where fish consumption is lower than average, should increase the amount of fish (especially fish rich in oils) in the diet (Food Standards Agency, 2006 and COT, 2004a). However, it has been a significant concern caused by arsenic content of seaweeds (R.N. Ratnaike., 2003).

Arsenic occurs in two oxidation states including a trivalent form, arsenite (As2O3; As III) and a pentavalent form, arsenate (As2O5; As V). Arsenic exists in different chemical forms, or 'species'. These are either as 'free' inorganic arsenic species such as As(III) or As(V), or as arsenic present in organic molecules such as arsenobetaine and arsenosugars (Francesconi and Kuehnelt, 2004). Inorganic arsenic has no known beneficial effect, and long-term exposure may be harmful to health.

Risk assessment is defined as the evaluation of the probability of known or potential adverse health effects arising from human or animal exposure to the identified hazards (FAO/WHO, 1996., FAO/WHO, 2000., EFSA, 2008). The OECD Test Guideline for in vivo toxicological studies is described that the experimental material to test and the practical conditions used to test such as target animal species, housing, number of doses administered gender and number of animals, etc (Organisation for Economic Co-Operation and Development, 1998). The appropriate methods used to measure the responses of phenotype (body weight, food consumption, clinical biochemistry, etc.) in test animals throughout the test are also provided (Chelsea, 2012). Based on this protocol, a proposal for a new guideline on fish embryo toxicity for the testing of chemicals has been described to the OECD by the German Federal Environment Agency (Braunbeck and Lammer, 2006., Kirsten, 2011). One of the proposed alternatives has been used for the early life stages of fish to the experimental model (Claudia W, 2000., Nagel, 2002., Hutchinson et al., 2003., Benoit Fraysse, 2006). The scope of fish embryo tests as an animal alternative method in hazard and risk assessment and scientific research has been reviewed (Embry, 2010).

As an animal model, the zebrafish (*Danio rerio*) has been widely used in studies on molecular genetics and development biology, drug discovery, and toxicology because of their physiological similarity to mammals (Kimmel, 1995., Hertog, 2005). Moreover, the zebrafish

has been used to evaluate the toxicity of a chemical which was essential to identify the endpoints of toxicity and their dose-response relationship, elucidate the mechanisms of toxicity, and determine the toxic dynamics of the chemical (Adrian, 2005., Sun, 2013). The zebrafish has numerous advantages as a toxicological model species. For example, their comparatively small size, fecundity, large clutches, low cost, and rapid embryonic development in vivo (Eisen, 1996., Fishman, 1999). In addition, the zebrafish has a very short development, plan of the basic body is laid out 24 h post-fertilization (hpf), embryos hatch approximately 2-3 days post-fertilization (dpf) and they complete maturity at about a 3 months. Furthermore, one female can spawn about 100 eggs per day, which are fertilized by sperm released into the water by males (Kim, 2014).

Apoptosis is a controlled form of cell death that serves as a molecular point of regulation for biological processes. Cell selection by apoptosis occurs during normal physiological functions as well as toxicities and diseases. Insights into the mechanisms governing apoptosis and increasing appreciation of the relevance of apoptotic cell death are redirecting research in toxicology and carcinogenesis and are yielding novel therapeutic approaches for the control of toxicity, disease, and ultimately perhaps senescence (Apoptosis: molecular control point in toxicity, toxicology and applied pharmacology, 1994, G.B. Corcoran) To study the mechanism of arsenic induced apoptosis, we evaluated the expressions of p53, Bax, Bcl-xL, caspase-3, -9, and caspase-8 by RT-PCR and western blot analysis in the zebrafish model.

For these reasons, zebrafish model were used to investigate the developmental toxicity induced by organic and inorganic arsenic compounds and algae arsenic. To evaluate the toxicity of arsenic from seaweeds (*Ecklonia cava*, *Undaria pinnatifida*, and *Hzikia fusiformis*), the zebrafish was used in a model for *in vivo*.

Part Ⅰ.

Comparison of toxicity in zebrafish for organic and inorganic arsenic compounds

Part Ⅰ.

Comparison of the toxicity of organic and inorganic arsenic compounds in zebrafish

1. Abstract

One of the most toxic metals, As is widespread in the environment with a compound of various types. Arsenic as significant risk substance has been ranked by Agency for Toxic Substances and Disease Registry (ATSDR). Zebrafish (*Danio rerio*) has been developed into an important model organism for biomdeical research over the last decades. Also, zebarafish have advantages over mammals as an animal model for evaluating toxicity. Therefore, we investigated the comparison between inorganic arsenic compounds and organic arsenic compounds through survival rates, heart-beat rate, yolk sac edema size, cell death, ROS production and morphology in zebrafish embryos. 4-hour post-fertilization (hpf) zebrafish embryos were exposed to various concentrations of arsenic compounds (0.25, 0.5, and 1ppm) until 96 hpf. Also, adult zebrafish was investigated in this study through survival rates, morphology, real-time PCR and western blot. Adult zebrafish were exposed to various concentrations of arsenic compounds (5, 10, and 20ppm) for 96 hours. Through the results, zebrafish was strongly affected by exposure from inorganic arsenic compounds. Furthermore, zebrafish can serve as a reliable model for the toxicity of arsenic compounds.

2. Material and methods

2.1. Materials

Each test arsenic compounds, reagent name, chemical formula, and the use of the compounds are listed in Table 1. Six of the organic arsenic compounds are Acetarssone (Act), Arsenobetaine (AsB), Arsenocholine Bromide (AsCB), Methylarsonic acid (MAA), Roxarsone (ROX), Dimethylarsinic acid (DMA), and Triphenylarsine (AsPh3). Two of the inorganic arsenic compounds are Sodium arsenite (Asi) and Sodium arsenate diabasic heptahydrate (Asa).

Table 1-1. The list of organic and inorganic arsenic compounds

Reagent	Chemical Formula	Use	
Acetarsone	$C_8H_{10}AsNO_5$	Antiprotozoal agent	**Act**
Arsenobetaine	$C_5H_{11}AsO_2$	Seaweed	**AsB**
Arsenocholine Bromide	$C_5H_{14}AsBrO$	Marine organism	**AsCB**
Methylarsonic acid	CH_5AsO_3	Germicide	**MAA**
Roxarsone	$C_6AsNH_6O_6$	Controlling parasites	**ROX**
Dimethlyarsinic acid	$(CH_3)_2As(O)OH$	Seaweed	**DMA**
Triphenylarsine	$As(C_6H_5)_3$	Ligand and a reagent	**AsPh3**
Sodium arsenite	$NaAsO_2$	Pesticide, Insecticide	**Asi**
Sodium arsenate diabasic heptahydrate	$Na_2HAsO_4{\cdot}7H_2O$	Antiseptic	**Asa**

2.2. Origin and maintenance of zebrafish

Adult zebrafish were purchased from a commercial dealer (Seoul Aquarium, Korea) and 15 fish were kept in a 3L acrylic tank at 28.5±1◦C with a 14/10h light/dark cycle. Zebrafish were fed two times a day (Tetra GmgH D-49304 Melle Made in Germany). The day before 1 female and 2 males interbreed. Embryos were mated and natural spawning, induced in the morning by turning on the light. Collection of embryos was completed within 30 min in petri dishes (containing media).

2.3. Waterborne exposure of embryos to arsenic compounds

The embryonic toxicity test was followed the OECD (Organization for Economic Cooperation and Development) guideline 210. As from approximately 3-4 hour post-fertilization (3-4 hpf), the embryos (n=15) were transferred to individual wells of 12-well plates containing 900 mL embryo media. Three concentrations of arsenic compounds were chosen for this test; 0.25, 0.5, and 1ppm. Arsenic compounds were added to each of the wells for up to 96 hpf. Then, embryos were rinsed using fresh embryos media.

2.4. Waterborne exposure of adult to arsenic compounds

The fish acute toxicity test was followed the OECD guideline 203. Adult zebrafish of similar length and age were randomly distributed to each of tanks containing 1 L fresh water. Three concentrations of arsenic compounds were chosen for this test; 5, 10, and 20ppm. Arsenic compounds were exposed to each of tanks for up to 96 hpf. Then, enbryos were rinsed using fresh embryos media.

2.5. Measurement of survival rate, heartbeat rate and yolk sac edema size in zebrafish

embryos

The survival rates were measured up to 7 dpf after exposed to arsenic compounds. The heartbeat rate of both atrium and ventricle was recorded at 48 hpf for 1 min under the microscope. At 48 hpf, lateral views of anesthetized larvae were imaged using a microscope for size of yolk sac edema. The outlines of the yolk sac edema were traced, and the area within each tracing was assessed by ISCapture for windows.

2.6. Morphology observation in zebrafish embryos

The 3 embryos were randomly selected from each well. Thus, zebrafish embryos layered in a glass slide containing embryo media, and evaluated for malformation using the microscope at 24, 48, 72, 96, 120, and 144 hpf.

2.7. Measurement of cell death by sample treatment and image analysis

Cell apoptosis was identified in live embryos using acridine orange satining. Acridine orange; a nucleic acid-selective metachromatic stain is useful for studying apoptosis patterns. At 72 hpf of exposure to arsenic compounds (0.25, 0.5 and 1ppm), the zebrafish larvae was transferred to 96-well plate and treated with acridine orange solutions (7 μg/ml), and the plated were incubated for 30 min in the dark at 28.5±1◦C. After incubation, the zebrafish larvae were rinsed in the fresh embryo media. Before examination, the larvae were anesthetized with 0.03% MS-222. Apoptotic cells were observed and photographed under the microscope CoolSNAP-Pro dolor digital camera (Olympus, Japan). Individual zebrafish larvae fluorescence intensity was quantified using the image J program.

2.8. Measurement of ROS production by sample treatment and image analysis

Generation of ROS production in the zebrafish larvae was measured using an oxidation-sensitive fluorescent probe dye, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). At 72 hpf, the zebrafish larvae was transferred to 96-well plate and treated with DCFH-DA solution (20 μg/ml) and incubated for 1 h in the dark at 28.5±1˚C. After incubation, the zebrafish larvae were rinsed in the fresh embryo media. Before examination, the larvae were anesthetized with 0.03% MS-222. ROS production was observed and photographed under the microscope CoolSNAP-Pro dolor digital camera (Olympus, Japan). Individual zebrafish larvae fluorescence intensity was quantified using the image J program.

2.9. Histological evaluation in the liver of adult zebrafish

After 7 days, the liver of adult zebrafish for histological analysis were preserved in Bouin's solution for approximately 24 h and subsequently transferred to 70% ethanol for storage. Later, fixed zebrafish were dehydrated, embedded in paraffin, and sectioned at 7 μm. Standard histological slides were prepared by using hematoxylin and eosin. The liver of adult zebrafish was determined under the microscope.

2.10. Quantitative real-time PCR

As from approximately 3-4 hpf, the embryos (n = 30) were transferred to individual wells of 6-well plates containing 2,850 μL of embryo media. Embryos were treated with concentration of arsenic compounds (20ppm) was added to the plate for up to 96 hpf. The zebrafish larvae were transferred into Eppendorf tube, and then washed twice with heated PBS in 28.5±1°C. After that, keep in -70±1˚C overnight.

Larvae and liver tissues were homogenized in TRIzol reagent (ambion, USA), and total RNA was extracted according to the manufacturer's instructions. After which real-time PCR

was performed with cDNAs and gene-specific primer pairs (Table 2.) mixed with SYBR Green PCR master mix (Takara) in an Light Cycler real-time PCR system. PCR condition were as follows: denatured for 5 min at 95◦C and then amplified using 50 cycles of 30 s at 95◦C, 20 s at 55◦C and 20 s at 72◦C.

Table 1-2. Primer sets employed in real-time PCR analysis

Gene name	Sequence of the primer (5'-3')
β-actin	Forward-GCTGACAGGATGCAGAAGGA Reverse-TAGAAGCATTTGCGGTGGAC
p53	Forward-GGGCAATCAGCGAGCAAA Reverse-ACTGACCTTCCTGAGTCTCCA
Bax	Forward-GGCTATTTCAACCAGGGTTCC Reverse-TGCGAATCACCAATGCTGT
Bcl-2	Forward-TCACTCGTTCAGACCCTCAT Reverse-ACGCTTTCCACGCACAT
Caspase 3	Forward-CCGCTGCCCATCACTA Reverse-ATCCTTTCACGACCATCT
Caspase 8	Forward-GATGAGAACCTGACAAGCGGTGATG Reverse-GCTCATCCAGTCGCAGAATCAGGT
Caspase 9	Forward-AAATACATAGCAAGGCAACC Reverse-CACAGGGAATCAAGAAAGG

2.11 Western blot analysis of apoptosis pathway in zebrafish

As from approximately 3-4 hpf, the embryos (n = 30) were transferred to individual wells of 6-well plates containing 2,850 μL of embryo media. Embryos were treated with concentration of arsenic compounds (20ppm) was added to the plate for up to 96 hpf. The zebrafish larvae were transferred into Eppendorf tube, and then washed twice with heated PBS in 28.5±1°C. After that, keep in -70±1°C overnight.

After 96 hpf, liver tissues of adult zebrafish were homogenized in lysis buffer, and hepatic protein was extracted. The protein concentrations were measured using BCA^{TM} protein assay kit (Theremo scientific,Waltham,USA).The lysate, which contained 30 μg of protein, was subjected to electrophoresis on a 10% sodium dodecyl sulfate –polyacrylamide gel. The gel was transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) using a glycine transfer buffer [192 mM glycine, 25mM Tris-HCL (pH 8.8), 20% methanol (v/v)]. The membranes were blocked with 5% blotting-grade blocker in TBS buffer containing 0.2% Tween 20 (TBST) for 3 h. Then the membranes were incubated with polyclonal anti-rabbit p53 Ab (1 : 500), Bcl-xL Ab (1:500), Bax Ab (1:250), Cleaved caspase-3 Ab (1:500), Cleaved caspase-9 Ab (1:500), caspase-8 Ab (1:500), and polyclonal anti-mouse β-actine Ab (1:500) overnight at 4°C. The membranes were washed with TTBS and incubated with the goat anti-rabbit IgG HRP conjugated secondary antibody at a 1:3,000 dilution in TBST that contained 5% blotting-grade blocker for 90 min at room temperature. After three times washing with TBST, the immunoreactive proteins were visualized with an ECL Western blotting detection reagent (Cyanagen Srl, Bologna, Italy) and Fusion Solo chemiluminescence reader and quantified using FusionCapt software (VILBER LOURMAT, France).

2.12 Statistical analysis

All experiments were conducted in triplicate (n=3) and an one-way analysis of variance (ANOVA) test (using SPSS 12.0 statistical software) was used to analyse the data. Significant differences between the means of parameters were determined by using the Duncan test to analyze. *P*-values of less than 0.05 ($P < 0.05$) was considered as significant.

3. Results

3.1. Survival rates. heartbeat rates, and yolk sac edema sizes in zebrafish embryos/larvae after exposure arsenic compounds

To evaluate the potential toxicity of zebrafish embryos exposure to arsenic compounds (0.25, 0.5, and 1ppm), we measured the survival rate until 7 dpf, as shown in Fig. 1-1. In this study, zebrafish embryos were exposed to arsenic compounds for 96 hours. After 96 h of exposure, the survival rates in embryos were reduced by the treatment of organic and inorganic arsenic compounds, compared with the control group at 0.25, 0.5, and 1ppm. The survival rates of the treated groups was recorded as 83.3 ± 3.3%, 63.3 ± 3.3%, 70.0 ± 10.0%, 30.0 ± 10.0%, 63.3 ± 3.3%, 50.0 ±3.3%, 40.0 ± 6.7%, 36.7 ± 3.3%, 16.7 ± 3.3% and 26.3 ± 3.3% relative to 86.7 ± 6.7% in the control group at 1ppm. Inorganic arsenic compounds were dramatically reduced as compared to an organic arsenic compounds. After 7 days of exposure, inorganic arsenic compounds were no survival at 1ppm. However, marine-derived organic arsenic compounds (AsB, AsCB, and DMA) were recorded as 33.3 ± 0.0%, 20.0 ± 0.0%, and 16.7 ± 3.3% compared with the control group. The other survival rates of the lower concentrations were also significantly reduced to exposure arsenic compounds, compared with the control group. Therefore, zebrafish embryos were strongly affected by exposure from inorganic arsenic compounds. The heart-beating was evaluated the embryos hatched at 2 dpf. As shown in Fig.1-2., the heart-beat rates in larvae were increased or decreased to 102.4 ± 2.2%, 101.6 ± 2.0%, 103.4 ± 1.7%, 108.2 ± 2.4%, 96.8 ± 5.6%, 105.6 ± 4.3%, 95.6 ± 3.9%, 93.4 ± 5.7%, 91.6 ± 5.2%, and 93 ± 4.2% compared with the control group at 1ppm. The other survival rates of the lower concentrations were also significantly reduced to exposure arsenic compounds, compared with the control group. And inorganic arsenic compounds were

dramatically reduced as compared to an organic arsenic compounds at all thecentrations groups. Thus, this result showed that inorganic arsenic compounds-treated groups were strongly damaged from any toxicity. Altered yolk sac edema size were also measured in larvae exposed to organic and inorganic arsenic compounds. At 48 hpf, the outlines of the yolk sac edema were traced, and the area within each tracing was assessed in the zebrafish larvae. The yolk sac edema sizes in larvae were 98.9 ± 2.9%, 97.8 ± 2.5%, 98.5 ± 4.1%, 109.4 ± 2.4%, 106.7 ± 4.8%, 105.1 ± 2.4%, 104.1 ± 3.7%, 105.1 ± 5.0%, 120.7 ± 4.6%, and 113.2 ± 3.8% compared with the control group at 1ppm, as shown in Fig.1-3. Especially, the size of yolk sac edema in zebrafish larvae were sgnificantly increased or decreased by treatment of inorganic arsenic compounds (Asi and Asa) at the lower concentrations treated. Accordning to those results, inorganic arsenic compounds-treated groups were strongly affected any harmful toxicity than organic arsenic compounds.

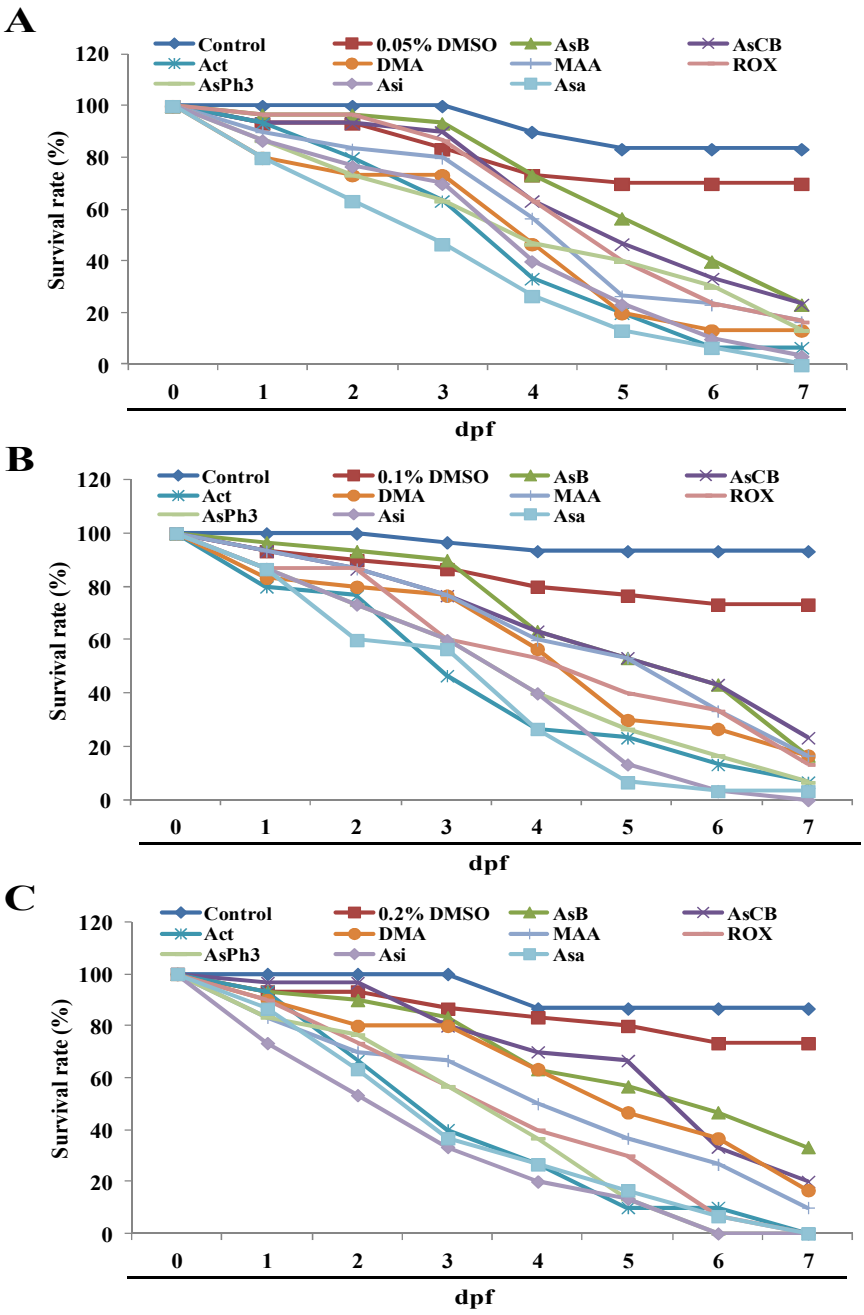

Fig. 1-1. Measurement of survival rates after treating with arsenic compounds. (A) 0.25ppm arsenic compounds, (B) 0.5ppm arsenic compounds, and (C) 1ppm arsenic compounds. The zebrafish were treated with various of concentrations of arsenic compounds. And then we measured survivorship of zebrafish for 7dpf. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

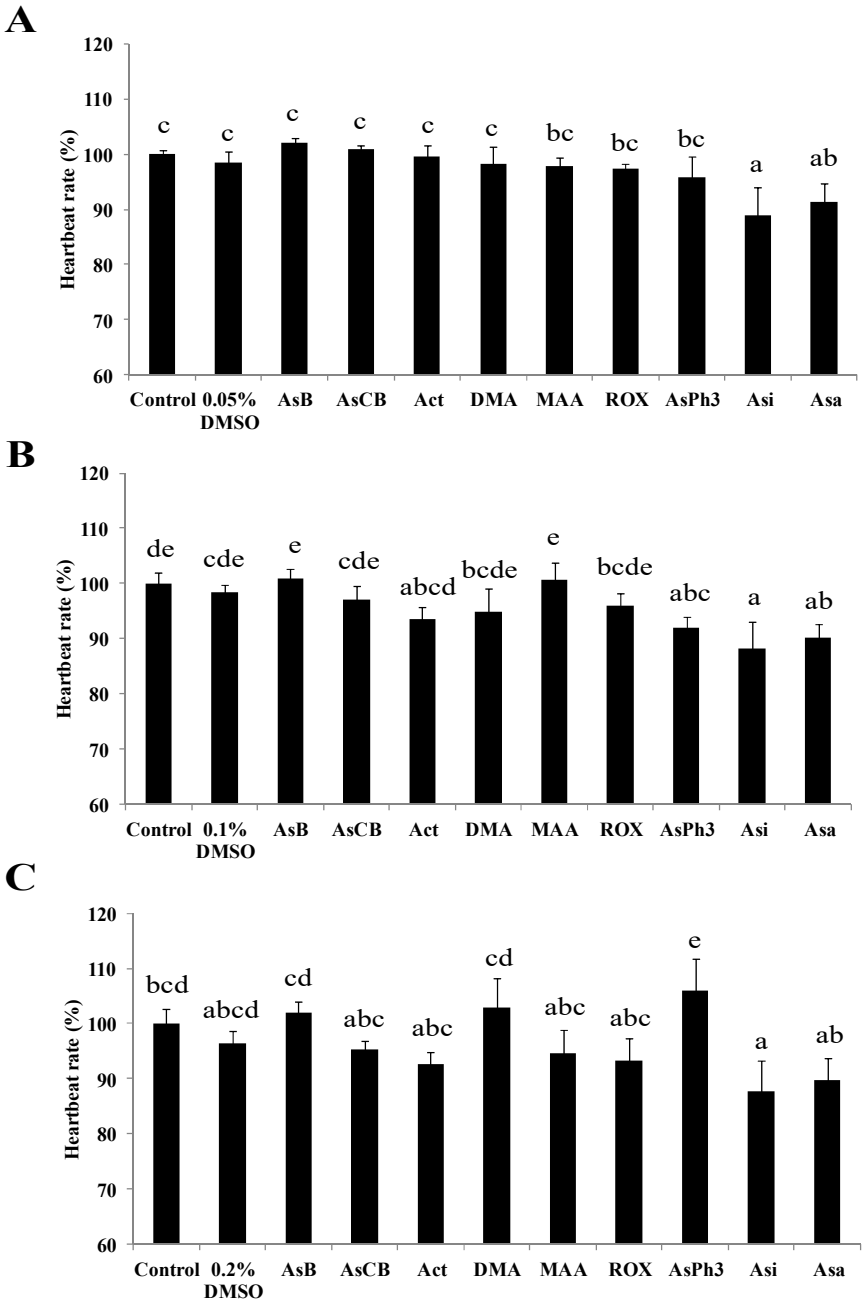

Fig. 1-2. Measurement of toxicity of the arsenic compounds by the heartbeat rate. (A) 0.25ppm arsenic compounds, (B) 0.5ppm arsenic compounds, and (C) 1ppm arsenic compounds. The heartbeat rate of both atrium and ventricle were counted at 48 hpf by under the microscope for 1 min. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

