GENOTOXIC AND HEMATOLOGICAL EFFECTS OF ALUMINIUM AND LEAD EXPOSURE IN RED HYBRID TILAPIA (*Oreochromis sp.*)

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GENOTOXIC AND HEMATOLOGICAL EFFECTS OF ALUMINIUM AND LEAD EXPOSURE IN RED HYBRID TILAPIA (*Oreochromis sp.)*

ABSTRACT

This study was conducted to investigate the genotoxic effects of single aluminium (Al), 25.00 mg/l, single lead (Pb), 18.75 mg/l and binary mixtures of Al and Pb (25.00 mg/l + 18.75 mg/l) and to determine their hematological effects on the tilapia fish as test subjects after 96 h exposure to these heavy metals. *Oreochromis sp.* was selected as the test subjects because of its adaptability to the environment and it can be easily found as it is the most common species of freshwater fish being cultured and farmed. Two different methods, micronucleus test and comet assay, were used for the genotoxicity studies while complete blood count (CBC) test was used for the hematological studies. Results showed that 18.75 mg/l of Pb was able to significantly induce the highest erythrocytes with nuclear abnormalities and highest erythrocytes with comets compared to the other samples. Regarding the absolute blood measurement index, it could be seen that there were significant differences in red blood cell (RBC) which is 0.000 and white blood cells (WBC), 0.010, which is ($p<0.05$) of Pb at 18.75 mg/l compared to the other samples and control. This study showed that the micronucleus test, comet assay test and complete blood count test were able to support the results of the genotoxic and hematology study whereby Pb created a bigger impact on the samples compared to Al and the mixtures of Al and Pb. In addition, this study strengthen the different methods as complementary to each other in assessing the effects of genotoxicity and the hematological effects of *Oreochromis sp.* exposed to heavy metals of Al and Pb.

Keywords: genotoxic effects, *Oreochromis sp*, hematological studies, heavy metals.

KESAN GENOTOKSIK DAN HEMATOLOGI DARIPADA PENDEDAHAN ALUMINIUM DAN PLUMBUM TERHADAP TILAPIA KACUKAN MERAH

ABSTRAK

Kajian ini dijalankan untuk mengkaji kesan genotoksik tunggal aluminium (Al), 25 mg/l, tunggal plumbum (Pb), 18.75 mg/l dan campuran binari diantara Al dan Pb, (25 mg/l + 18.75 mg/l), untuk menentukan kesan hematologi pada ikan tilapia sebagai subjek ujian selepas didedahkan kepada kedua-dua logam berat selama 96 jam. *Oreochromis sp.* dipilih sebagai subjek ujian kerana kesesuaiannya terhadap alam sekitar dan mudah dijumpai kerana ia adalah spesies ikan air tawar yang paling lazim dibiakkan dan diternakkan. Dua kaedah yang berbeza iaitu ujian mikronukleus dan asset komet digunakan untuk kajian genotoksik manakala kaedah ujian kiraan darah yang lengkap digunakan untuk kajian hemotologi. Keputusan menunjukkan kepekatan plumbum 18.75 mg/l mampu menggerakkan frekuensi eritrosit dengan kelainan nuklear tertinggi dan eritrosit dengan komet yang tertinggi secara signifikan jika dibandingkan dengan kumpulan kawalan yang lain. Mengikut indeks pengukuran darah mutlak, ia dapat dilihat bahawa terdapat perbezaan yang signifikan dalam sel darah merah (RBC), iaitu 0.000 dan sel darah putih (WBC), 0.010, ($p<0.05$) Pb pada 18.75 mg/l berbanding dengan sampel dan kawalan lain. Kajian ini menunjukkan ujian mikronukleus, asset komet dan ujian kiraan darah yang lengkap dapat membantu memberikan keputusan kajian genotoksik dan hematologi dimana Pb memberikan kesan yang besar berbanding dengan Al dan campuran Al dan Pb. Selain itu, kajian ini menguatkan kaedah yang berbeza sebagai pelengkap antara satu sama lain dalam menilai kesan genotoksik dan kesan hematologi terhadap *Oreochromis sp.* yang didedahkan kepada logam berat Al dan Pb.

Kata kunci: kesan genotoksik, *Oreochromis sp*, kesan hematologi, logam berat.

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LIST OF SYMBOLS AND ABBREVIATIONS

g	:	gram
mg/l	:	milligram per litre
mg/ml	:	milligram per millilitre
µg/l	:	microgram per litre
µg/ml	:	microgram per millilitre
µl	:	microlitre
AL	:	aluminium
ANOVA	:	analysis of variance
CBC	:	complete blood count
DNA	:	deoxyribonucleic acid
EDTA	:	ethylenediaminetetraacetic acid
HCL	:	hydrochloric acid
HCT	:	hematocrit
HGB	:	hemoglobin
LMP	:	low melting point
MCH	:	mean corpuscular hemoglobin
MCHC	:	mean corpuscular hemoglobin concentration
MCV	:	mean corpuscular volume
MN	:	micronuclei
NA	:	nuclear abnormalities
PB	:	lead
PBS	:	phosphate buffered saline

RBC	:	red blood cell
RNA	:	ribonucleic acid
ROS	:	reactive oxygen species
SD	:	standard deviation
SE	:	standard error
SPSS	:	statistical package for the social sciences
TDD	:	total DNA damage
V	:	voltan
WBC	:	white blood cell

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

A few decades ago, the heavy metals usage has increased tremendously. The flow of metallic elements inescapably expanded in the aquatic environment (Yang & Rose, 2003). The constant increase of heavy metals diffusion in natural waters because of numerous activities of agricultural and industrial have made the materials of genetic of exposed organisms including fish damaged and then producing further effects of genotoxicity (Francoise et al., 2011). Genotoxicity is a deleterious action which affects a cell genetic material that affecting its integrity (WHO 1997; Environ Health Perspect, 1996). Aquatic environment that has been polluted describes the introduction of contaminants with the potentials of carcinogenic, mutagenic and teratogenic into its media of principal and the resident organisms' genome (Fagr et al., 2008).

The pollutants will be accumulated by fish from the dissolved phase and from the food and sediments. The phases and stages of the food chain for the aquatic organisms will be affected by these pollutants which can make the whole ecosystem interrupted.

In Malaysia, the rivers of Sungai Pengorak and Pantai Pengorak, Kuantan, Pahang have been found to contain arsenic metalloid and heavy metals. As stated in the News Straits Times in August 2015, 101.5 mg/kg of arsenic was caught in fish water sources around the area which above the allowable level of 1 mg/kg. Meanwhile besides, other heavy metals such as aluminium, nickel, copper, magnesium, lead, zinc and iron were detected in the fish sample.

This study focused on the metals aluminium (Al) and lead (Pb). Al is an abundant metal and the third most common metal on earth (Authman, 2011). It is commonly treated as the greatest poisonous when in the form of soluble ionic (Walton et al., 2009). Heavy metal of Pb has been identified as a persistent and concern hazardous substance

(Sfakianakis et al., 2015). Both are related to heavy metals which result the same amount of toxicity when the concentration is excessive.

There are studies that have been carried out on the single effects of Al and Pb alone. Only few studies in the tilapia, *Oreochromis sp.* on the mixture of both of heavy metals have been done. Therefore, the objectives of these studies are focusing to single and binary mixtures of both heavy metals.

1.2 Objectives

The objectives of the research are:

1. To determine the effects of genotoxicity on tilapia exposed to single and binary mixtures of aluminium and lead by using the micronucleus test and comet assay.
2. To determine the hematological effects on tilapia exposed to single and binary mixtures of aluminium and lead.

CHAPTER 2: LITERATURE REVIEW

2.1 *Oreochromis sp.*

The chosen test organism must be relatively sensitive to environmental pollution and other toxicants. All test organism preferably similar age or similar size, free from disease and obtained from the same source (EIFAC, 1976). *Oreochromis sp.* was chosen as the test subject in this study. The hybrid red tilapia is considered as the most important for commercial aquaculture. The ease adaptation to culture conditions and confinement, salinity tolerance and its attractive color that enhances the retail value (Brol et al., 2017; He et al., 2017; Nakphet et al., 2017) are among the principal reasons for its popularity. A freshwater fish, tilapia is a common species of freshwater fish being well-bred and farmed due to their changeability to the surrounding of the environment, tasty and affordable selling price in the market (Olurin & Aderibigbe, 2006). The species can stand for adaptation to various surroundings including when the water quality is poor. Tilapia can live in the temperature from 13.5ºC to 33ºC (Cheung et al., 2004). *Oreochromis sp.* has sensitivity to detect the changes of environmental surrounding them and to respond to the changes respectively (Almeida et al., 2001). Therefore, tilapia has a great potential as a good biomarker against pollution due to its features which can endure harsh environmental circumstances (Baysoy et al., 2012).