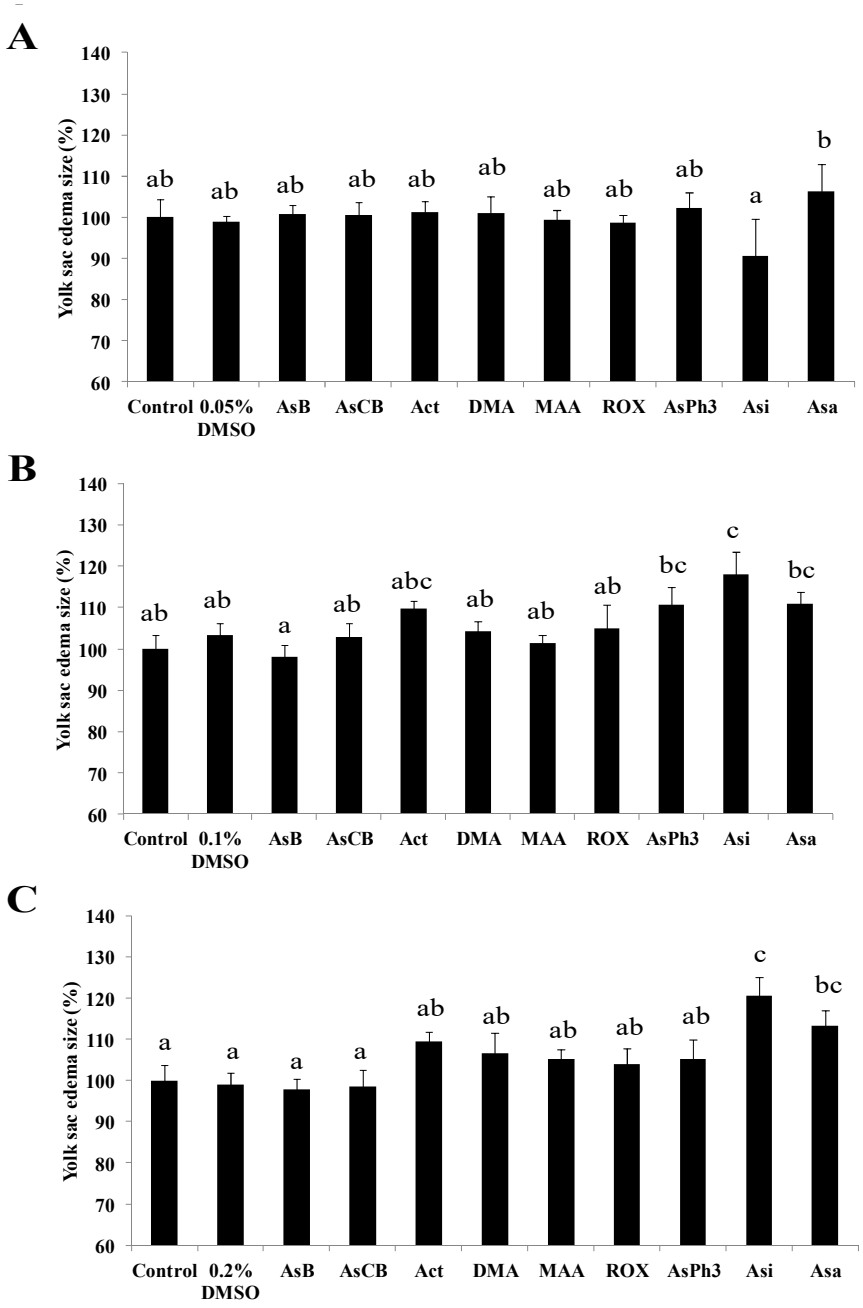

Fig. 1-3. Measurement of toxicity of the arsenic compounds by the yolk sac edema size. (A) 0.25ppm arsenic compounds, (B) 0.5ppm arsenic compounds, and (C) 1ppm arsenic compounds. The yolk sac edema size was measured at 48 hpf using the microscope. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.2. Morphological changes in zebrafish embryos and larvae

After fertilization, we observed the developmental abnomalities of zebrafish embryos and larvae that had been exposed to arsenic compounds from 1 dpf to 6 dpf by morphology, as shown in Fig.1-4, -5, -6, -7, -8, and -9. The developmental abnormalities included a yolk sac edema, pericardial edema, tail malformation. Most of zebrrafish was similarities in the symptoms exposed by organic arsenic compounds except the control group. However, inorganic arsenic compounds-treated zebrafish groups were difference in the symptoms such as early hatching, slowly developmental process, and death at 1 dpf. Marine-derived organic arsenic compound (AsB) was a little increased the yolk sac edema at all concentrations but no serious. Zebrafish embryos and larvae were strongly affected by exposure from inorganic arsenic compounds compared with the organic arsenic compounds.

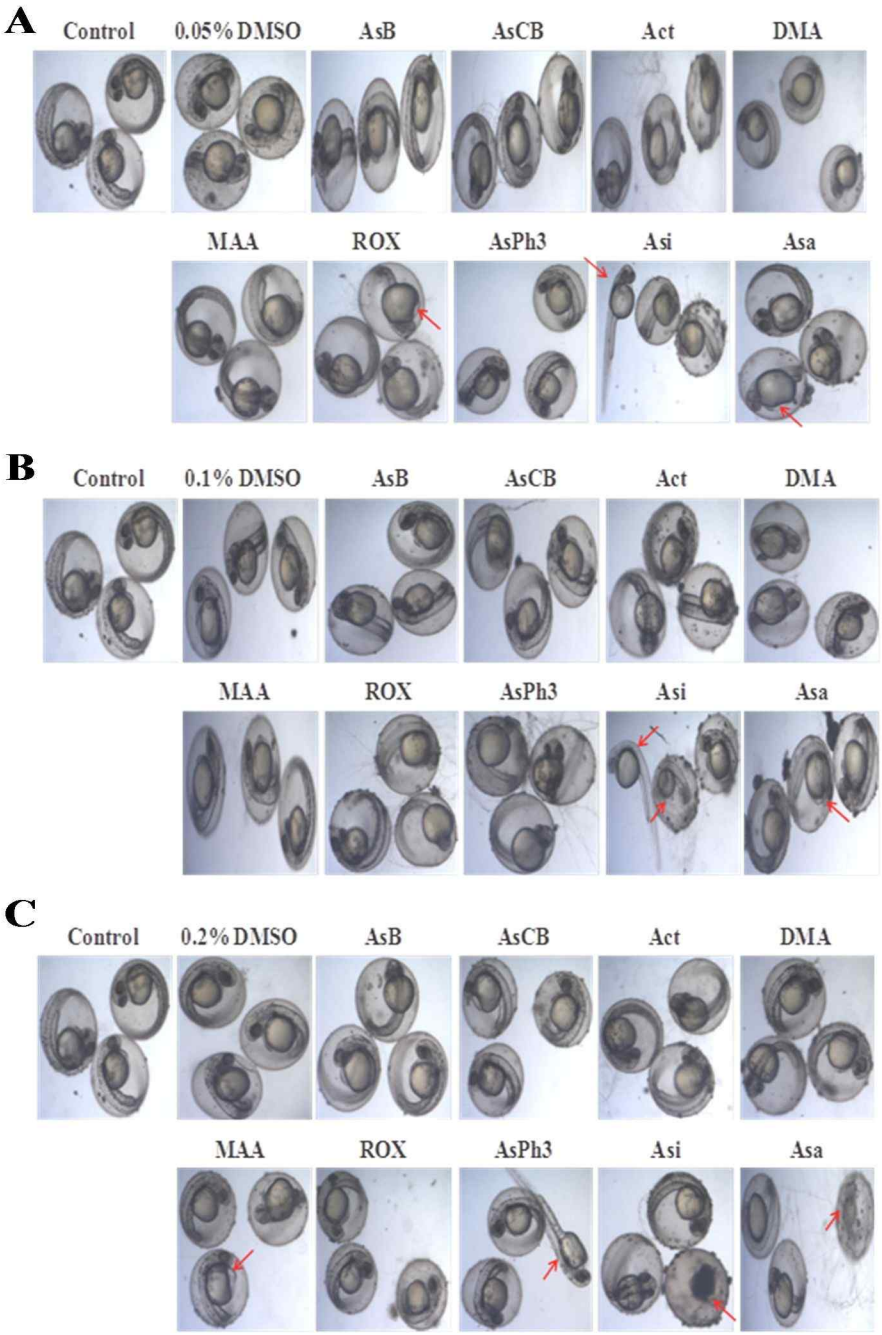

Fig. 1-4. Measurement of toxicity of the organic and inorganic arsenic compounds by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 24 hpf.

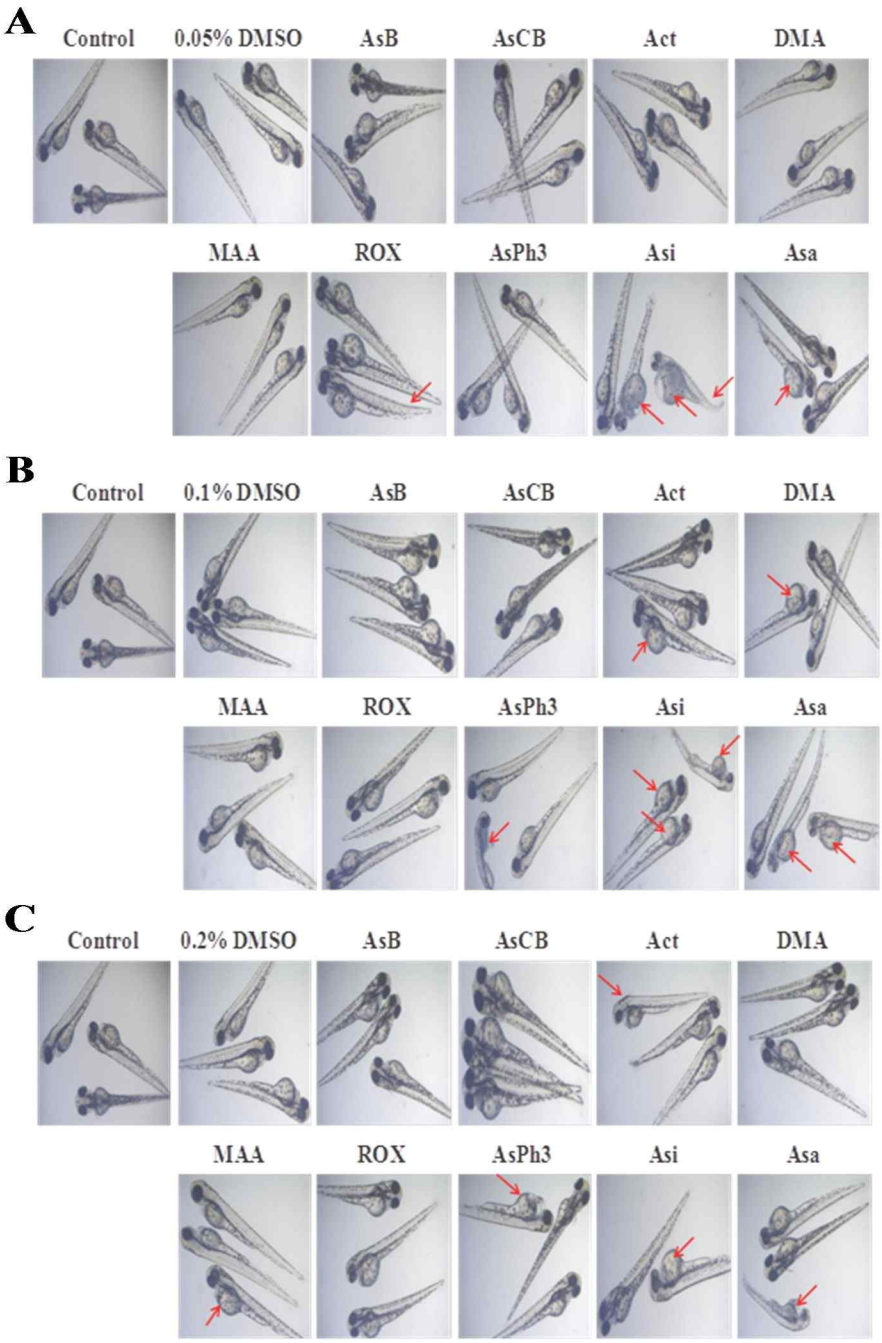

Fig. 1-5. Measurement of toxicity of the organic and inorganic arsenic compounds by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 48 hpf.

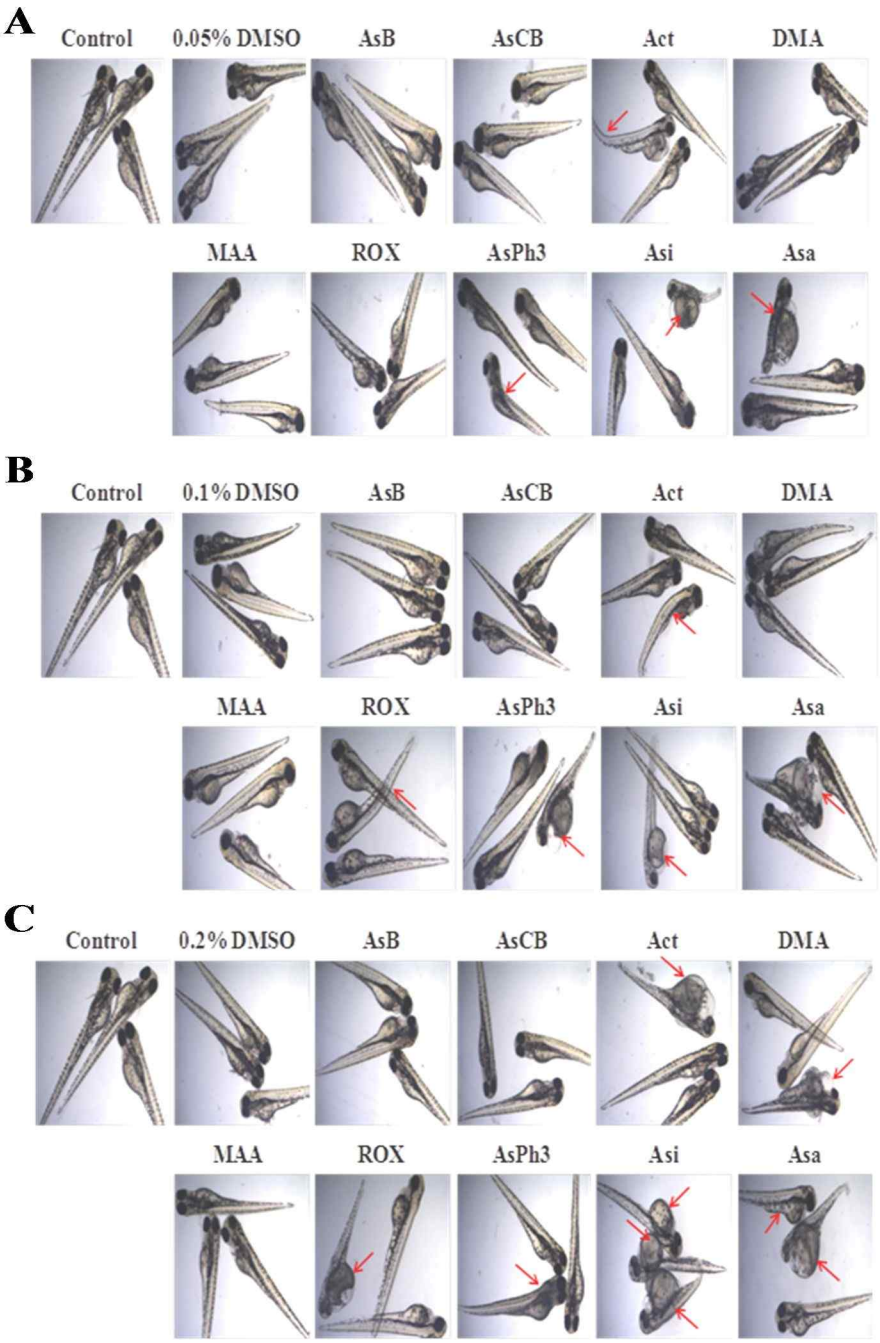

Fig. 1-6. Measurement of toxicity of the organic and inorganic arsenic compounds by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 72 hpf.

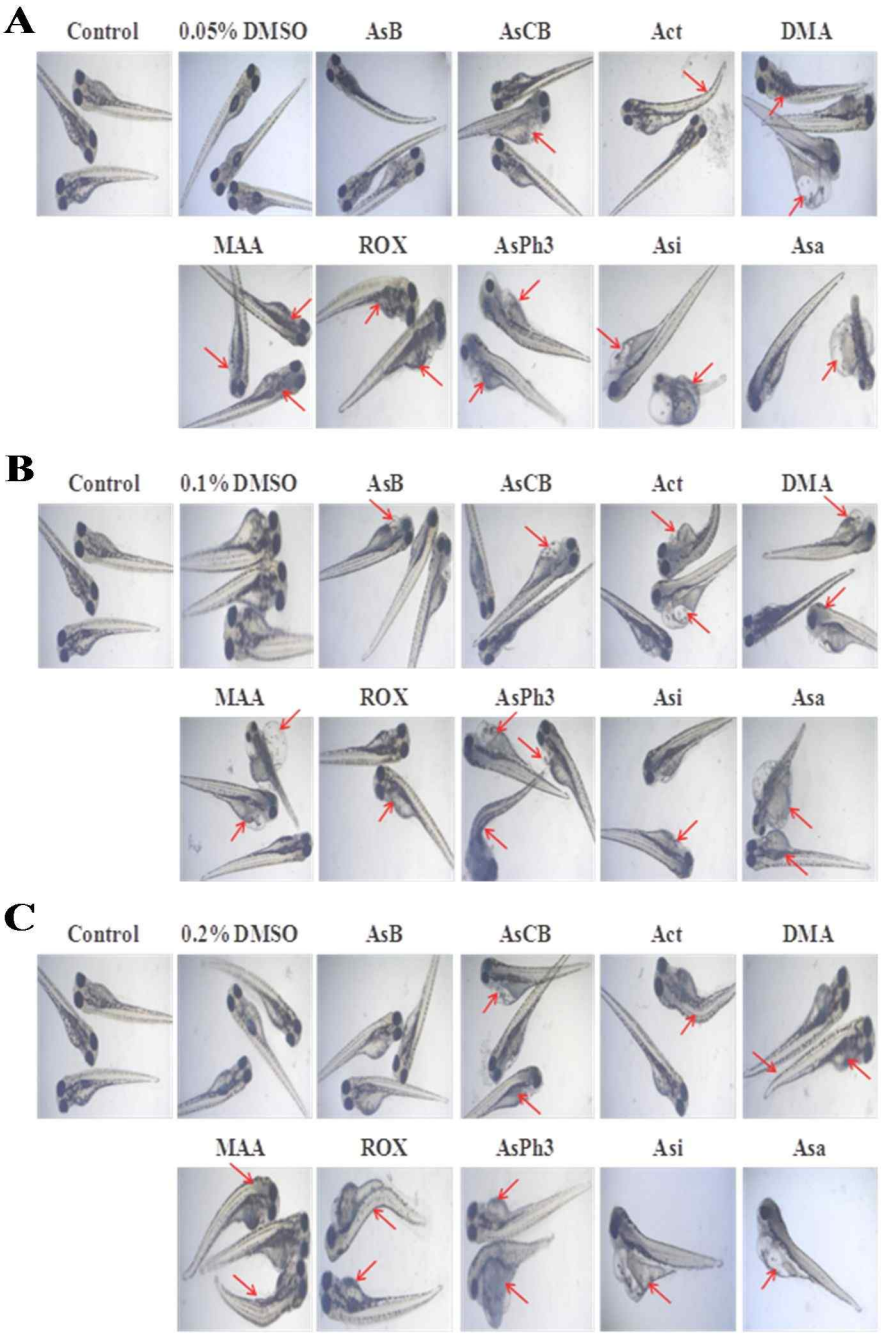

Fig. 1-7. Measurement of toxicity of the organic and inorganic arsenic compounds by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 96 hpf.

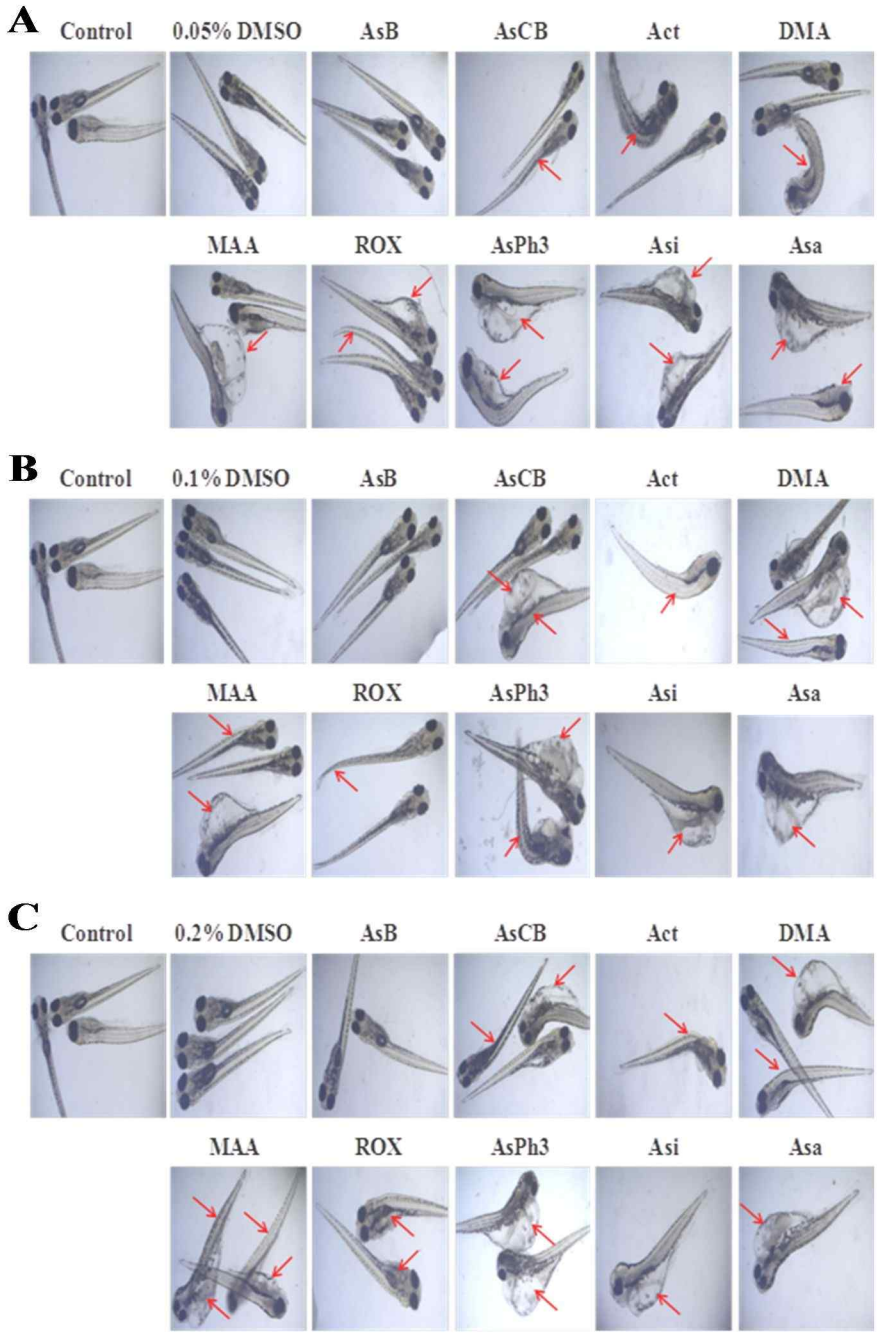

Fig. 1-8. Measurement of toxicity of the organic and inorganic arsenic compounds by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 120 hpf.

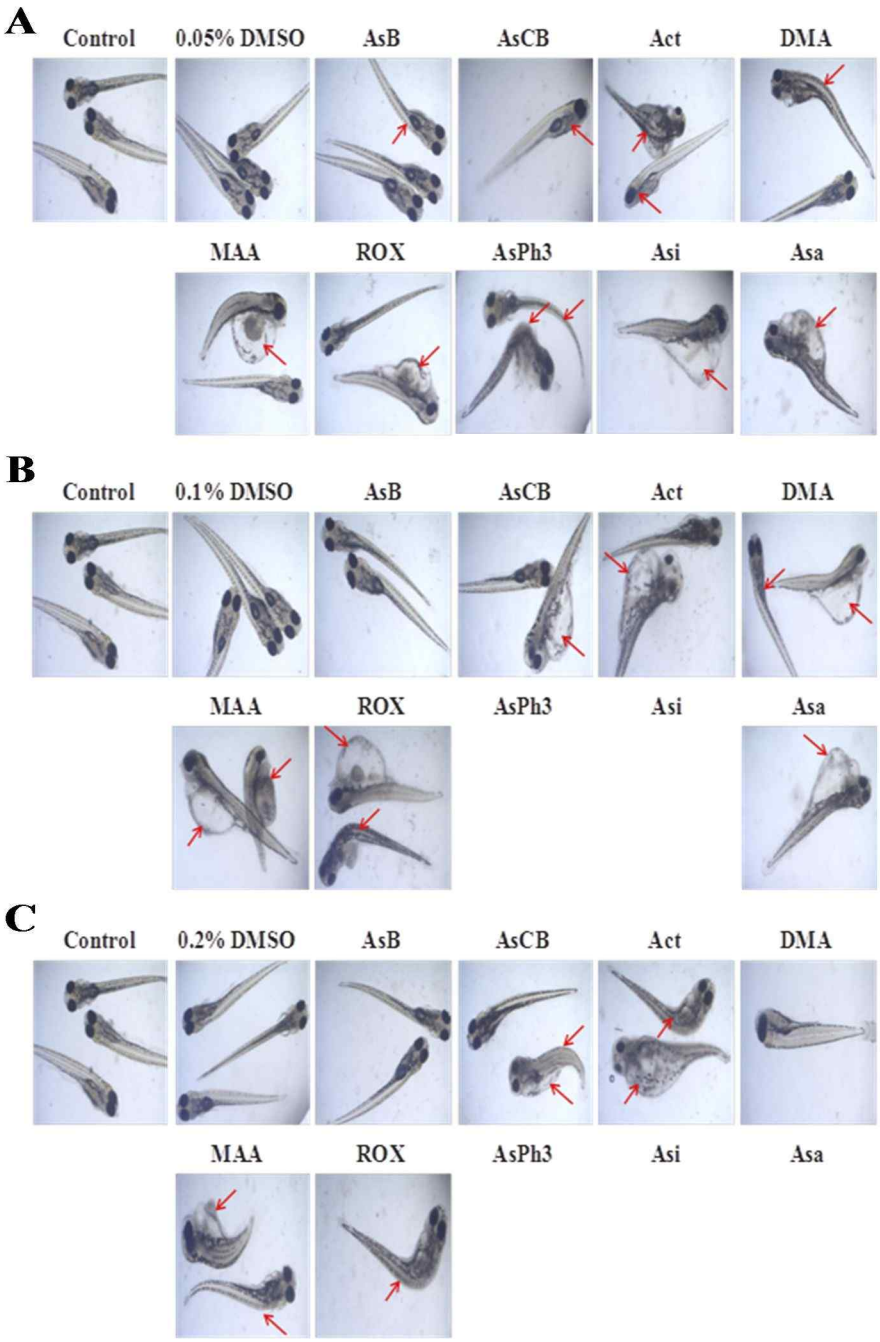

Fig. 1-9. Measurement of toxicity of the organic and inorganic arsenic compounds by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 144 hpf.

3.3. Toxicity of arsenic compounds by AO staining in zebrafish larvae

To determine if samples lead to increased cell death, embryos were treated from 3 hpf to 24 hpf to graded arsenic compounds concentrations by types. Cell death induced by the toxicity of the samples was evaluated via acridine orange for 30 min to stain dying cells. Lavae exposed the types of organic and inorganic arsenic compounds with the concentrations had an increase in cell death at 0.25ppm, 0.5ppm, and 1ppm. The level of cell death in an inorganic arsenic compounds were more than 1.6-fold and 1.5-fold at 1pom. The lower concentrations were also significantly increased to exposure inorganic arsenic compounds, compared with the control group.

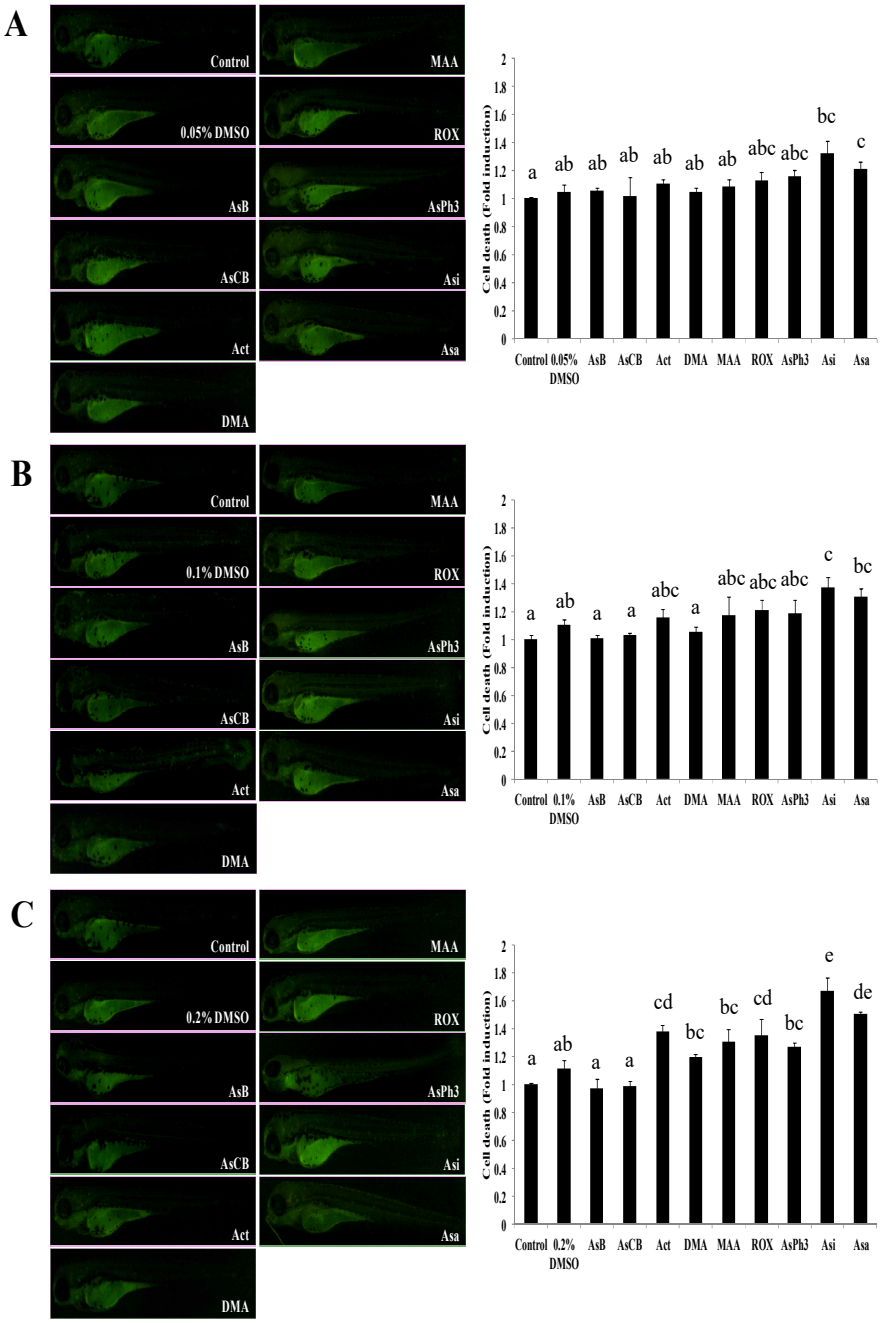

Fig. 1-10. Measurement of toxicity of the organic and inorganic arsenic compounds by the cell death. (A) 0.25ppm arsenic compounds, (B) 0.5ppm arsenic compounds, and (C) 1ppm arsenic compounds. The cell death was measured by image analysis using fluorescence microscope and levels using Image J. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.4. Toxicity of arsenic compounds by DCFH-DA staining in zebrafish larvae

The abilities of arsenic compounds to cause oxidative stress-induced apoptosis in zebrafish larvae were assessed by measuring ROS production. Larvae exposed types of organic and inorganic arsenic compounds with the concentrations had an increase in ROS production at 0.25ppm, 0.5ppm, and 1ppm. Zebrafish larvae were increased more than 10% at all the concentrations. However, marine-derived organic arsenic compounds (AsB and AsCB) were recorded as 104.5 ± 2.5% and 107.6 ± 4.3% compared with the control group. The lower concentrations sere also significantly increased to exposure inorganic arsenic compounds, compared with the control group.

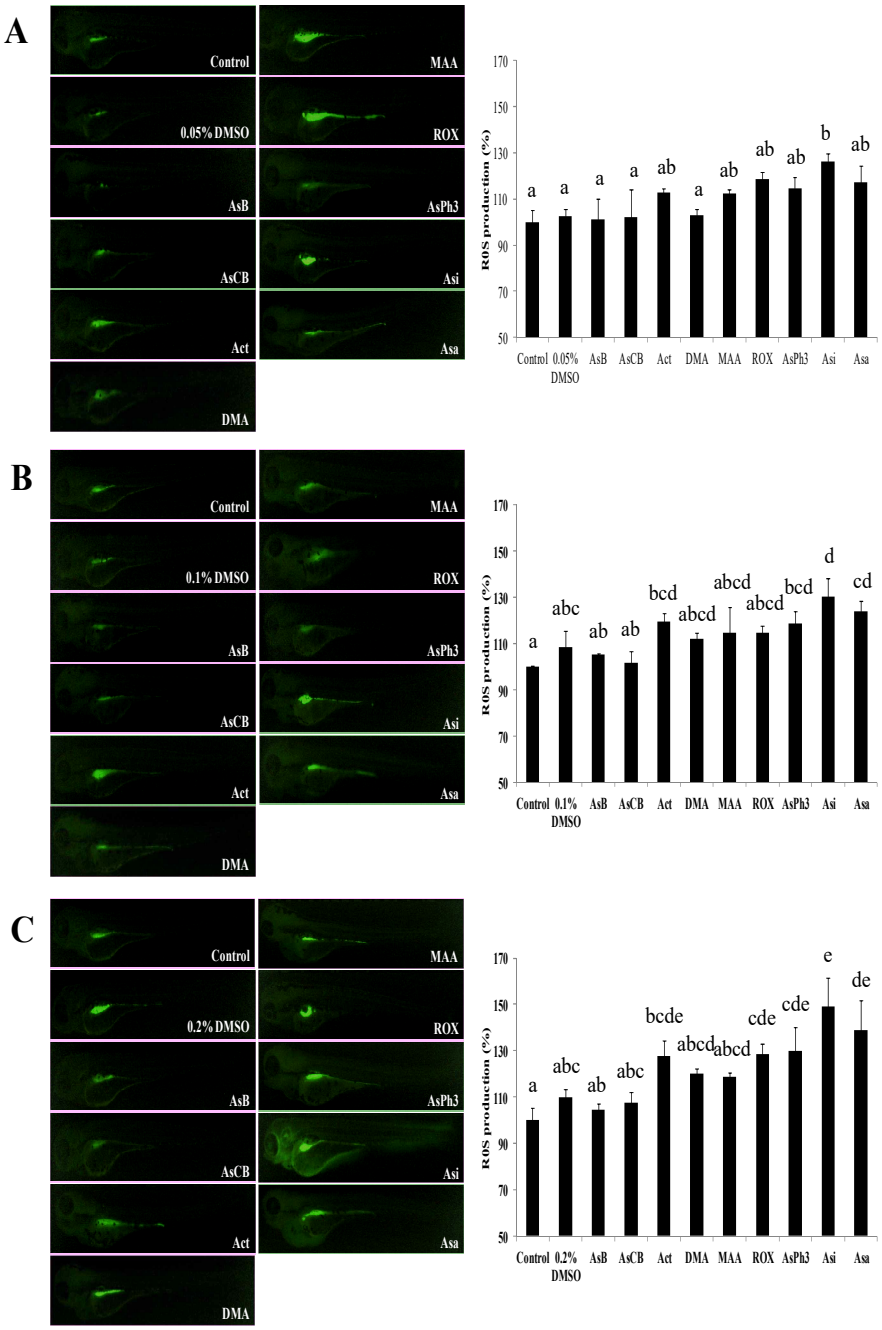

Fig. 1-11. Measurement of toxicity of the organic and inorganic arsenic compounds by the ROS production. (A) 0.25ppm arsenic compounds, (B) 0.5ppm arsenic compounds, and (C) 1ppm arsenic compounds. The ROS production was measured by image analysis using fluorescence microscope and levels using Image J. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.5. Gene expression

The mRNA levels of p53, an important regulator of apoptosis induction, were changed significantly in the 20ppm with increases of 0.8-, 1.0-, 1.1-, 1.3-, 1.1-, 0.9-, 1.1-, 1.6-, and 1.4- fold compared with the control group, as shown in Fig.1-12. As for the induction patterns of Bax, the Bcl2 associated X protein, it was found that mRNA levels dramatically increased to 1.1-, 1.0-, 1.0-, 1.5-, 1.3-, 1.3-, 1.1-, 1.1-, 2.3-, and 1.7-fold compared with the control group. To assess whether arsenic compounds induces apoptosis via the caspase pathway, the gene expression of caspase-3, caspase-8, and caspase-9 was shown in Fig.1-12. The gene expression of caspase-3, and caspase-9 activity was significantly up-regulated in the 20ppm. Similarly, the caspase-3 and caspase-9 activity was also significantly induced in the exposure groups. Also, the caspase-8 acticity was significantly induced in the exposure groups.

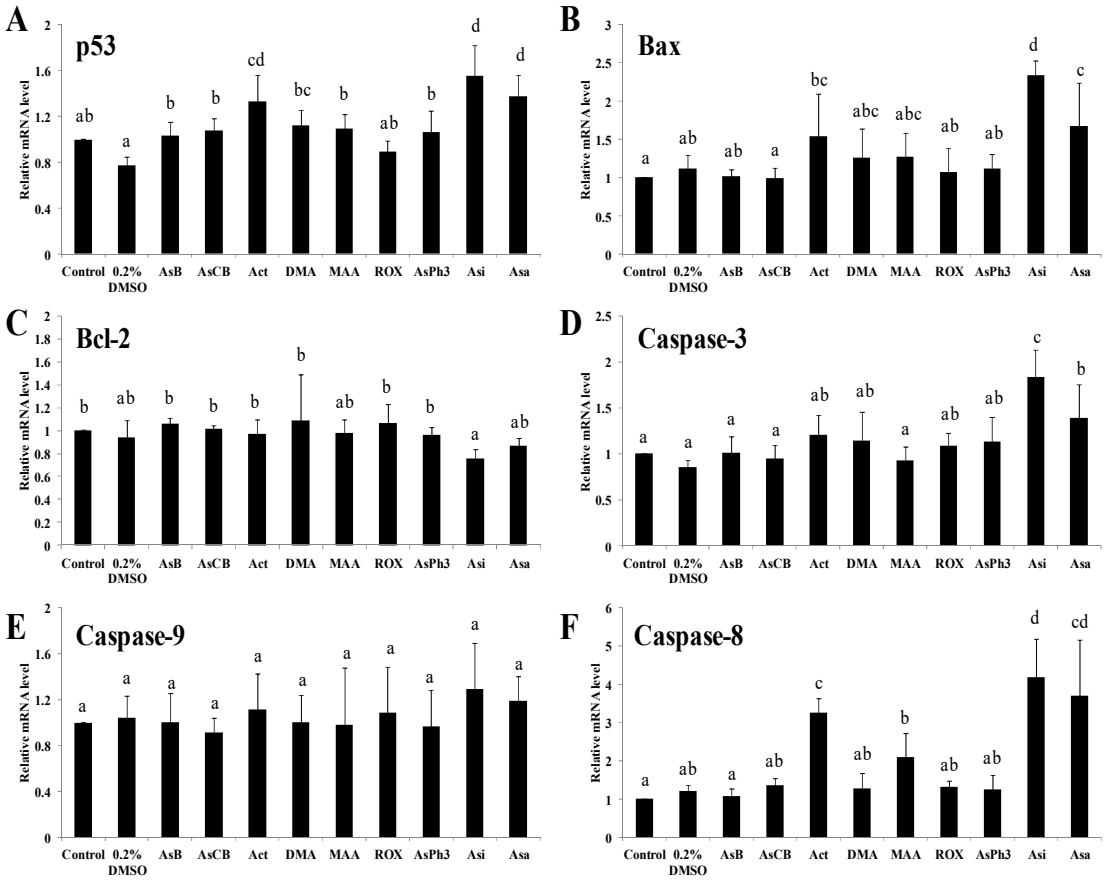

Fig. 1-12. Expression of p53 (A), Bax (B), Bcl-2 (C), Caspase-3 (D), Caspase-9 (E), and Caspase-8 (F) mRNA species in the embryos treated with arsenic compounds of 1ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. [a,b,c] Values having different superscript are significantly different at $P<0.05$.

3.6. Western blot

To study the mechanism of arsenic compounds induced apoptosis, we evaluated the expressions of p53, Bax, Bcl-xL, caspase-3, -9, and caspase-8 by western blot analysis. As shown in Fig.1-13, the expression levels of p53 and Bax, a pro-apoptotic protein was increased gradually with inorganic arsenic compounds. Also, the expresson levels of Bcl-xL, antiapoptotic protein were decreased with inorganic arsenic compounds as shown in Fig.1-14. The expression levels of Cleaved Caspase 3, Cleaved Caspse 9, and Caspase 8 in the embryos with arsenic compounds of 1ppm for 96 h and untreated controls was activated as shown in Fig.1-15. These results show that inorganic arsenic compounds-treated groups were strongly induced the toxicity than organic arsenic compounds through up and down regulation in apoptosis pathway. Therefore, we suggest that organic arsenic compounds did not induced the toxicity by apoptosis pathway.

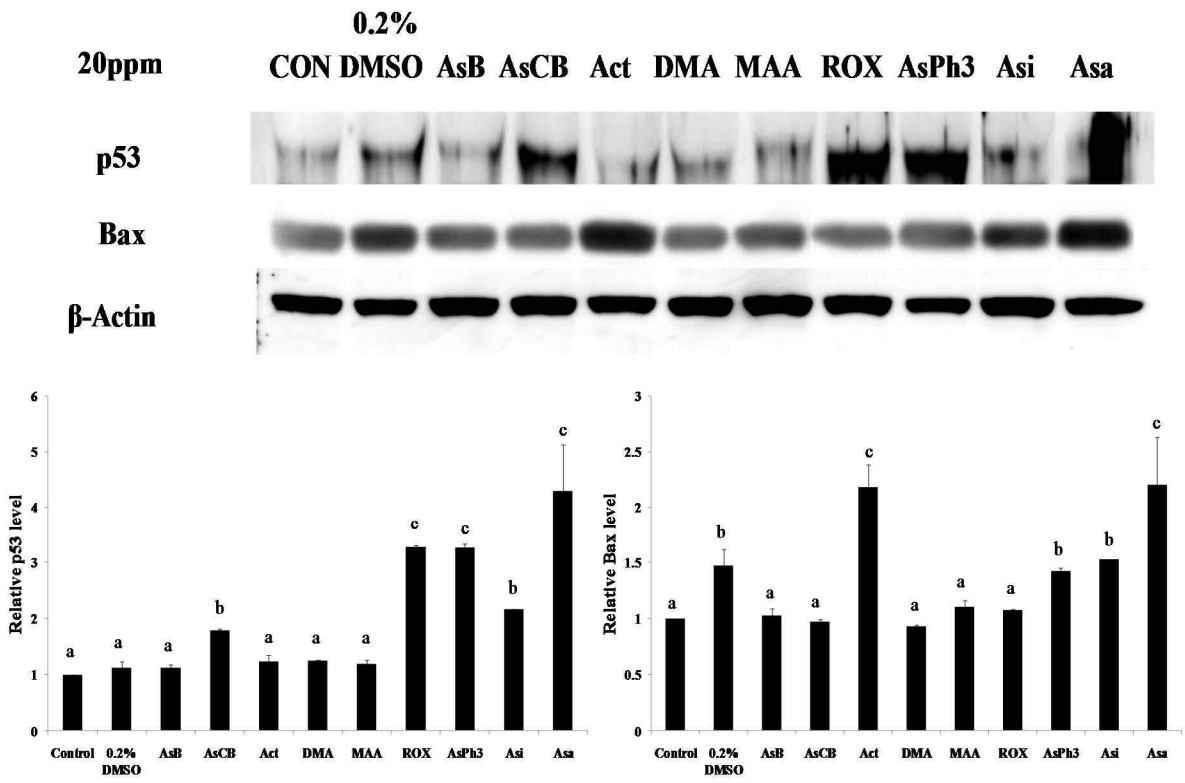

Fig. 1-13. Western blot analysis of p53 and Bax in the embryos with arsenic compounds of 1ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. [a,b,c] Values having different superscript are significantly different at $P<0.05$.