Figure 2.1: *Oreochromis sp.*

2.2 Heavy Metals

Heavy metals are defined as metallic chemical element and related with a very high density of metal which has high potential to contribute in toxicity especially in environmental surrounding (Srivastava & Goyal, 2010). They are found naturally from the earth at varying levels in all ground and surface water. A result from human activities will make the heavy metals become concentrated. It will become toxic and will enter the animal, plant and human tissues through inhalation, diet or manual handling as well as the food chains. Besides, they can bind to interrupt the function of vital cellular component. Since it is non-biodegradable, it can lead to increase the concentrations of metals which will contribute to toxicity in organism and will also affect the ecological system.

For an example, the heavy metals accumulated in the fish can enter the food chain (Saeed & Shaker, 2008) and will affect fish mortality, the rate of growth, and reproduction (Hayat et al., 2007). Studies have shown that various fish species that have been exposed to the heavy metals can cause polycythemia, anemia, and morphological changes in erythrocytes and inclusions in nucleus or cytoplasm (Clauss et al., 2008; Orun et al., 2008). Toxicity levels are different according to their maturity, types of species and size of species. The metals are depending on the size of the fish however not all metals are depending on the fish sizes. Different types of species also have different relationship in size of species with metal exposure (Cogun et al., 2003).

2.2.1 Aluminium (Al)

After oxygen and silicon, the third most abundant element and metal in the earth's surface is aluminium (Al) (Fernandez-Davila et al., 2012). It is broadly scattered and forms nearly 8.8% of the earth's layer (ATSDR, 2006). Al is distributed through the environment by sources of anthropogenic and natural processes. The activities of mining coal strip, acid rainfall, industrial wastes and treatment of water by using aluminium sulphate (alum) contributes Al to enter natural waters.

Studies have shown that Al can evoke oxidative stress through stimulation of ROS production in cells (Li et al., 2006; Sinha et al., 2007); lipid peroxidation induction; disruption of activity of antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase; and facilitation of protein oxidation (Almroth et al., 2005; Parvez & Raisuddin, 2005; Vlahogianni et al., 2007).

In fishes, Al may be associated with gill damage due to its deposition and changes in osmoregulation, as well as with oxidative stress in lymphocytes (Galar-Martinez et al., 2010; Garcia-Medina et al., 2010). According to Bondy & Cambell (2001), Al can cause direct damage to the mitochondrion and affect electron transport in the respiratory chain, increasing LPO and subsequently ROS production. Fernandez-Davilla et al., (2012) described a time-dependent increase in SOD activity in grass carp after 48 h of Al exposure.

2.2.2 Lead (Pb)

Lead is a universal metal that scattered all over the environment generally as the outcome from anthropogenic activities. Nowadays it is highly used as a batteries (71%), mainly for industrial batteries, vehicles and electricity backup systems (Skervfing & Bergdahl, 2007). Because of increasing anthropogenic sources of lead, lead has been strictly regulated and eliminated due to its toxicity.

Pb remains a status as a priority pollutant because it continues to disturb the environment regardless of its use become reduce (USEPA, 2006). Pb is listed as one of 129 priority pollutants by the Environmental Protection Agency and recorded as under the 25 hazardous substances which pose the greatest ability to threat human health at priority superfund sites. The main reason of Pb toxicity in fish is the water contamination (Rogers & Wood, 2004). Pb enters in various organs of fish such as gills, liver, digestive tract and spleen (Jezierska & Witeska, 2006). Changes in the blood parameters such as damage in the nervous system and severe damage to leucocytes and erythrocytes are some symptoms of chronic lead toxicity (El-Badawi, 2005).

0.07 to 1.78 mg/l of Pb were caught in the *cyperinidae* fish species which have high levels of Pb compared to the permissible levels stated in Malaysian Food Act. This might came from various sources that introduce heavy metals of Pb in the lake including agriculture activities such as rubber plantations, oil palm and activities of mining that use chemical fertilization (Ashraf et al., 2011).

2.3 Genotoxic effects on tilapia fish

Toxic substances released into aquatic environment from man-made activities, industrial and domestic are importance because of their potential of bioaccumulation and their competence to cause DNA damage. The one that cannot avoid among animal species from these contaminations are the fishes (Olaifa et al., 2004). Toxic substances in water which are heavy metals may decrease the populations of fish or even can contribute to entire fish population to extinct. The studies carried out on various fishes demonstrate that the fish growth and survival reduced in the presence of heavy metals.

Previous studies on the effects of Al and Pb on the tilapia, *Oreochromis sp.* suggested that both were sensitive to both metals. Metals that were exposed to fish can highly increase the reactive oxygen species (ROS) which lead to damage of tissue, inhibition of ATPase activity, osmoregulatory dysfunctions and oxidative stress (Atli & Canli, 2007). Fish exposed to Al relatively can cause ionic and osmotic balance to alter and problems in respiratory resulting from mucous coagulation on the gills and leading to cause severe fusion of filaments and lamellae (Abdel Latif, 2008). Some of the studies showed that the Pb toxicity can cause oxidative stress on aquatic animals (Zhang et al., 2007). Pb can constraint to inhibit the conductivity of impulse.