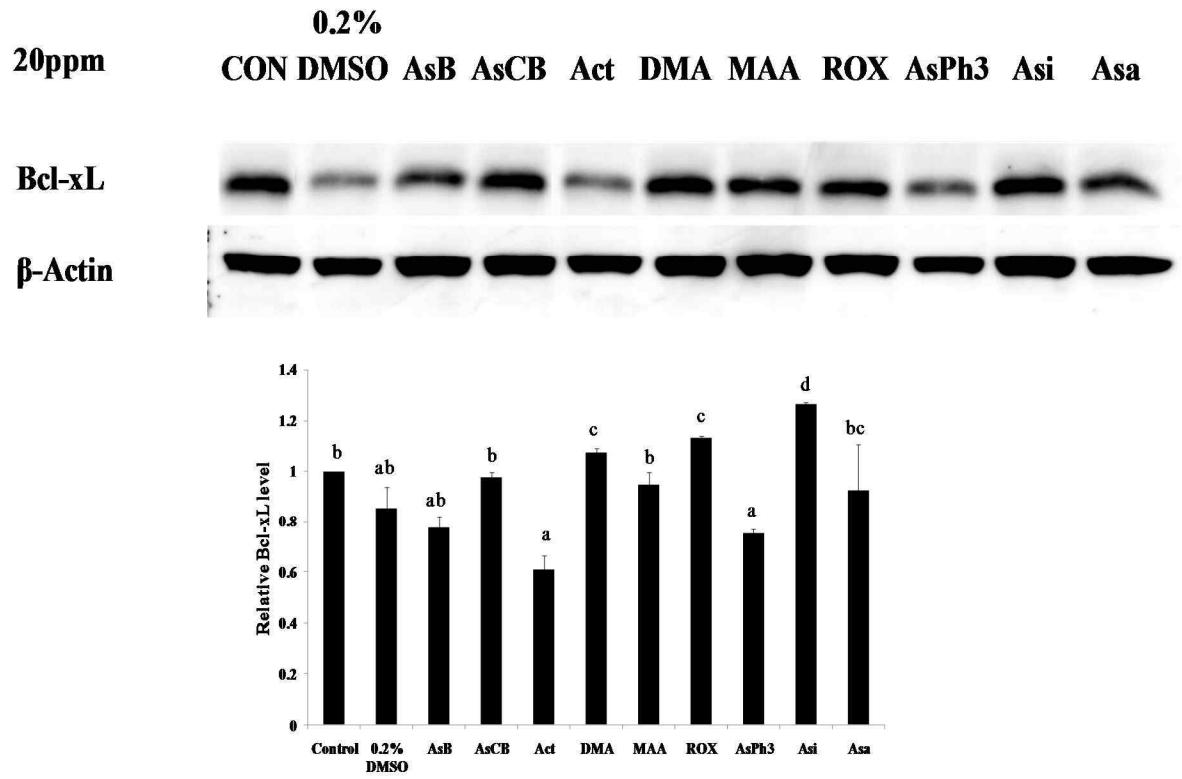

Fig. 1-14. Western blot analysis of Bcl-xL in the embryos with arsenic compounds of 1ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

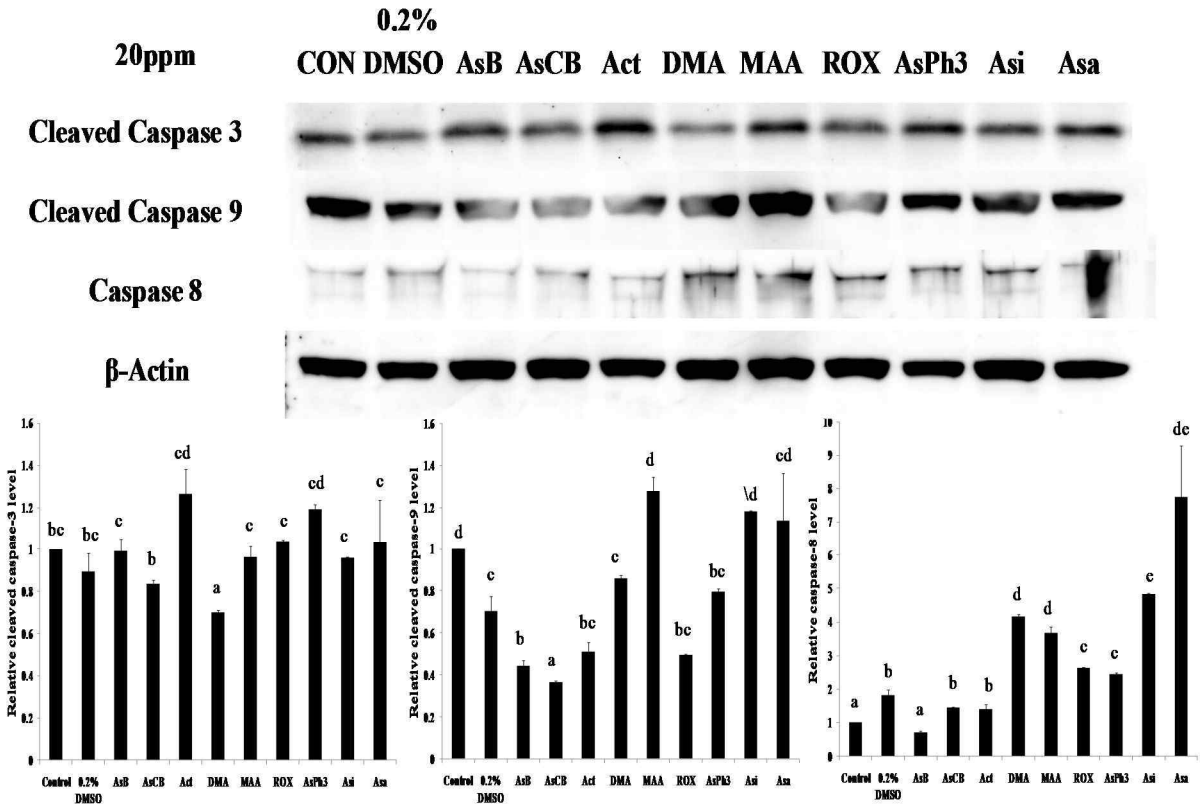

Fig. 1-15. Western blot analysis of Cleaved Caspase 3, Cleaved Caspse 9, and Caspase 8 in the embryos with arsenic compounds of 1ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.7. Survival rates in adult zebrafish after exposure arsenic compounds

To evaluate the potential toxicity of adult zebrafish exposure to arsenic compounds (5, 10, and 20ppm), we measured the survival rate until 7 dpf, as shown in Fig. 1-16. In this study, adult zebrafish were exposed to arsenic compounds for 96 hours. After 96 h of exposure, the survival rates in zebrafish were reduced by the treatment of organic and inorganic arsenic compounds, compared with the control group at 5, 10, and 20ppm. The survival rates of the treated groups was recorded as 90.0 ± 14.1%, 85.0 ± 21.2%, 90.0 ± 14.1%, 75.0 ± 7.1%, 80.0 ± 14.1%, 90.0 ± 14.1%, 50.0 ± 14.1%, and 65.0 ± 7.1% relative to 90.0 ± 14.1% in the control group at 20ppm. Inorganic arsenic compounds were significantly reduced as compared to the organic arsenic compounds. The other survival rates of the lower concentrations were also significantly reduced to exposure arsenic compounds, compared with the control group. Therefore, adult zebrafish were strongly affected by exposure from inorganic arsenic compounds.

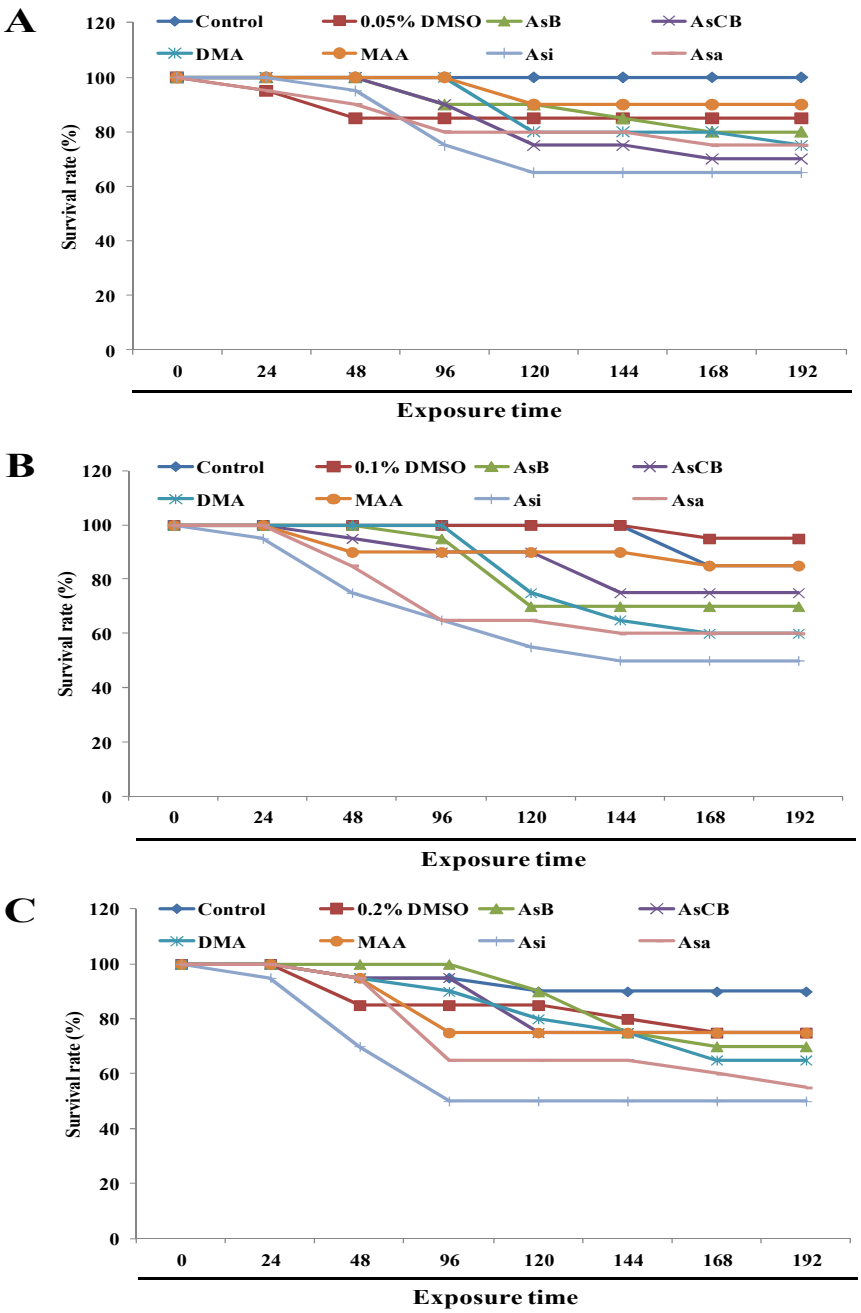

Fig. 1-16. Measurement of survival rates after treating with arsenic compounds. (A) 5ppm arsenic compounds, (B) 10ppm arsenic compounds, and (C) 20ppm arsenic compounds. The zebrafish were treated with various of concentrations of arsenic compounds. And then we measured survivorship of zebrafish for 7dpf. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.8. Morphological changes in adult zebrafish

Zebrafish have a single dorsal fin and no adipose fin. Their fins do not contain true spines but instead have hardened rays. We observed the symptoms of zebrafish have been exposed to arsenic compounds from 1 dpf to 6 dpf by morphology, as shown in Fig.1-17. Zebrafish exposed to arsenic compounds were appeared various symptoms such as short caudal fin, part of the heart changes the red color, and the belly of fish changes the green color. A dilated common bile duct was discerned grossly after 7days of exposure to arsenic compounds in the zebrafish. The bile duct of organic arsenic compound groups has no differences compared with the control group, as shown in Fig.1-18. However, the inorganic arsenic compound groups were strongly appeared to green color in the bile duct of zebrafish.

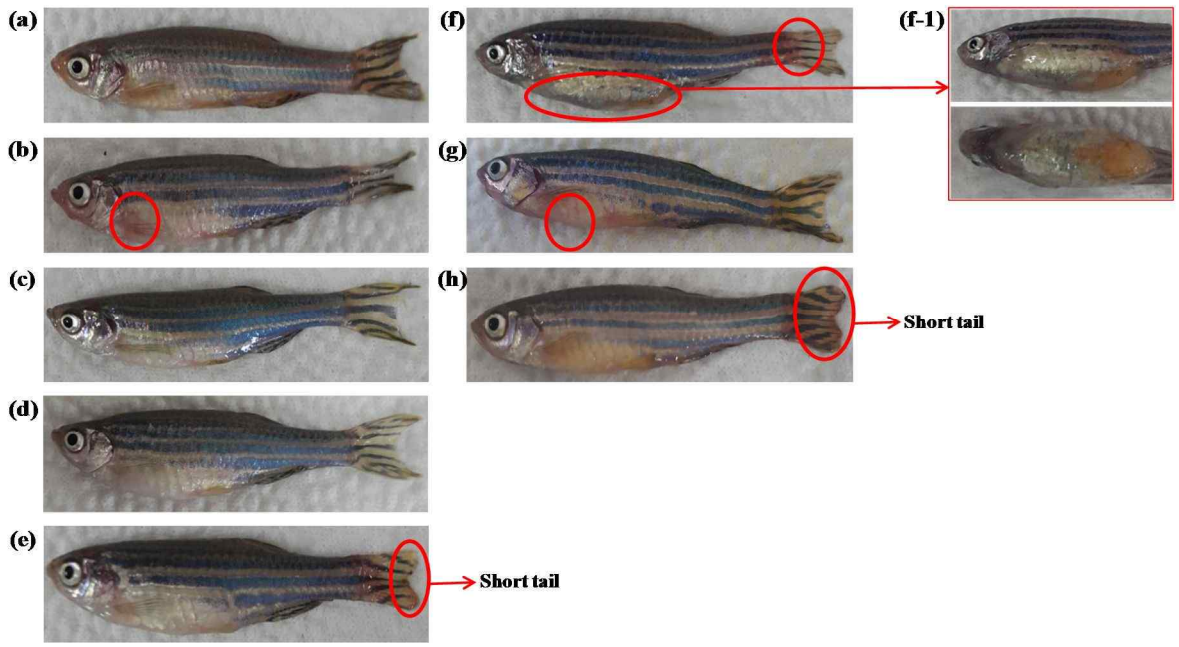

Fig. 1-17. Measurement of toxicity of the organic and inorganic arsenic compounds by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation in the adult zebrafish at 7 days post-exposure after treated arsenic compounds with 20ppm.

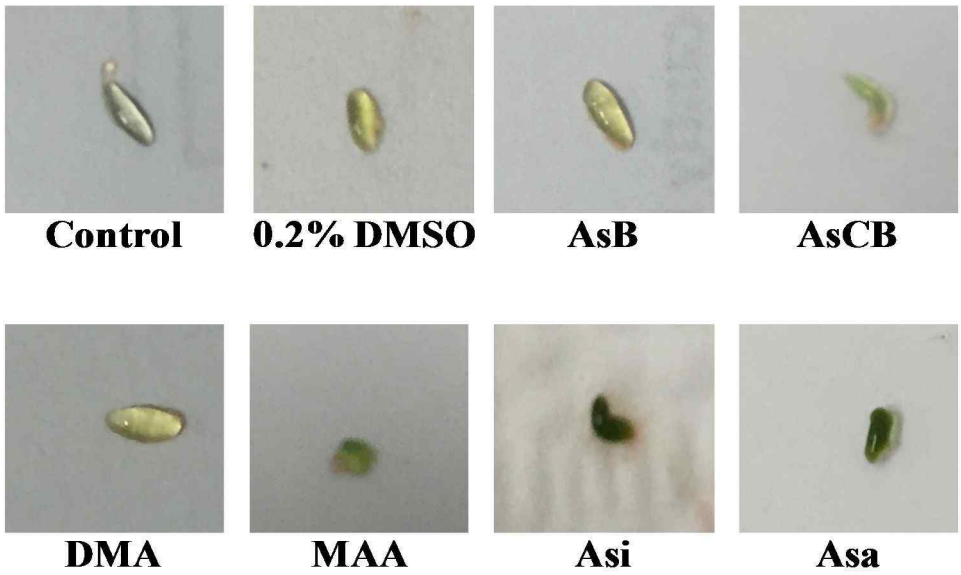

Fig. 1-18. The bill duct of zebrafish at 7 days post-exposure after treated arsenic compounds with 20ppm.

3.9. Histopathology in the liver of adult zebrafish

Livers of zebrafish exhibited histopathological changes following arsenic compounds treatment as compared to control. Exposure to 20ppm inorganic arsenic compounds obvious swelling of hepatic cells. And cell borders became obscure and hepatocelular vacuolar degeneration was observed. Also, nuclei enlargement and pycnosis were observed along with concomitant hepatocyte necrosis and cytolysis. The cellular structure was nearly collapsed in livers of zebrafish receiving the inorganic arsenic compounds. However, organic arsenic compounds-treated groups were no changes as compared to the control group.

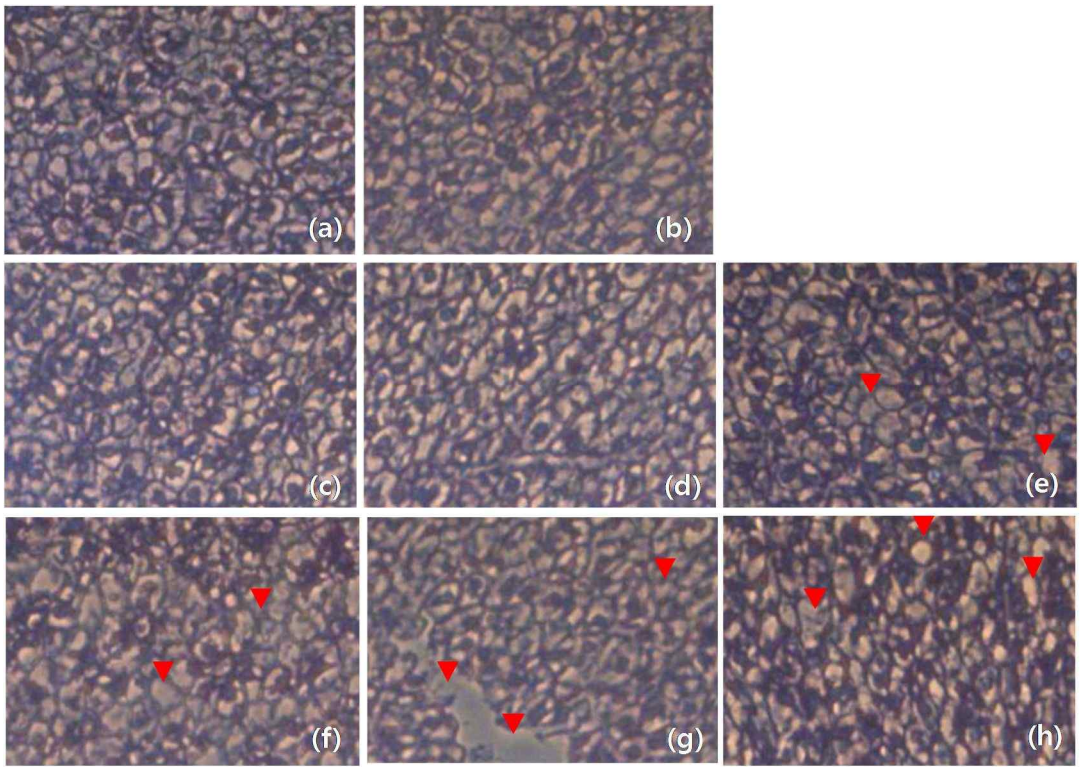

Fig. 1-19. Histopathological micrographs of liver in zebrafish at 7 days post-exposure after treated arsenic compouds. (a) Control, (b) 0.2% DMSO, (c) AsB, (d) AsCB, (e) DMA, (f) MAA, (g) Asi, and (h) Asa groups.

3.10. Gene expression in the liver of adult zebrafish

Livers of zebrafish exhibited changes of gene expression following arsenic compounds treatment as compared to control. The mRNA levels of p53, an important regulator of apoptosis induction, were changed significantly in the 20ppm with increases of 0.9-, 1.1-, 1.2-, 1.2-, 1.2-, 4.5- and 1.9-fold compared with the control group, as shown in Fig.1-20. As for the induction patterns of Bax, the Bcl2 associated X protein, it was found that mRNA levels dramatically increased to 1.0-, 0.9-, 1.0-, 0.8-, 1.0-, 1.6-, and 1.2-fold compared with the control group. To assess whether arsenic compounds induces apoptosis via the caspase pathway, the gene expression of caspase-3, caspase-8, and caspase-9 was shown in Fig.1-20. The gene expression of caspase-3, and caspase-9 activity was significantly up-regulated in the 20ppm. Similarly, the caspase-3 and caspase-9 activity was also significantly induced in the exposure groups. Also, the caspase-8 activity was significantly induced in the exposure groups.

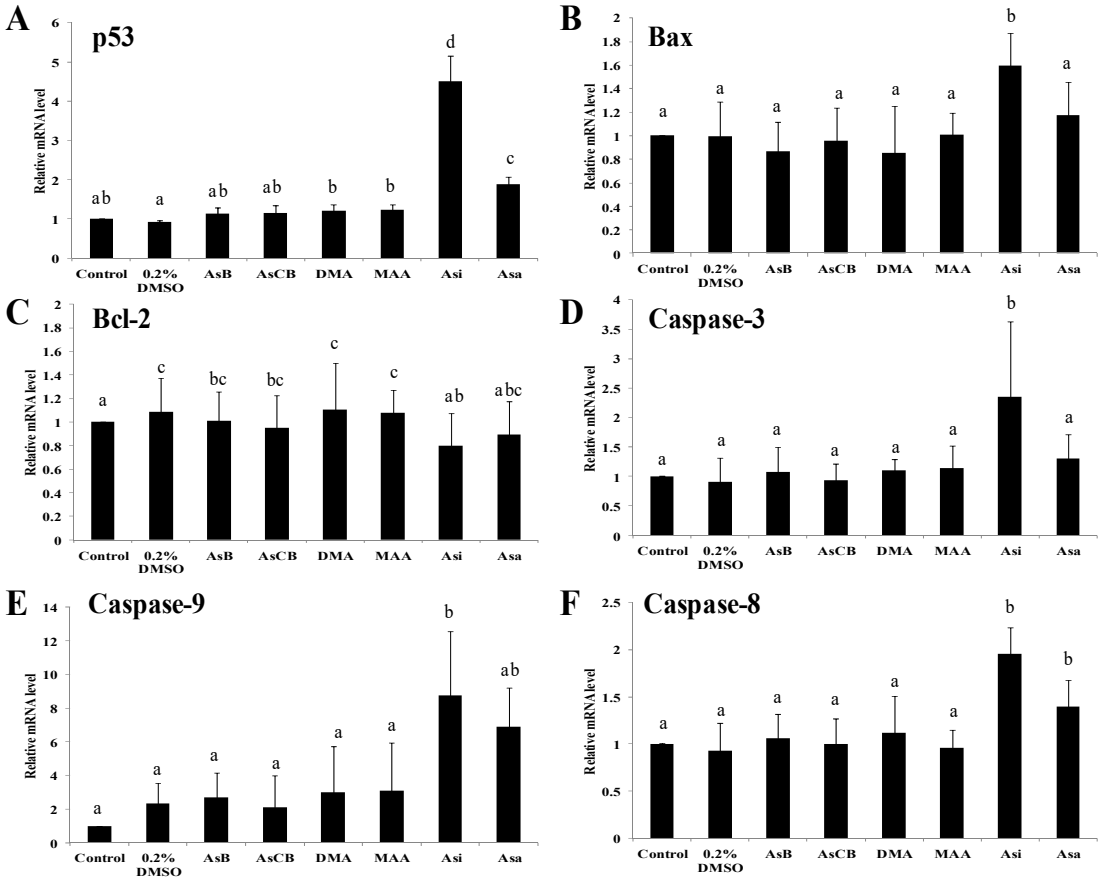

Fig. 1-20. Expression of p53 (A), Bax (B), Bcl-2 (C), Caspase-3 (D), Caspase-9 (E), and Caspase-8 (F) mRNA species in the liver tissues of adult zebrafish treated with arsenic compounds of 20ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. [a,b,c] Values having different superscript are significantly different at $P<0.05$.

3.11. Western blot in the liver of adult zebrafish

To study the mechanism of arsenic compounds induced apoptosis, we evaluated the expressions of p53, Bax, Bcl-xL, caspase-3, -9, and caspase-8 by western blot analysis. As shown in Fig.1-21, the expression levels of p53 and Bax, a pro-apoptotic protein was increased gradually with inorganic arsenic compounds. Also, the expression levels of Bcl-xL, antiapoptotic protein were decreased with inorganic arsenic compounds as shown in Fig.1-22. The expression levels of Cleaved Caspase 3, Cleaved Caspse 9, and Caspase 8 in the liver of adult zebrafish with arsenic compounds of 20ppm for 96 h and untreated controls was activated as shown in Fig.1-23. These results show that inorganic arsenic compounds-treated groups were strongly induced the toxicity than organic arsenic compounds through up and down regulation in apoptosis pathway. Therefore, we suggest that organic arsenic compounds did not induced the toxicity by apoptosis pathway.

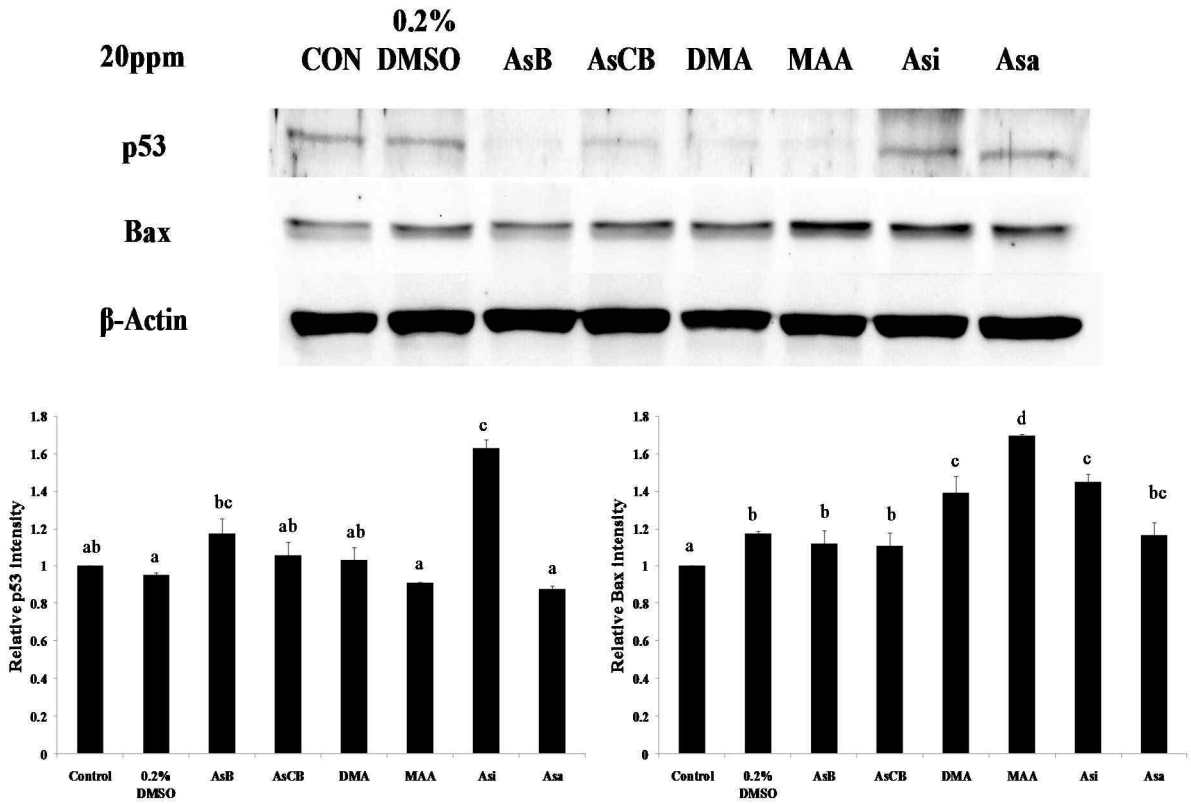

Fig. 1-21. Western blot analysis of p53 and Bax in the liver tissues of adult zebrafish with arsenic compounds of 20ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. [a,b,c] Values having different superscript are significantly different at $P<0.05$.

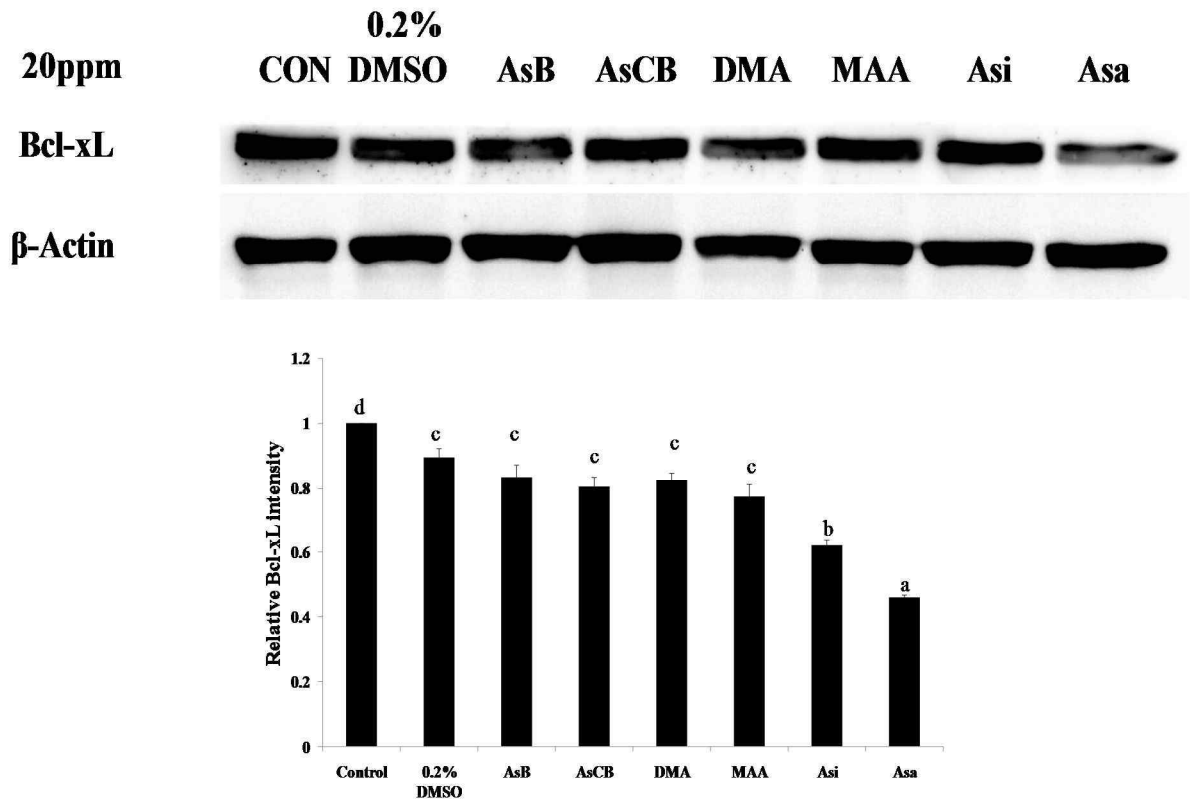

Fig. 1-22. Western blot analysis of Bcl-xL in the liver tissues of adult zebrafish with arsenic compounds of 20ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. [a,b,c] Values having different superscript are significantly different at $P<0.05$.

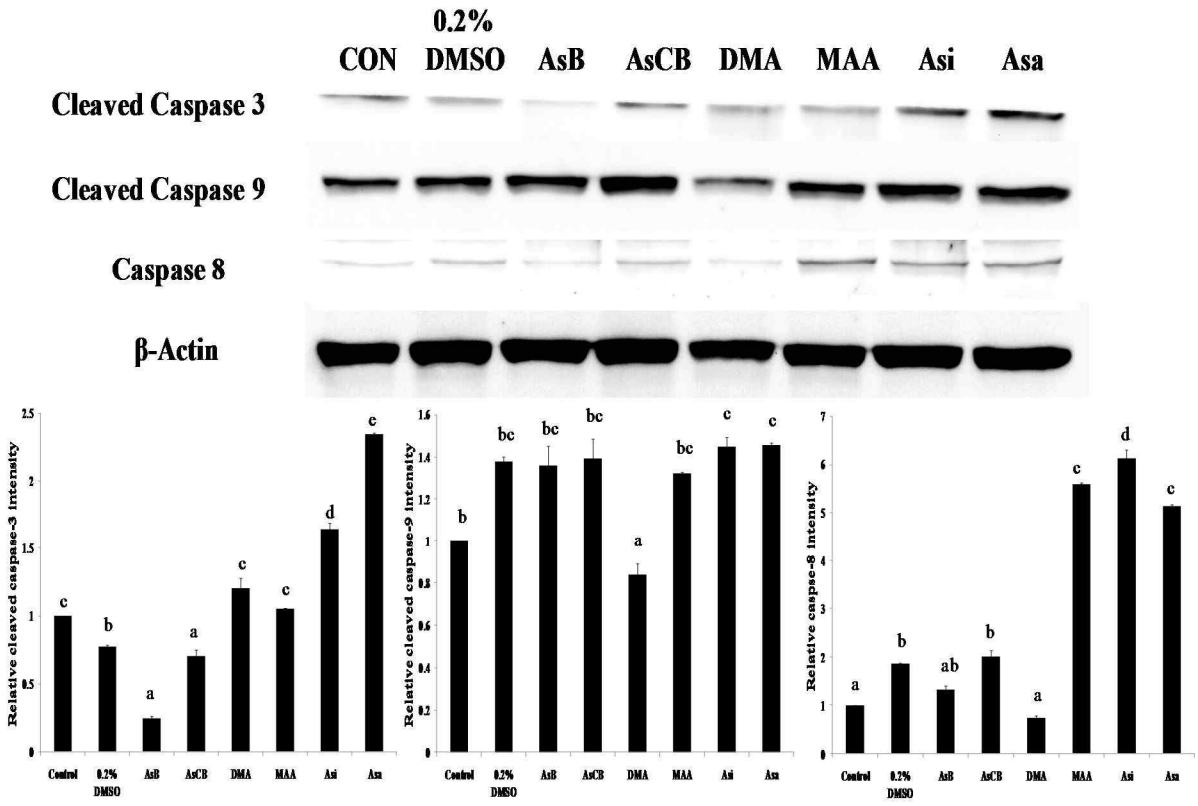

Fig. 1-23. Western blot analysis of Cleaved Caspase 3, Cleaved Caspse 9, and Caspase 8 in the liver tissues of adult zebrafish with arsenic compounds of 20ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

4. Discussion

In recent years, they were required appropriate data on the risk assessment for registration of chemicals, pesticides, biocides and pharmaceuticals in the legislation of the European and other industrialized countries (EMEA/CHMP, 2006). Although the number of animal experiments is expected, we need the information on the toxicity from vertebrates, in particular with fish. However, vertebrates such as rats, and mice were emerged to be an ethical problem. There is an interest in the zebrafish as an alternative animal model. Previous studies have shown that early life stages of zebrafish have been already being applied as high throughput screening systems for drug development and safety assessment (Nasevicius, 2001; Peterson, 2000; Richards, 2008). Our results showed that zebrafish affected by exposure to organic and inorganic arsenic compounds in various concentrations. Heart of the zebrafish is the first organ to develop and function (Hill, 2005). For physiological correlation, the heart-beating rate used to predict the metabolic diseases of fish (Thorarensen, 1996; Wang, 2014). It was indicated that organic and inorganic arsenic compounds exposures at 0.25, 0.5, and 1ppm could increase the heart-beating rates of embryos. This means zebrafish get damaged to the samples. The inorganic arsenic compounds groups were significantly affected by the toxicity.

The normal time, 24 hpf for development of circulation in zebrafish was observed in the zebrafish exposed to arsenic compounds. Blood flow was normal through arteries and veins at that time in groups (Ezdihar Hassoun, 2005). During the period from 24 to 72 hpf, the yolk sac is the site of the earliest blood flow and occurring the extensive vascular remodeling (Kimmel, 1995). The yolk sac edema sizes of the zebrafish were observed to increase, compared with the control group. Also, the results of the present study suggest that malformation may be due to a general response of zebrafish exposed to arsenic compounds.

In relation to the morphological malformations obtained, inorganic arsenic compounds-treated groups were significantly changed to the control group. This results suggested that they induced malformation. Malformations of the zebrafish have been reported, when exposed to toxicants, such as PBDE 47 (Lema, 2007), PFOS (Shi, 2008), TCDD (Antkiewicz, 2005' Henry, 1997).

The acridine orange staining demonstrated that abnormalities mainly occurred in the heart areas in zebrafish (Jun, 2009). A nucleic acid, acridine orange, is a selective fluorescent cationic dye useful for apoptotic cells. The cell death was treated by arsenic compounds in zebrafish, compared to the control group. But, inorganic arsenic compounds were remarkable difference in the cell death at the 1ppm.

It is well known that the production of ROS in zebrafish in response to contamination is intimately associated with apoptotic cell death. ROS-induced oxidative stress is thought to contribute to abnormal development during embryogenesis (Yamashita, 2003). In this study, it was observed that inorganic arsenic compounds induced ROS formation in zebrafish embryos at 1ppm.

We have characterized the hepatoxicity of arsenic compounds in zebrafish. Inorganic arsenic compounds treatment were induced hepatotoxicity in the liver of adult zebrafish.

Activation of transcription factor p53 in response to DNA damage can lead to cell cycle arrest or apoptosis (Langheinrish et al., 2002). p53 expression in tightly regulated at both the transcriptional and post-tranlational levels, but mainly at the post-translational level. In this study, inorganic arsenic compounds treatments caused DNA damage and induction of p53 in the liver tissues. The induction of p53 was mainly at the post-translational level, because p53 protein and mRNA level was increased.

Liver histopathology in adult zebrafish was determined in this study (hepatocyte cloudy

swelling and pyknosis) (Lynn P. Weber 2003). No previous study we are aware of has quantitied these classic histopathological parameters in organic and inorganic arsenic compounds-exposed zebrafish livers. The analysis of these parameters involves counting the number of cells affected per field of view and this was likely confounded by a concurrent, concentration-dependent increase in hepatocyte size.

In summary, this study demonstrates the occurrence of toxicity in zebrafish model exposed to organic and inorganic arsenic compounds. Zebrafish was exposed to inorganic arsenic compounds as indicated by increased malformation and reduced survival in the larvae. The mechanism of this toxicity appears to be the generation of ROS and the nosequent triggering of apoptosis genes. This study indicates that gene expression in zebrafish model provide a sensitive method of elucidating the toxicity mechanism, although only a few genes related to cell apoptosis pathway.

Part Ⅱ.

Toxicity assessment of algal arsenic in zebrafish model

Part Ⅱ.

Toxicity assessment of algal arsenic in zebrafish model

1. Abstract

Seaweeds are known to compose a variety of bioactive substances (polysaccharides, pigments, minerals, peptides, and polyphenols). However, it has been a significant concern caused by arsenic content of seaweeds. Therefore, the present study was carried out to evaluate toxicity of arsenic from *Ecklonia cava, Undaria pinnatifida,* and *Hizikia fusiformis* in zebrafish model. Arsenic extracted from *Ecklonia cava, Undaria pinnatifida,* and *Hizikia fusiformis* (ECAE, UPAE, and HFAE) by using 50% methanol in 1% HNO_3 solvent. ECAE, UPAE, and HFAE were added to each of the wells for up to 96 hpf. The survival rates of embryos were no significantly changed at 100, 500, and 1000ppm. At 1000ppm, embryos were significantly differences in morphology, heartbeat rate, yolk sac edema size, and fluorescence. Also, we were assessed toxicity of algae arsenic in adult zebrafish at 200, 400 and 800ppm. The survival rates of adult zebrafish were no significantly changed at all the concentrations. And, the bile duct of ECAE, UPAE and HFAE groups have no differences compared with the control group. However, zebrafish were affected by exposure to HFAE in the histopathology of liver. For these results, we were assessed the degress of toxicity of arsenic from seaweeds in the zebrafish model.

2. Material and methods

2.1. Materials

Ecklonia cava, Undaria pinnatifida, and *Hizikia fusiformis* of cultured Korean was purchased from a southern coastal area of Seongsan, South Korea. Salt, sand and epiphytes ere removed with tap water. Then, the 3 species of seaweeds were rinsed carefully with fresh water and freeze-dried. The dried seaweeds were ground and sieved through a 50-mech standard testing sieve.

2.2. Extraction arsenic from seaweeds

The dried, *Ecklonia cava, Undaria pinnatifida,* and *Hizikia fusiformis* powder (1 g) were extracted with 10 ml 50% methanol in 1% HNO_3 solvent. The mixture was extracted at 60°C for 2 h in the ultrasonic focalized bath. The samples were centrifuged for 10 min at 5,980*g, the supernatant was collected and the residue was re-extracted following the former procedure. The two combined extracts were mixed, filtered through a 0.45 μm nylon syringe filter. And evaporated to dryness using a evaporator and cold trap system. Each residue was kept frozen (-20◦C) prior to analysis. Three extracts were prepared from each sample.

Table 2-1. Total arsenic contents of ECAE, UPAE, and HFAE

(mg/kg)

	As	Cd	Hg	Pb
ECAE	**98.26**	**3.67**	**0.14**	**1.27**
UPAE	**69.85**	**2.74**	**0.12**	**0.42**
HFAE	**189.88**	**1.92**	**0.1**	**1.87**

ECAE : *Ecklonia cava* arsenic extracts
UPAE : *Undaria innatifid* arsenic extracts
HFAE: *Hizikia fusiform arsenic extracts*

2.3. Origin and maintenance of zebrafish

Adult zebrafish were purchased from a commercial dealer (Seoul Aquarium, Korea) and 15 fish were kept in a 3L acrylic tank at 28.5±1°C with a 14/10h light/dark cycle. Zebrafish were fed two times a day (Tetra GmgH D-49304 Melle Made in Germany). The day before 1 female and 2 males interbreed. Embryos were mated and natural spawning, induced in the morning by turning on the light. Collection of embryos was completed within 30 min in petri dishes (containing media).

2.4. Waterborne exposure of embryos to arsenic extracts

The embryonic toxicity test was followed the OECD (Organization for Economic Cooperation and Development) guideline 210. As from approximately 3-4 hour post-fertilization (3-4 hpf), the embryos (n=15) were transferred to individual wells of 12-well plates containing 900 mL embryo media. Three concentrations of arsenic extract from seaweeds were chosen for this test; 100, 500, and 1000ppm. Arsenic extracts were added to each of the wells for up to 96 hpf. Then, embryos were rinsed using fresh embryos media.

2.5. Waterborne exposure of adult to arsenic extracts

The fish acute toxicity test was followed the OECD guideline 203. Adult zebrafish of similar length and age were randomly distributed to each of tanks containing 1 L fresh water. Three concentrations of arsenic extracts were chosen for this test; 800, 1600, and 2400ppm. Arsenic extracts were exposed to each of tanks for up to 96 hpf. Then, embryos were rinsed using fresh embryos media.

2.6. Measurement of survival rate, heartbeat rate and yolk sac edema size in zebrafish

embryos

The survival rates were measured up to 7 dpf after exposed to arsenic extracts. The heartbeat rate of both atrium and ventricle was recorded at 48 hpf for 1 min under the microscope. At 48 hpf, lateral views of anesthetized larvae were imaged using a microscope for size of yolk sac edema. The outlines of the yolk sac edema were traced, and the area within each tracing was assessed by ISCapture for windows.

2.7. Morphology observation in zebrafish embryos

The 3 embryos were randomly selected from each well. Thus, zebrafish embryos layered in a glass slide containing embryo media, and evaluated for malformation using the microscope at 24, 48, 72, 96, 120, and 144 hpf.

2.8. Measurement of cell death by sample treatment and image analysis

Cell apoptosis was identified in live embryos using acridine orange staining. Acridine orange; a nucleic acid-selective metachromatic stain is useful for studying apoptosis patterns. At 72 hpf of exposure to algae arsenic (100, 500 and 1000ppm), the zebrafish larvae was transferred to 96-well plate and treated with acridine orange solutions (7 μg/ml), and the plated were incubated for 30 min in the dark at 28.5±1◦C. After incubation, the zebrafish larvae were rinsed in the fresh embryo media. Before examination, the larvae were anesthetized with 0.03% MS-222. Apoptotic cells were observed and photographed under the microscope CoolSNAP-Pro dolor digital camera (Olympus, Japan). Individual zebrafish larvae fluorescence intensity was quantified using the image J program.

2.9. Measurement of ROS production by sample treatment and image analysis

Generation of ROS production in the zebrafish larvae was measured using an oxidation-sensitive fluorescent probe dye, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). At 72 hpf, the zebrafish larvae was transferred to 96-well plate and treated with DCFH-DA solution (20 μg/ml) and incubated for 1 h in the dark at 28.5±1◦C. After incubation, the zebrafish larvae were rinsed in the fresh embryo media. Before examination, the larvae were anesthetized with 0.03% MS-222. ROS production was observation and photographed under the microscope CoolSNAP-Pro dolor digital camera (Olympus, Japan). Individual zebrafish larvae fluorescence intensity was quantified using the image J program.

2.10. Histological evaluation in the liver of adult zebrafish

After 7 days, the liver of adult zebrafish for histological analysis were preserved in Bouin's solution for approximately 24 h and subsequently transferred to 70% ethanol for storage. Later, fixed zebrafish were dehydrated, embedded in paraffin, and sectioned at 7 μm. Standard histological slides were prepared by using hematoxylin and eosin. The liver of adult zebrafish was determined under the microscope.

2.11. Quantitative real-time PCR

As from approximately 3-4 hpf, the embryos (n = 30) were transferred to individual wells of 6-well plates containing 2,850 μL of embryo media. Embryos were treated with concentration of algae arsenic (100, and 500ppm) was added to the plate for up to 96 hpf. The zebrafish larvae were transferred into Eppendorf tube, and then washed twice with heated PBS in 28.5±1°C. After that, keep in -70±1˚C overnight.

Larvae and liver tissues were homogenized in TRIzol reagent (ambion, USA), and total RNA was extracted according to the manufacturer's instructions. After which real-time PCR

was performed with cDNAs and gene-specific primer pairs (Table 2.) mixed with SYBR Green PCR master mix (Takara) in an Light Cycler real-time PCR system. PCR condition were as follows: denatured for 5 min at 95◦C and then amplified using 50 cycles of 30 s at 95◦C, 20 s at 55◦C and 20 s at 72◦C.

Table 2-2. Primer sets employed in real-time PCR analysis

Gene name	Sequence of the primer (5'-3')
β-actin	Forward-GCTGACAGGATGCAGAAGGA Reverse-TAGAAGCATTTGCGGTGGAC
p53	Forward-GGGCAATCAGCGAGCAAA Reverse-ACTGACCTTCCTGAGTCTCCA
Bax	Forward-GGCTATTTCAACCAGGGTTCC Reverse-TGCGAATCACCAATGCTGT
Bcl-2	Forward-TCACTCGTTCAGACCCTCAT Reverse-ACGCTTTCCACGCACAT
Caspase 3	Forward-CCGCTGCCCATCACTA Reverse-ATCCTTTCACGACCATCT
Caspase 8	Forward-GATGAGAACCTGACAAGCGGTGATG Reverse-GCTCATCCAGTCGCAGAATCAGGT
Caspase 9	Forward-AAATACATAGCAAGGCAACC Reverse-CACAGGGAATCAAGAAAGG

2.12. Statistical analysis

All experiments were conducted in triplicate (n=3) and an one-way analysis of variance (ANOVA) test (using SPSS 12.0 statistical software) was used to analyse the data. Significant differences between the means of parameters were determined by using the Duncan test to analyze. *P*-values of less than 0.05 ($P < 0.05$) was considered as significant.

3. Results

3.1. Survival rates. heartbeat rates, and yolk sac edema sizes in zebrafish embryos/larvae after exposure arsenic extracts

To evaluate the potential toxicity of zebrafish embryos exposure to ECAE, UPAE, and HFAE (100, 500, and 1000ppm), we measured the survival rate until 7 dpf, as shown in Fig. 2-1. In this study, zebrafish embryos were exposed to arsenic extracts for 96 hours. After 96 h of exposure, the survival rates in embryos were reduced by the ECAE, UPAE, and HFAE compared with the control group at 100, 500, and 1000ppm. The survival rates of the treated groups were recorded as 83.3 ± 3.3%, 53.3 ± 13.3%, and 53.3 ± 0.0% relative to 86.7 ± 6.7% in the control group at 1000ppm. The other survival rates of the lower concentrations were also significantly reduced to exposure ECAE, UPAE, and HFAE, compared with the control group. Therefore, zebrafish embryos were no affected by exposure from ECAE, UPAE, and HFAE at 100ppm. The heart-beating was evaluated the embryos hatched at 2 dpf. As shown in Fig.2-2., the heart-beat rates in larvae were decreased to 83.4 ± 3.1%, 87.9 ± 4.1%, and 84.1 ± 4.8% compared with the control group at 1000ppm. The other survival rates of the lower concentrations were also no significantly increased to exposure ECAE, UPAE, and HFAE, compared with the control group. Altered yolk sac edema size were also measured in larvae exposed to ECAE, UPAE, and HFAE. At 48 hpf, the outlines of the yolk sac edema were traced, and the area within each tracing was assessed in the zebrafish larvae. The yolk sac edema sizes in larvae were dramatically increased in larvae exposed to ECAE, UPAE, and HFAE at 1000ppm as shown in Fig.2-3. But, the other yolk sac edema sizes of the 100ppm groups were no significantly increased to exposure ECAE, UPAE, and HFAE, compared with the control group.