2.4 Hematological effects on tilapia fish

Hematological parameters are very important in determining health and physiological status of the fish (Clauss et al., 2008; Adeyemo et al., 2009). In addition, these parameters reflect the changes in the organism correctly and play an important role in the detection of disease and metabolism of fish living in different ecological environments (Cengizler & Şahan, 2000). The changes in internal or external environment of animals have strong correlation with haematological levels which can either increases or decreases when exposed to the pollutants.

According to the previous study, fish exposed to Al resulted a higher significant of hematocrit (HCT), total erythrocytes counts, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) whereas significantly lower in mean corpuscular volume (MCV) (Alwan et al., 2009). Previous study demonstrated that by using low concentrations such as as 0.52 mg/l Al reduced the growth of fish (Bjerknes et al., 2003). Different fish species that have been exposed to Al showed physiological alterations regularly which were generally associated to hematologic (Barcarolli et al., 2004), respiratory, and metabolic (Brodeur et al., 2001).

2.5 Micronucleus Test (MN Test)

MN test becomes one of the most widely method used and it was later adjusted by Hooftman and de Raat (1982). Cytoplasmic chromatin-containing bodies are micronuclei which chromosomes lag and fragments of acentric chromosome are formed during anaphase and are failed to divide into daughter cell nuclei during cell division (Palhares & Grisolia, 2002; Fagr et al., 2008). Abnormalities of spindle and chromosome which cause formation of micronucleus emerged as the damage in genetic.

These types of damage by chemicals have been detected by MN test and have been broadly used (Palhares & Grisolia, 2002). The occurrence of micronuclei in fish serves as an index of these types of damage and counting of micronuclei is less technically and faster demanding than scoring in aberrations of chromosomal (Hartwell et al., 2000; Fagr et al., 2008).

2.6 Comet Assay (CA)

In genotoxicity studies, comet assay is widely used as one of the best methods to detect the damage in fish in evaluating the risk of genetic correlated with exposure of xenobiotic (Nagarani et al., 2012). To detect single or double strand of DNA breakage,

physical and chemical agents induced in individual eukaryotic cells and alkali-labile sites, CA is used as it is fast technique, responsive and reliable (Kim et al., 2002). Peripheral erythrocytes of many types of fish species has applied this assay effectively exposed to different toxicants of genetic (Matsumoto et al., 2006). To maintain the quality of environment and health of human, resident species in freshwater ecosystem can help in monitoring the xenobiotics.

There may be some limitations in this assay application, although the CA is suitable for genotoxicity studies in any nucleated eukaryotic cells as that were done by Sharma et al. (2007). According to Lee and Steinert, (2003) to determine the damage of DNA in the comet assay, blood cells were chosen. However, the data presented in this genotoxicity tests must be first checked if they have a relation with the overall credibility and the sensitivity of the viability of the test system. Therefore, as a part of the recommended guidelines used by Matsumoto and Colus (2000) the utilization of negative control group has been utilized.

CHAPTER 3: METHODOLOGY

3.1 Sample Preparation

Oreochromis sp. (weight range 80 – 100 g), were obtained from Razham Agro Trading, Beranang, Selangor. The twenty fishes were reared under controlled conditions for 4 days in 100L tanks. Each aquarium tank was served with circulated, dechlorinated and aerated tap water. The fishes were taken and transferred individually and acclimatized into a 20L tank for a week before exposed them to heavy metals. The fishes were fed with food pellet during acclimatization two times per day.

Heavy metals of Al and Pb were used in this study. The three fishes were placed per group and exposed to a sub-lethal concentration of 25 mg/l for Al ($1/4^{th}$ of 96h Lc_{50} value of Al), 18.75 mg/l for Pb ($1/4^{th}$ of 96h Lc_{50} value of Pb) and 25 mg/l + 18.75 mg/l for the mixtures of both and another one fish without any metal added was used as a control. These concentrations were selected based on sub-lethal concentration findings. There was 50% mortality at 100 mg/l for Al and 75 mg/l for Pb within 96 h which determined the Lc_{50} value.