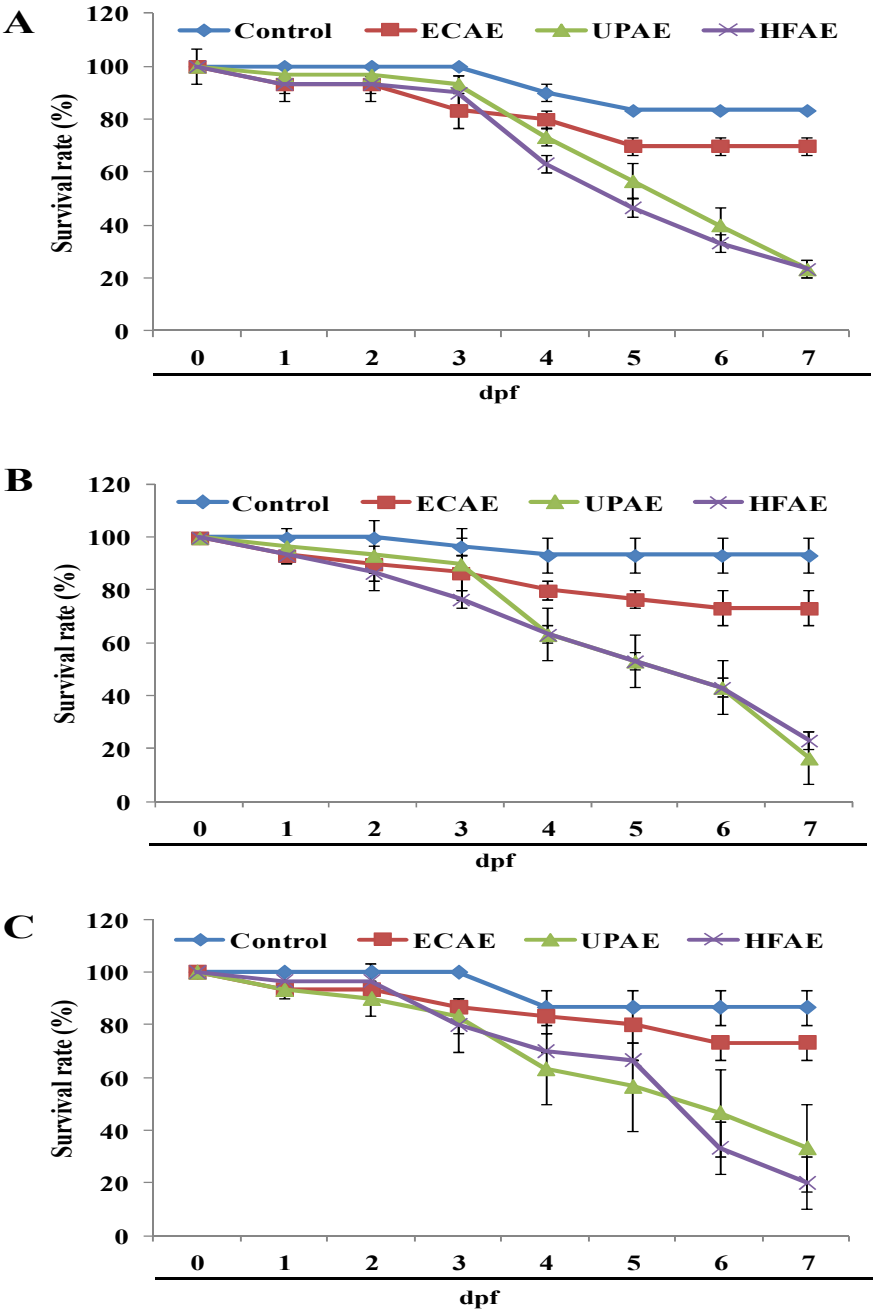

Fig. 2-1. Measurement of survival rates after treating with the algae arsenic. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The zebrafish were treated with various of concentrations of arsenic extracts. And then we measured survivorship of zebrafish for 7dpf. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

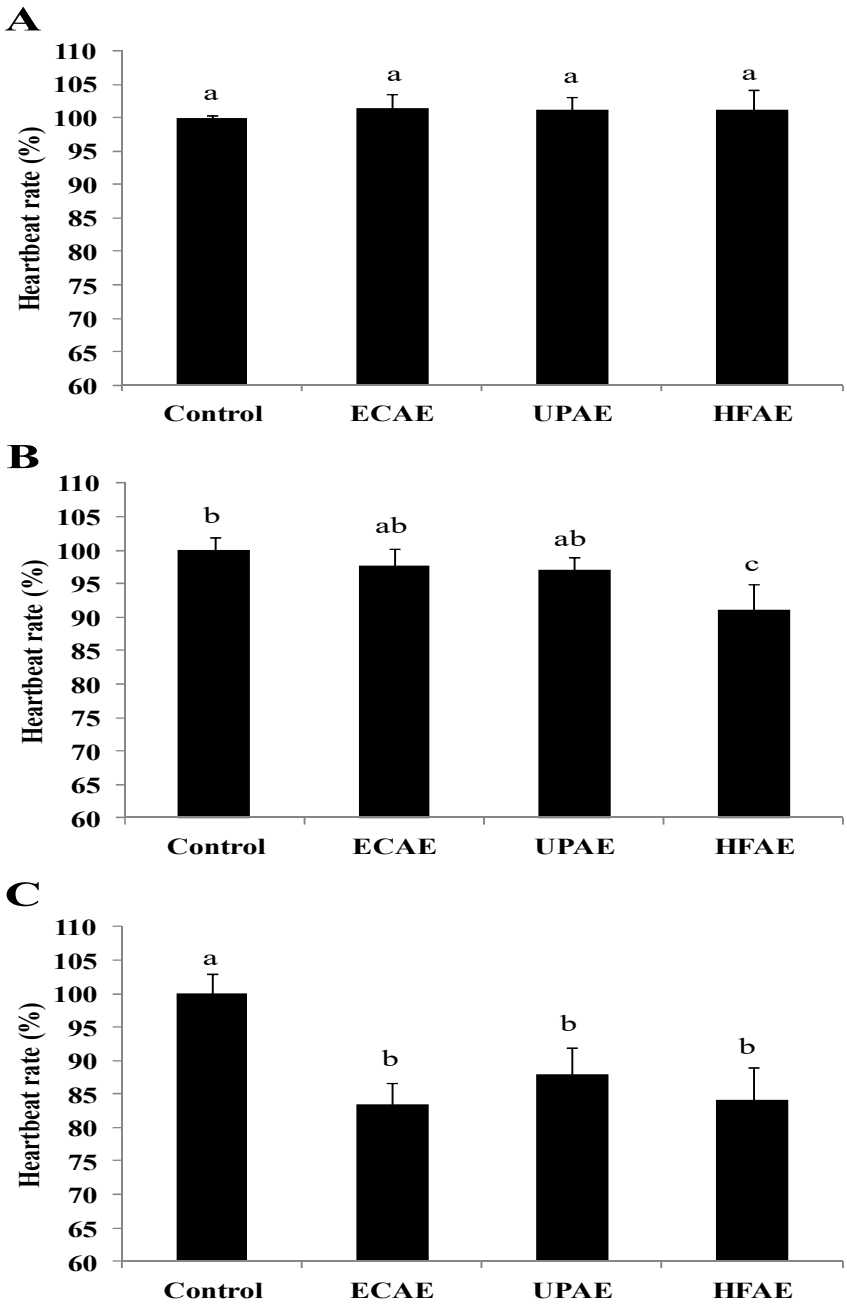

Fig. 2-2. Measurement of toxicity of the algae arsenic by the heartbeat rate. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The heartbeat rate of both atrium and ventricle were counted at 48 hpf by under the microscope for 1 min. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

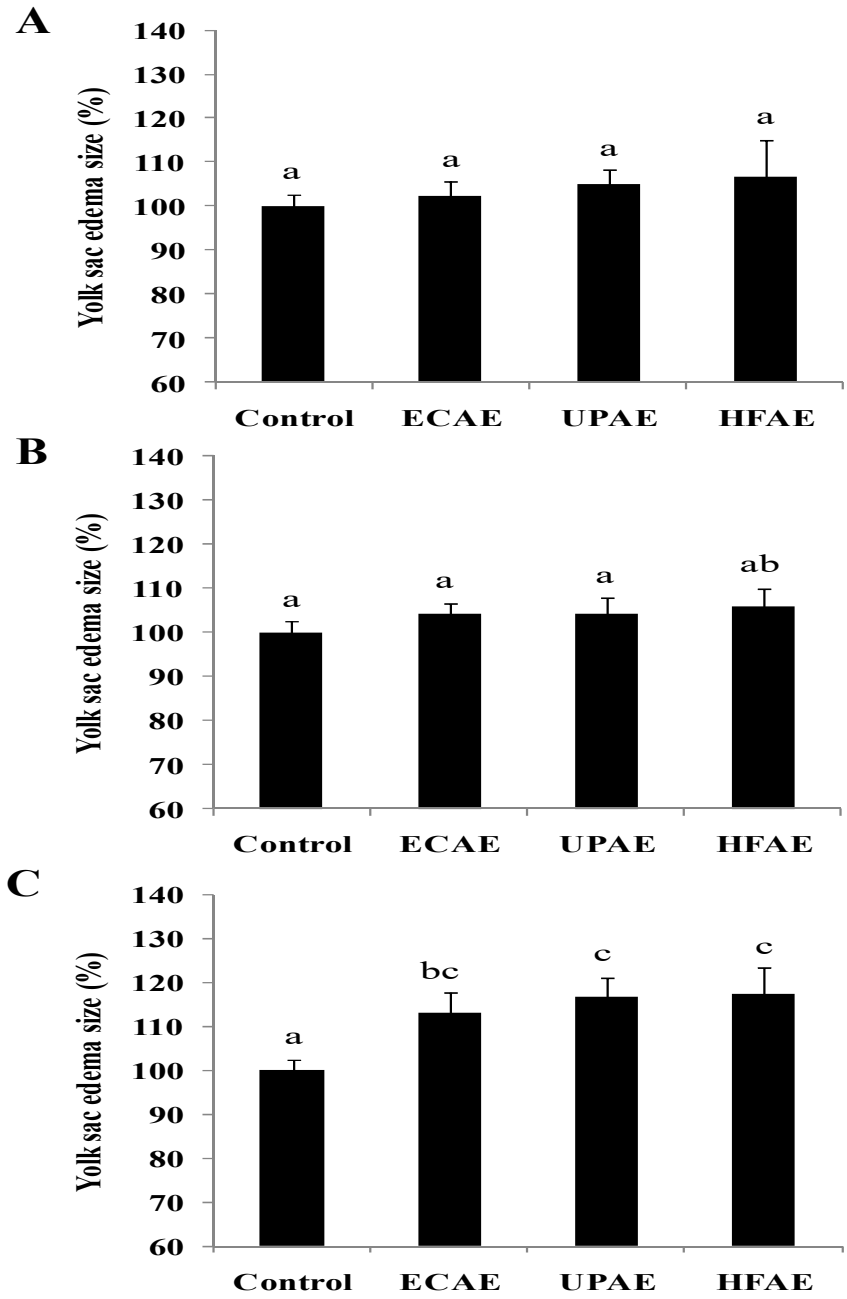

Fig. 2-3. Measurement of toxicity of the algae arsenic by the yolk sac edema size. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The yolk sac edema size was measured at 48 hpf using the microscope. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.2. Morphological changes in zebrafish embryos and larvae

After fertilization, we observed the developmental abnomalities of zebrafish embryos and larvae that had been exposed to ECAE, UPAE, and HFAE from 1 dpf to 6 dpf by morphology, as shown in Fig.2-4, -5, -6, -7, -8, and -9. The developmental abnormalities included a yolk sac edema, pericardial edema, tail malformation. Most of zebrrafish was similarities in the symptoms exposed by each of arsenic extracts except the control group. However, HFAE-treated zebrafish groups were difference in the symptoms such as early hatching, slowly developmental process, and death at 1000ppm. Zerafsih was a little increased the yolk sac edema at 100ppm but no serious. Zebrafish embryos and larvae were strongly affected by exposure from arsenic extracts of 1000ppm compared with the control group.

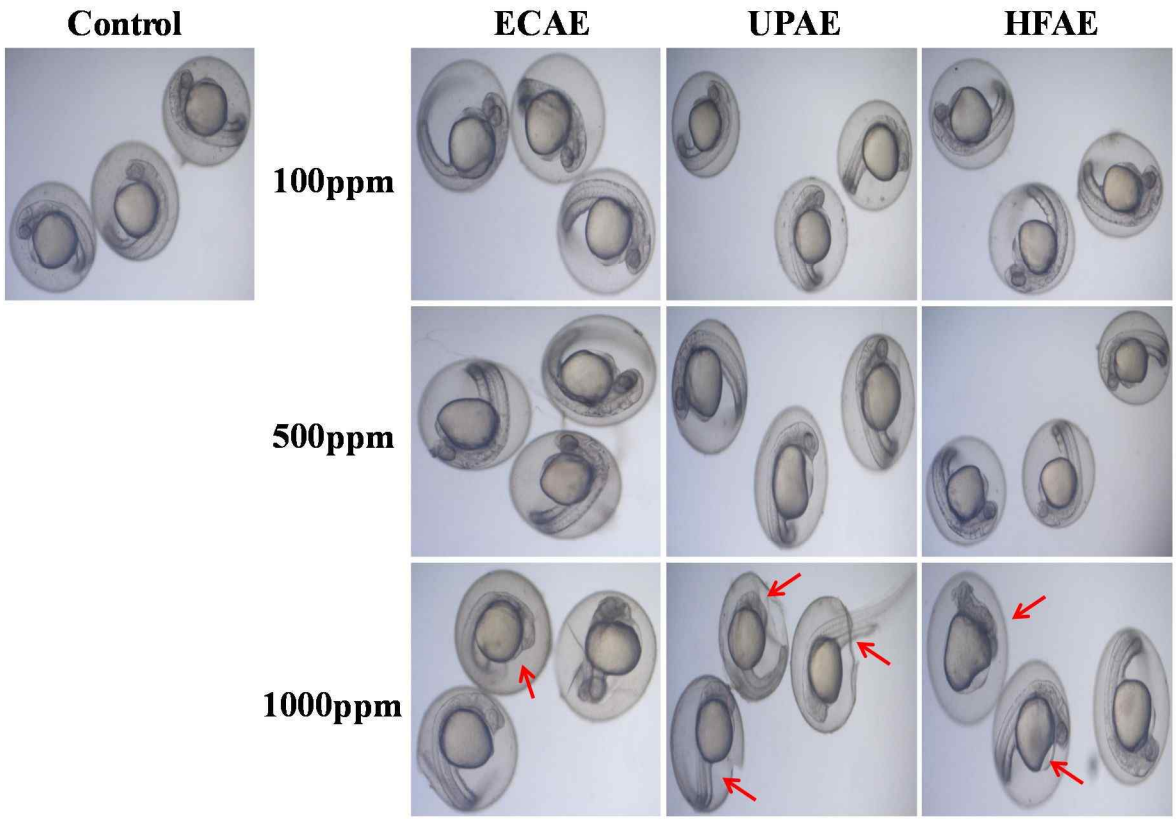

Fig.2-4. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 24 hpf

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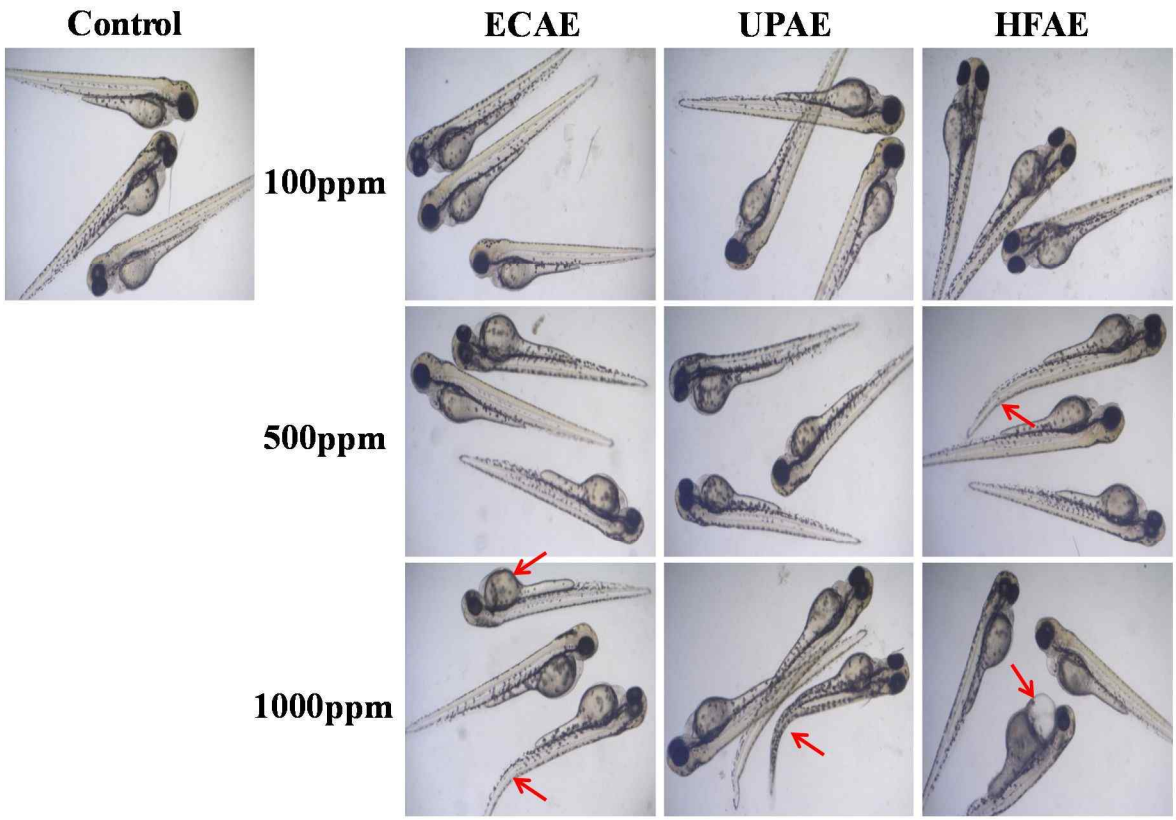

Fig.2-5. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 48 hpf.

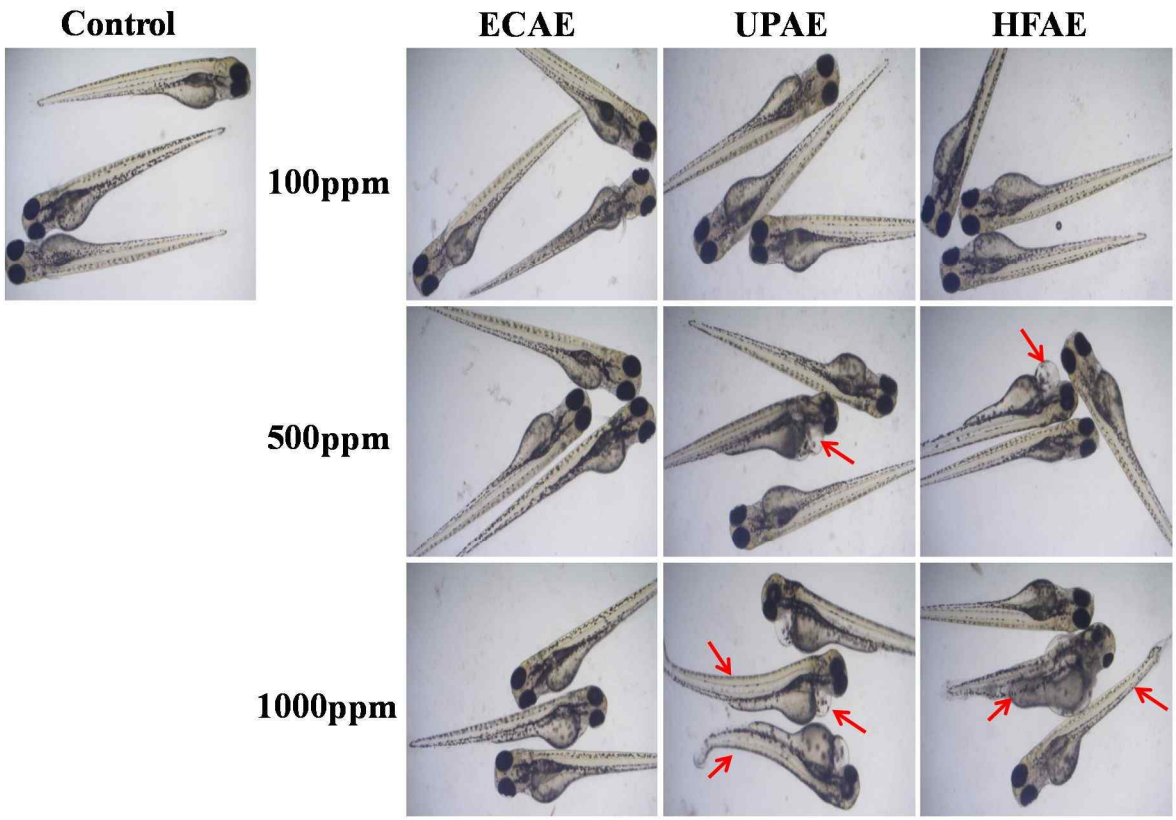

Fig.2-6. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 72 hpf.

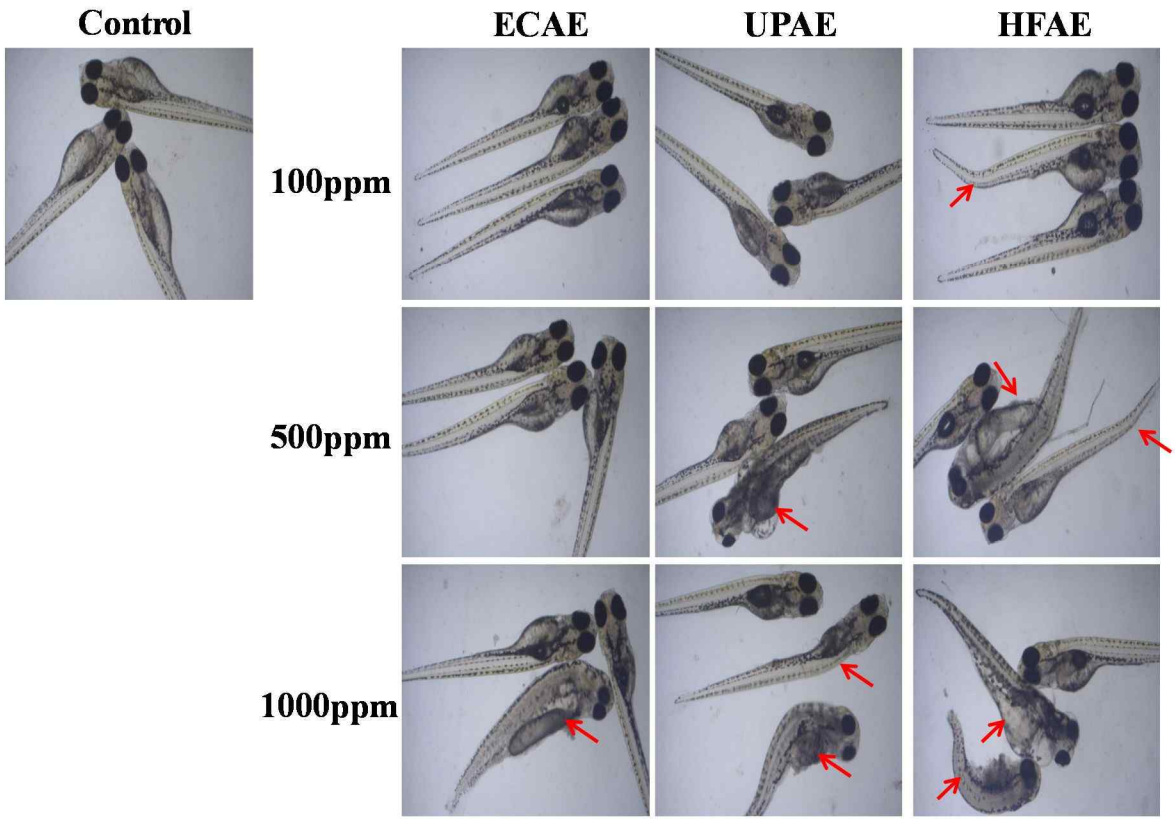

Fig.2-7. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 96 hpf.

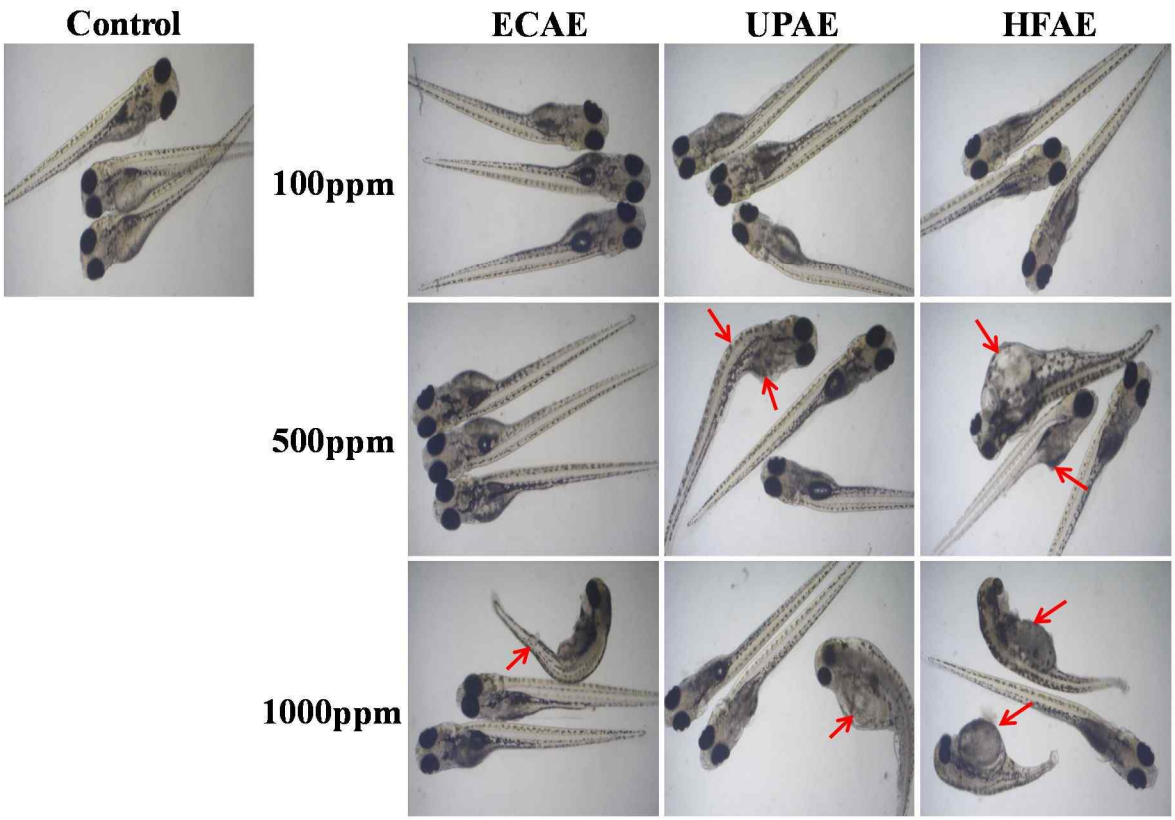

Fig.2-8. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 120 hpf.

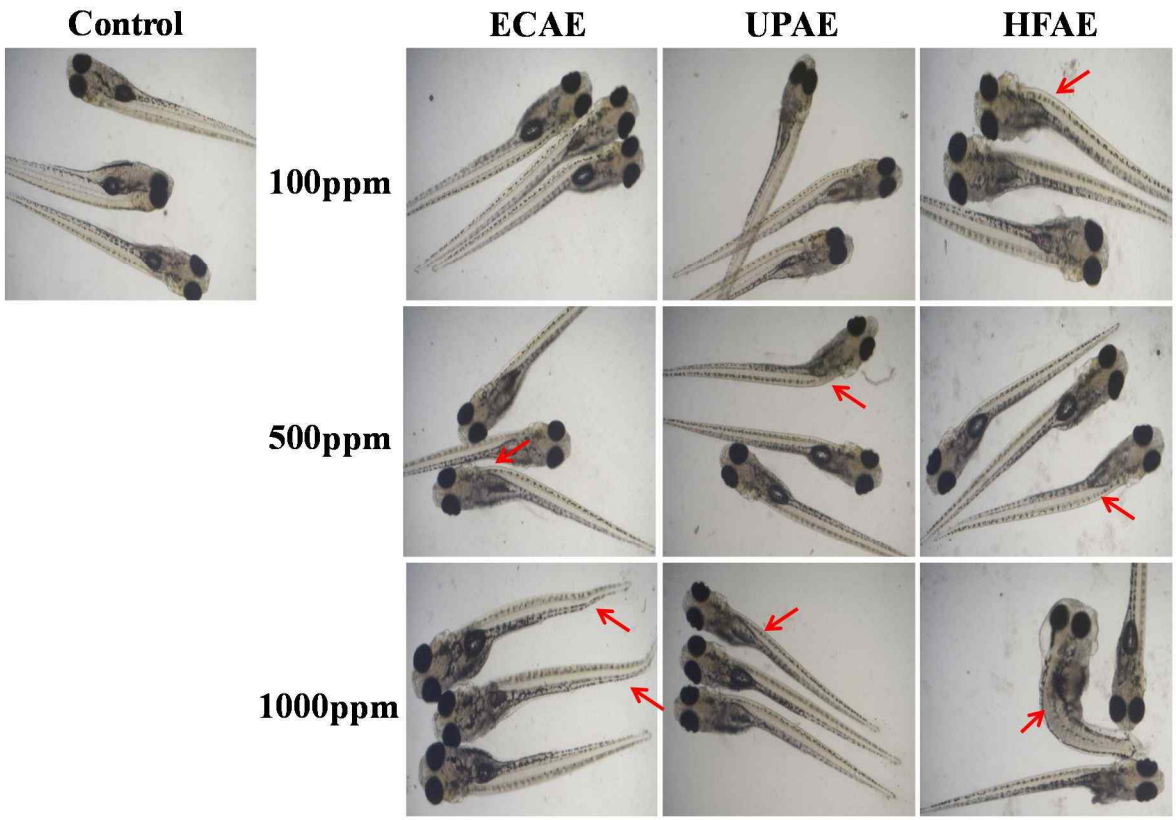

Fig.2-9. Measurement of toxicity of the ECAE, UPAE, and HFAE by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation at 144 hpf.

3.3. Toxicity of algae arsenic by AO staining in zebrafish larvae

To determine if samples lead to increased cell death, embryos were treated from 3 hpf to 24 hpf to graded ECAE, UPAE, and HFAE concentrations by types. Cell death induced by the toxicity of the samples was evaluated via acridine orange for 30 min to stain dying cells. Larvae exposed the types of ECAE, UPAE, and HFAE with the concentrations had an increase in cell death at 100ppm, 500ppm, and 1000ppm. The level of cell death in an ECAE, UPAE, and HFAE were more than 1.3-fold, 1.4-fold and 1.5-fold at 1000ppm. The lower concentrations were also significantly increased to exposure ECAE, UPAE, and HFAE, compared with the control group.

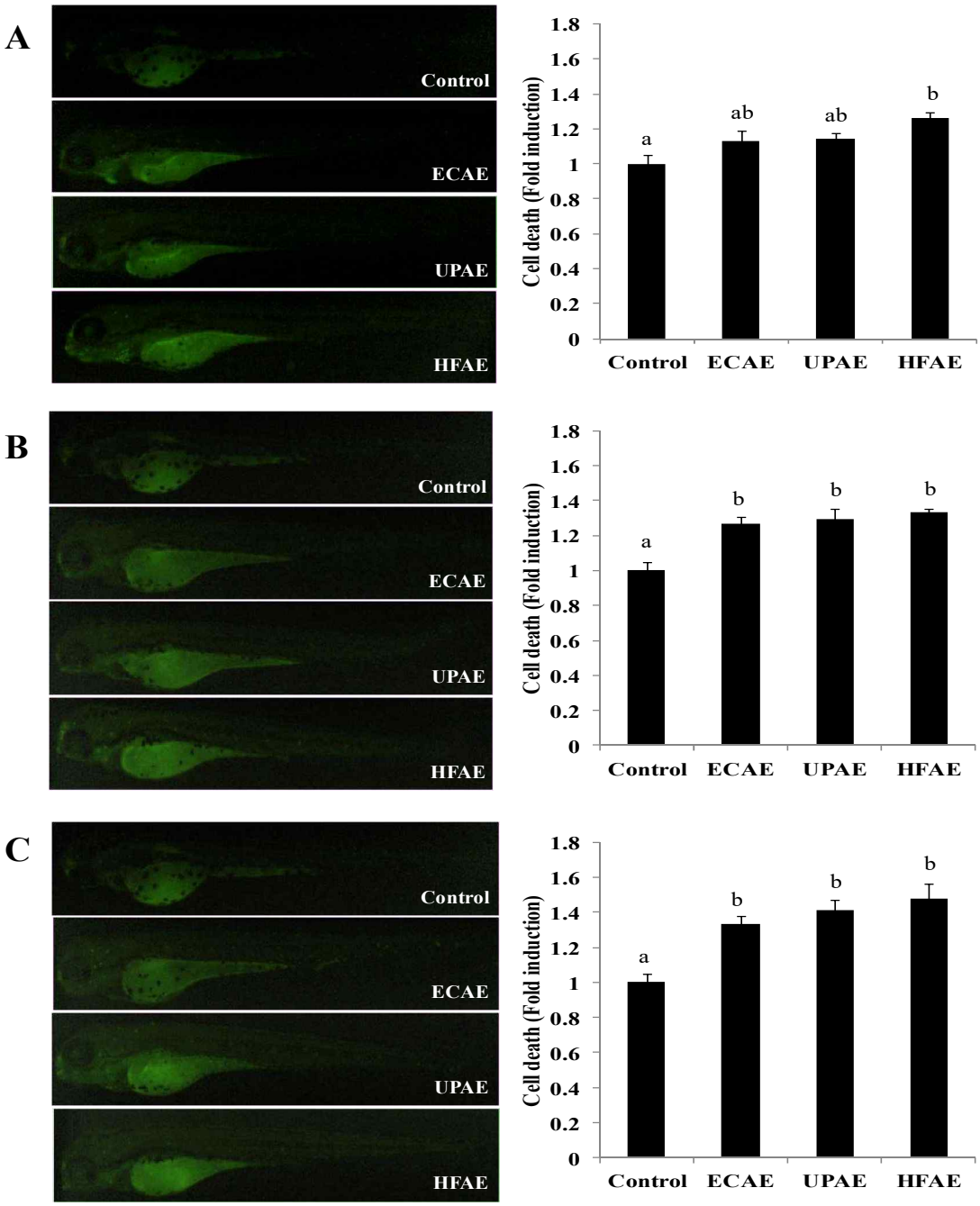

Fig. 2-10. Measurement of toxicity of algae arsenic by the cell death. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The cell death was measured by image analysis using fluorescence microscope and levels using Image J. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.4. Toxicity of algae arsenic by DCFH-DA staining in zebrafish larvae

The abilities of algae arsenic to cause oxidative stress-induced apoptosis in zebrafish larvae were assessed by measured ROS production. Larvae exposed types of ECAE, UPAE, and HFAE with the concentrations had an increase in ROS production at 100ppm, 500ppm, and 1000ppm. Zebrafish larvae were increased more than 10% at all the concentrations. HFAE-treated group was significantly increased to 132 ± 3.4%, 141.4 ± 2.0%, and 146 ± 3.3% compared with the control group at the all concentrations.

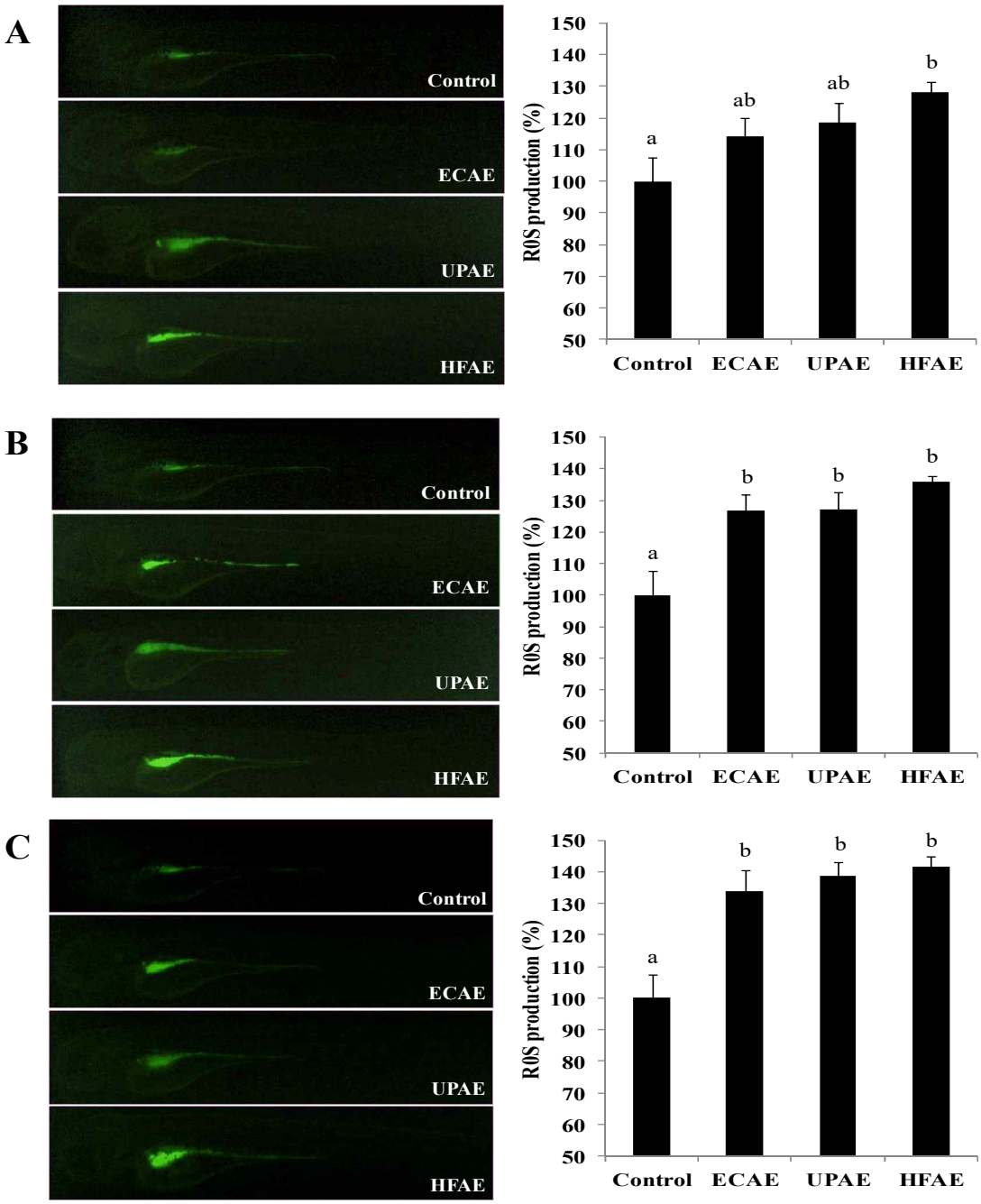

Fig. 2-11. Measurement of toxicity of the algae arsenic by the ROS production. (A) 100 ppm algae arsenic, (B) 500ppm algae arsenic, and (C) 1000ppm algae arsenic. The ROS production was measured by image analysis using fluorescence microscope and levels using Image J. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.5. Gene expression

The mRNA levels of p53, an important regulator of apoptosis induction, were changed significantly in the 500ppm with increases of 1.3- and 1.4- fold compared with the control group, as shown in Fig.2-12. As for the induction patterns of Bax, the Bcl2 associated X protein, it was found that mRNA levels dramatically increased to 1.3- and 1.5-fold compared with the control group. To assess whether algae arsenic induces apoptosis via the caspase pathway, the gene expression of caspase-3, caspase-8, and caspase-9 was shown in Fig.2-12. The gene expression of caspase-3, and caspase-9 activity was significantly up-regulated in the 500ppm. Similarly, the caspase-3 and caspase-9 activity was no significantly changed in the exposure groups. Also, the caspase-8 activity was significantly increased in the UPAE and HFAE groups.

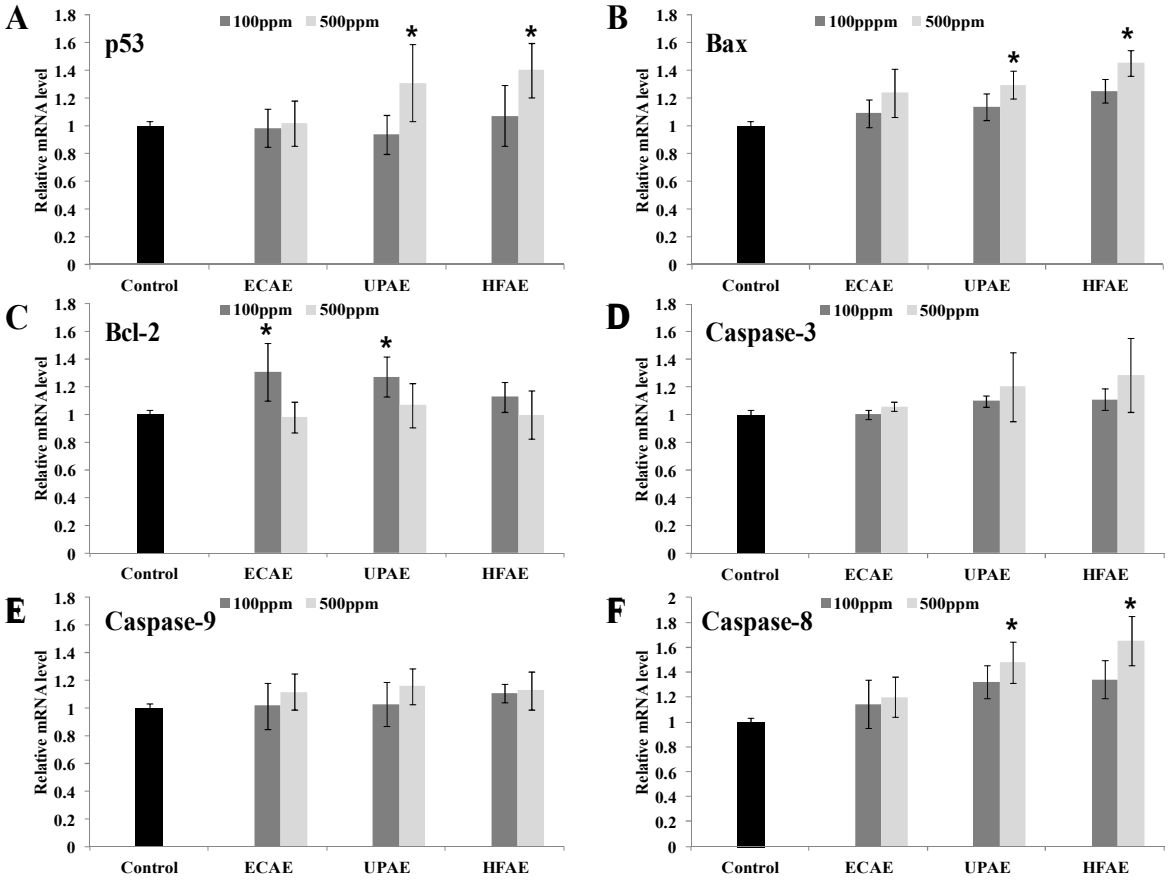

Fig. 2-12. Expression of p53 (A), Bax (B), Bcl-2 (C), Caspase-3 (D), Caspase-9 (E), and Caspase-8 (F) mRNA species in the embryos treated with algae arsenic of 100 and 500ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. [a,b,c] Values having different superscript are significantly different at $P<0.05$.

3.5. Survival rates in adult zebrafish after exposure arsenic extracts

To evaluate the potential toxicity of adult zebrafish exposure to ECAE, UPAE, and HFAE (200, 400, and 800ppm), we measured the survival rate until 7 dpf, as shown in Fig.2-13. In this study, adult zebrafish were exposed to arsenic extracts for 96 hours. After 96 h of exposure, the survival rates in zebrafish were no changed by the treatment of ECAE, UPAE, and HFAE, compared with the control group at 200, 400, and 800ppm. Therefore, adult zebrafish in the survival rates were any affected by exposure to arsenic extracts; ECAE, UPAE, and HFAE.

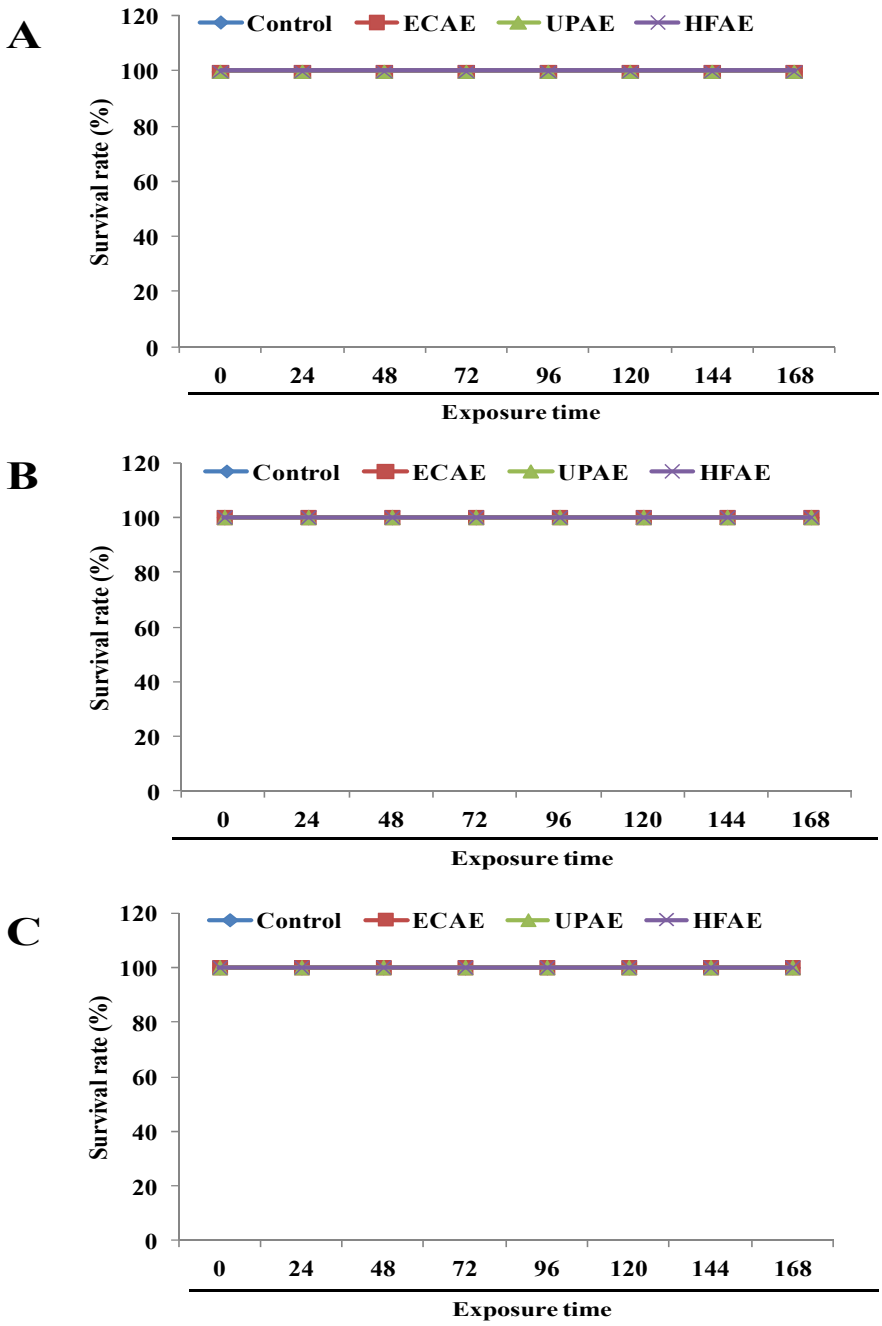

Fig.2-13. Measurement of survival rates after treating with algae arsenic. (A) 200ppm algae arsenic, (B) 400ppm algae arsenic, and (C) 800ppm algae arsenic. The zebrafish were treated with vatious of concentrations of arsenic extracts. And then we measured survivorship of zebrafish for 7dpf. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

3.6. Morphological changes in adult zebrafish

Zebrafish have a single dorsal fin and no adipose fin. Their fins do not contain true spines but instead have hardened rays. We observed the symptoms of zebrafish have been exposed to algae arsenic from 1 dpf to 6 dpf by morphology, as shown in Fig.2-14. Zebrafish exposed to ECAE, UPAE, and HFAE were no appeared various symptoms such as short caudal fin, part of the heart change the red color, and the belly of fish changes the green color. A dilated common bile duct was discerned grossly after 7days of exposure to ECAE, UPAE, and HFAE in the zebrafish, as shown in Fig.2-15. The bile duct of ECAE, UPAE, and HFAE groups has no differences compared with the control group.

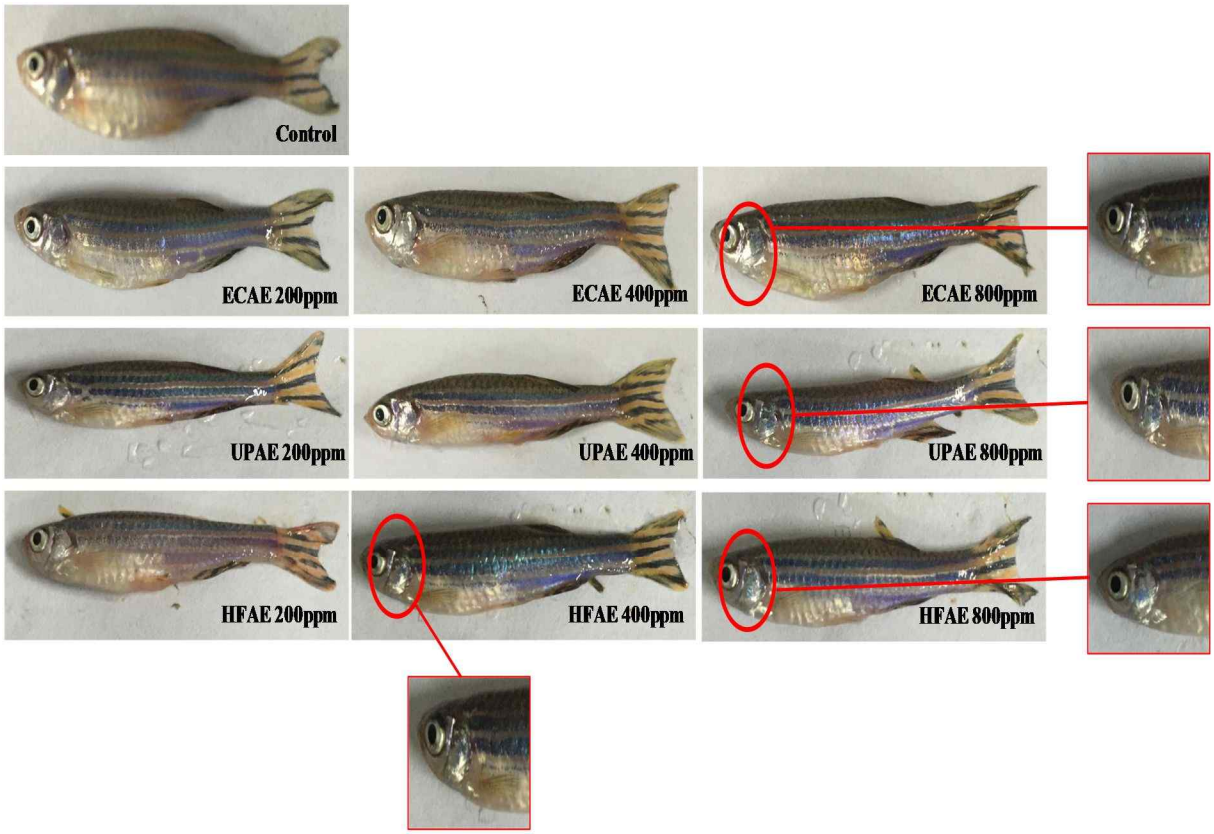

Fig. 2-14. Measurement of toxicity of algae arsenic by the morphologic features. The images were taken under a microscope for evaluation of the visual observation and malformation in the adult zebrafish at 7 days post-exposure after treated arsenic extracts with various concentrations.

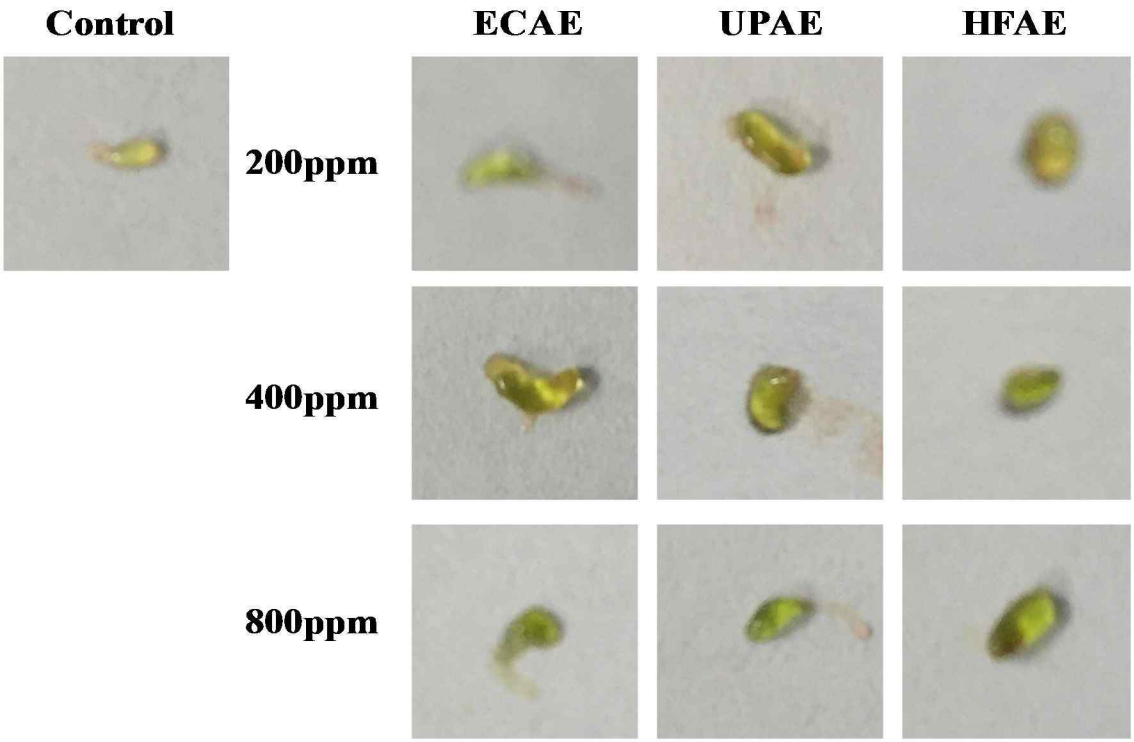

Fig. 2-15. The bill duct of zebrafish at 7 days post-exposure after treated ECAE, UPAE, and HFAE with various concentrations.

3.7. Histopathology in the liver of adult zebrafish

Livers of zebrafish exhibited histopathological changes following ECAE, UPAE, and HFAE treatment as compared to control. Exposure to 800ppm HFAE obvious swelling of hepatic cells. And cell borders became obscure and hepatocelular vacuolar degeneration was observed. Also, nuclei enlargement and pycnosis were observed along with concomitant hepatocyte necrosis and cytolysis. The cellular structure was nearly collapsed in livers of zebrafish receiving the highest dose of HFAE.

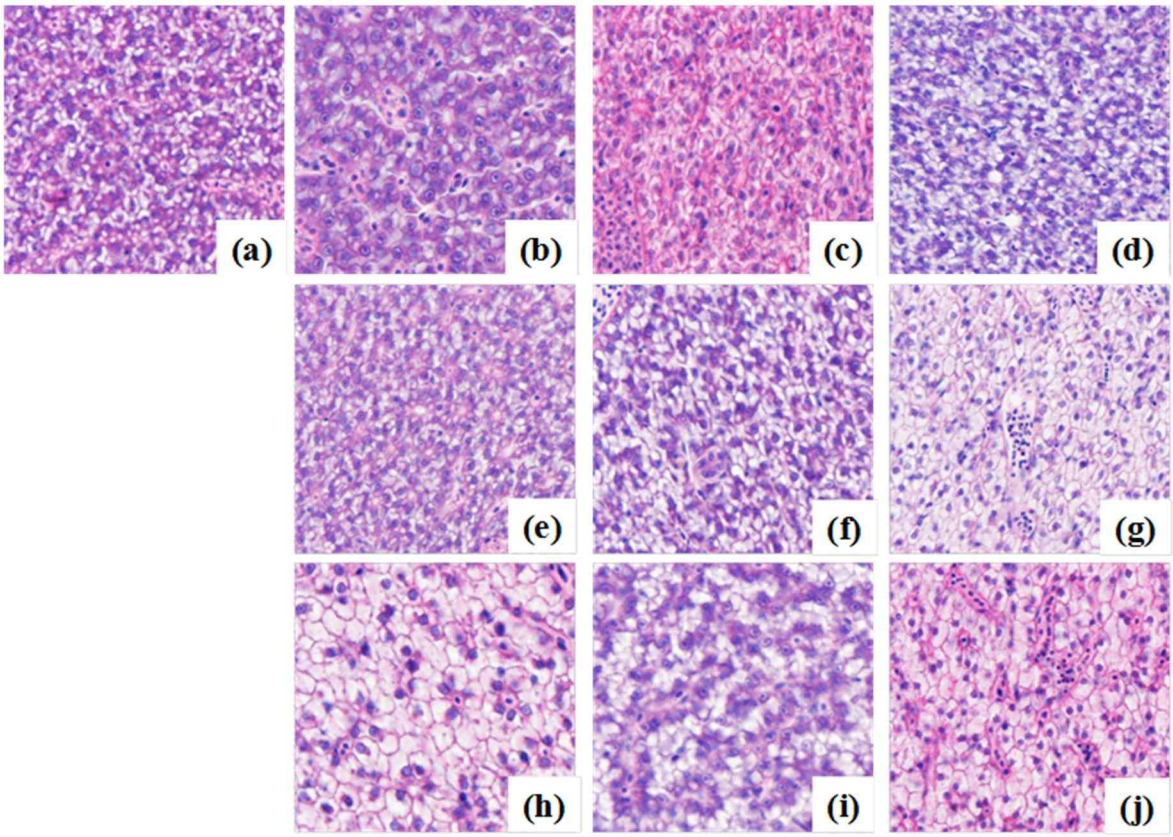

Fig. 2-16. Histopathological micrographs of liver in zebrafish at 7 days post-exposure after treated algae arsenic. (a) Control, (b) ECAE 200ppm (c) UPAE 200ppm (d) HFAE 200ppm, (e) ECAE 400ppm, (f) UPAE 400ppm, (g) HFAE 400ppm, (h) ECAE 800ppm (i) UPAE 800ppm, and (j) HFAE 800ppm groups.

3.8. Gene expression

The mRNA levels of p53, an important regulator of apoptosis induction, were changed significantly in the 800ppm with increases of 1.2-, 1,2- and 1.5- fold compared with the control group, as shown in Fig. 2-17. As for the induction patterns of Bax, the Bcl2 associated X protein, it was found that mRNA levels dramatically increased to 1.2-, 1.3-, and 1.5-fold compared with the control group. To assess whether algae arsenic induces apoptosis via the caspase pathway, the gene expression of caspase-3, caspase-8, and caspase-9 was shown in Fig. 2-17. The gene expression of caspase-3, and caspase-9 activity was significantly up-regulated in the 800ppm. Similarly, the caspase-3 and caspase-9 activity was also significantly induced in the HFAE group. Also, the caspase-8 activity was significantly increased in the UPAE and HFAE groups.

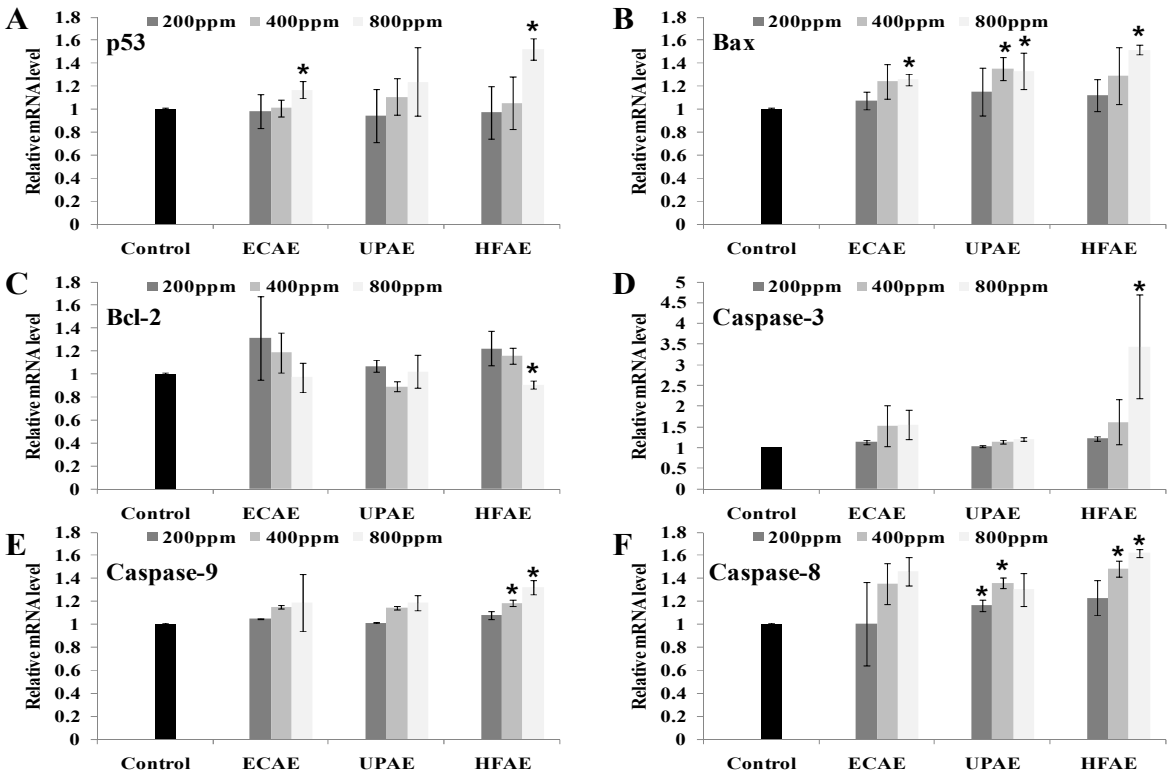

Fig. 2-17. Expression of p53 (A), Bax (B), Bcl-2 (C), Caspase-3 (D), Caspase-9 (E), and Caspase-8 (F) mRNA species in the liver tissues of adult zebrafish treated with algae arsenic of 200, 400, and 800ppm for 96 h and untreated controls. Experiments were performed in triplicate and data are expressed as mean ± SD. ^{a,b,c} Values having different superscript are significantly different at $P<0.05$.

4. Discussion

The number of animal experiments is expected, we need the information on the toxicity from vertebrates, in particular with fish. However, vertebrates such as rats, and mice were emerged to be an ethical problem. There is an interest in the zebrafish as an alternative animal model. Previous studies have shown that early life stages of zebrafish have been already being applied as high through put screening systems for drug development and safety assessment (Nasevicius, 2001., Peterson, 2000., Richards, 2008).

Our results showed that zebrafish affected by exposure to ECAE, UPAE, and HFAE in various concentrations. Heart of the zebrafish is the first organ to develop and function (Hill, 2005). For physiological correlation, the heart-beating rate used to predict the metabolic diseases of fish (Thorarensen, 1996., Wang, 2014). It was indicated that ECAE, UPAE, and HFAE exposures at 100, 500, and 1000ppm could increase the heart-beating rates of embryos. This means zebrafish get damaged to the samples, but, the damaged levels were almost no affected by the toxicity. Because the rates were increased within 10%.

The normal time, 24 hpf for development of circulation in zebrafish was observed in the zebrafish exposed to algae arsenic. Blood flow was normal through arteries and veins at that time in groups (Ezdihar Hassoun, 2005). During the period from 24 to 72 hpf, the yolk sac is the site of the earliest blood flow and occurring the extensive vascular remodeling (Kimmel, 1995). The yolk sac edema sizes of the zebrafish were observed to increase, compared with the control group. Also, the results of the present study suggest that malformation may be due to a general response of zebrafish exposed to ECAE, UPAE, and HFAE.

In relation to the morphological malformations obtained, algae arsenic-treated groups were significantly changed to the control group except 100ppm groups. These results suggested

that they induced malformation. Malformations of the zebrafish have been reported, when exposed to toxicants, such as PBDE 47 (Lema, 2007), PFOS (Shi, 2008), TCDD (Antkiewicz, 2005; Henry, 1997).

The acridine orange staining demonstrated that abnormalities mainly occurred in the heart areas in zebrafish (Jun, 2009). A nucleic acid, acridine orange, is a selective fluorescent cationic dye useful for apoptotic cells. The cell death was treated by ECAE, UPAE, and HFAE in zebrafish, compared to the control group. But, ECAE, UPAE, and HFAE were remarkable difference in the cell death at the 1000ppm.

It is well known that the production of ROS in zebrafish in response to contamination is intimately associated with apoptotic cell death. ROS-induced oxidative stress is thought to contribute to abnormal development during embryogenesis (Yamashita, 2003). In this study, it was observed that ECAE, UPAE, and HFAE were induced ROS formation in zebrafish embryos at all the concentrations.

We have characterized the hepatoxicity of algae arsenic in zebrafish. HFAE treatment was induced hepatotoxicity in the liver of adult zebrafish at 800ppm. Activation of transcription factor p53 in response to DNA damage can lead to cell cycle arrest or apoptosis (Langheinrish et al., 2002). p53 expression in tightly regulated at both the transcriptional and post-tranlational levels, but mainly at the post-translational level. In this study, algae arsenic treatments caused DNA damage and induction of p53 in the liver tissues. The induction of p53 was mainly at the post-translational level, because p53 protein and mRNA level was increased.

Liver histopathology in adult zebrafish was determined in this study (hepatocyte cloudy swelling and pyknosis) (Lynn P. Weber 2003). No previous study we are aware of has quantitied these classic histopathological parameters in marine algal arsenic-exposed

zebrafish livers. The analysis of these parameters involves counting the number of cells affected per field of view and this was likely confounded by a concurrent, concentration-dependent increase in hepatocyte size.

In summary, this study demonstrates the occurrence of toxicity in zebrafish model exposed to algae arsenic. Zebrafish was exposed to algae arsenic as indicated by increased malformation and reduced survival in the larvae. The mechanism of this toxicity appears to be the generation of ROS and the no sequent triggering of apoptosis genes.

5. Conclusion

In this study, we are in accordance with OECD guide lines was confirmed that organic arsenic compounds compared to inorganic arsenic compounds have no toxicity. Acceptable daily provisional intake of arsenic is known as the 2 μg/kg. An adult with a body weight of 60 kg in acceptable daily provisional intake of arsenic is 120 μg/kg. Among the samples, the highest arsenic content was identified to HFAE at 100ppm (5.4mg/kg), and for an adult with a body weight of 60 kg was within an acceptable level as 22.2 g/day when compared with the acceptable daily provisional intake, and would be considered safe with respect to health-hazardous effects.

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ACKNOWLEDGEMENT

부족한 저에게 관심을 주시고 연구의 길을 걸을 수 있도록 이끌어주셔서 2년이라는 석사과정을 마치고 본 논문이 있기까지 세심한 가르침과 사랑을 주신 전유진 교수님께 진심으로 감사드립니다. 또한 바쁘신 와중에도 이 논문이 좀 더 나은 방향으로 나아 갈 수 있게 심사해 주시고 관심을 보여주신 송춘복 교수님, 이제희 교수님, 허문수 교수님, 여인규 교수님, 정준범 교수님, 이승헌 교수님께도 깊은 감사를 드립니다.

제가 실험실 생활을 잘 해나갈 수 있게 이끌어주시고 좋은 말씀을 해주신 우리 실험실원들에게 감사의 표현을 하려 합니다. 잘하고 있냐며 응원해주시는 수진오빠, 연구 관련하여 조언해주시고 항상 반겨주시는 길남오빠, 무심한 듯 항상 잘 챙겨주시는 승홍오빠, 처음 실험실에 왔을 때 세포실험, 동물실험 등 많은 실험과 조언해주시는 석천오빠, 성명오빠, 여러 상황에 따른 조언을 아낌없이 해주시는 긴내언니, 항상 제게 도움을 주는 좋은 말과 응원을 해주시는 원우오빠, 모르는 부분이 있을 때마다 웃으면서 알려주며 아낌없는 조언까지 해주시는 지혁오빠, 주영언니, 처음 실험실에 왔을 때나 미국에 계시고 있는 지금까지도 제가 실험실 생활하는데 있어 많은 도움을 주시는 민철오빠, 처음부터 많이 친해지지 못해 힘

들었지만 지금은 누구보다 제가 실험실 생활하면서 기대고 있는 은아언니, 나래언니, 겉으로 무서운 거 같지만 아무 말 없이 도와주시는 재영오빠, 짜증내면서도 도와주는 준성오빠, 학부생 때부터 같이 생활하여 추억 많고 불평 없이 항상 도와주는 현수오빠, 항상 힘들고 짜증나고 지칠 때마다 쓰러지지 않게 도와주고 묵묵히 들어주며 항상 제 편이 되어준 우리 서영언니, 2년이라는 시간 동안 함께 연구하고, 항상 뒤에서 도와주는 나의 동기 윤택오빠, 상담할 때마다 해결책을 주는 은이언니, 잔소리만 한 기억밖에 없는 것 같아 미안한 형호오빠, 외국에서 와서 실험실 잘 적응하며 다방면으로 도움 주는 왕뢰, 온건, 샤누라, 산지와, 네할, 그리고 졸업하고 스리랑카 소식 들려주는 칼파, 락말, 이제 대학원 생활 시작할 파이팅 해주고 싶은 바로, 이제 더 친해져야 할 효근이까지 해양생물자원이용공학 연구실 식구들께 감사드립니다.

실험하고 늦게 들어오는 날에도 불구하고 뜬 눈으로 기다려주시고 아낌없이 많은 사랑과 제가 쓰러지지 않도록 밀어주시는 우리 아빠, 엄마 사랑합니다. 그리고, 직장생활 하는데 있어 힘들어도 고민하나 들어주지 못해서 미안한 하나뿐인 동생 지원이, 앞으로는 서로 이야기 많이 하고 남들 부럽지 않은 돈독한 자매가 되자. 저의 대학원 생활을 언제나 응원해주시는 할머니, 할아버지, 외할머니, 큰이모부,

큰이모, 작은이모부, 작은이모 모두 저를 위한 배려와 사랑에 감사드립니다.

감사한 분들이 많은데, 이 곳에 모두 거명하지 않더라고 여러 가지 모습으로 도움을 주신 분들에게 모두 감사드리며 항상 건강하고 좋은 일만 있기를 바랍니다.

사랑합니다.