Table 3.1: **Metal concentrations used for treatment on *Oreochromis sp.***

Metals	$\boldsymbol{Lc_{50}}$	$\boldsymbol{1/4^{th}}$ **of** $\boldsymbol{Lc_{50}}$
Al	100.00	25.00
Pb	75.00	18.75

Three replicates of experiment were conducted in this study. After exposed to 96 hours, the fishes were anaesthetized by 50 µl/l clove oil for 10 minutes and heparinized syringes were used to obtain the blood from the caudal vein for this genotoxicity and hematology studies. The blood was collected in 1 ml of purple vacutainer tubes that contains EDTA to prevent any blood clotting.

Figure 3.1: Sources of tilapia from Razham Agro Trading, Beranang, Selangor.

3.2 Chemical Preparation

59% Aluminium sulphate and 99% of lead (II) acetate were purchased from Sigma Aldrich which available in powder forms respectively. Al and Pb were weighed and diluted in water to make 1 L stock solution. The concentration of the stock solution prepared was 5%. Both Al and Pb are soluble in water.

3.3 Genotoxicity studies

Two different assays were used to figure out the genotoxicity of Al, Pb and their binary mixture in *Oreochromis sp.*, which were (i) micronucleus test and (ii) comet assay.

3.3.1 Micronucleus Test

The analysis was performed after 96 h of heavy metals exposure. Three slides were prepared for each specimen. The fishes were anesthetized with 2-phenoxyethanol, weighed and dissected. Heparinized syringes by Terumo were used to obtain the blood from the caudal vein. Collected blood was smeared on clean microscope slides. The slides were left dried at room temperature. The slides then were fixed in absolute ethanol for 15 minutes and left air dried for 24 hours at room temperature. 5% Giemsa were used to stain the dried slides for 15 minutes. Distilled water was used to wash and remove the excess stained. The slides were left air dried for 6 hours and were further analyzed under the light microscope by Leica.

For the statistical analysis, the data from micronuclei and nuclear abnormalities analysis were analysed by SPSS software. All data were expressed as mean±SD. The one-way ANOVA, followed by Tukey Multiple Comparisons test was employed to compare mean differences in frequency between the control and different exposure groups and time.

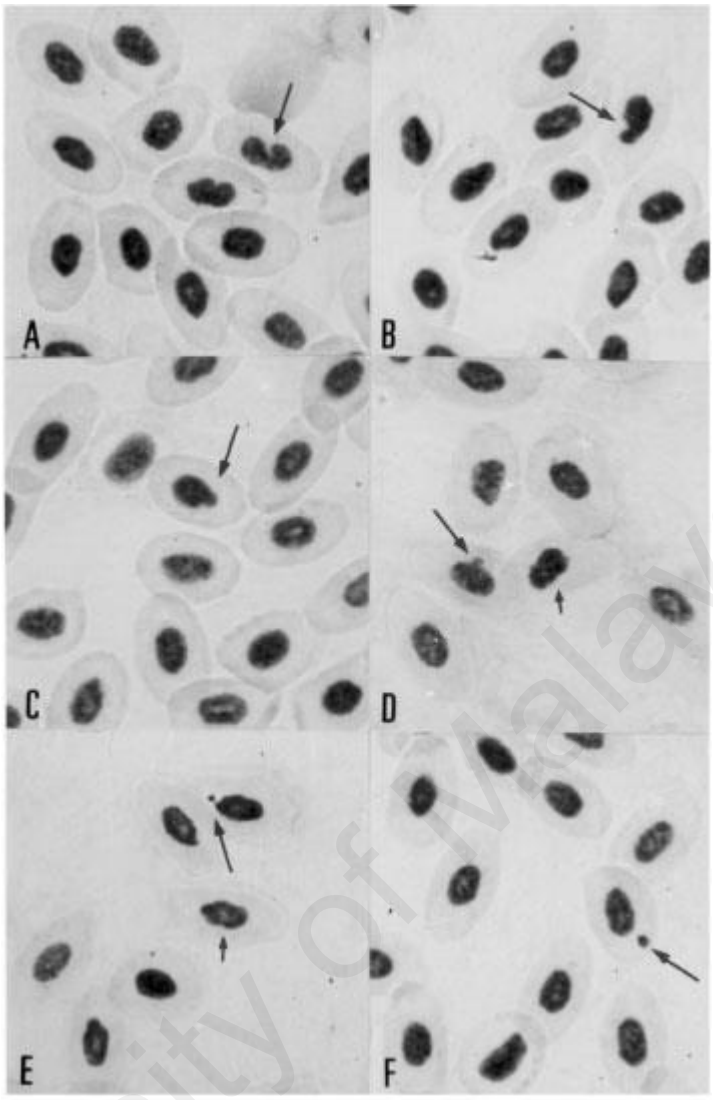

Figure 3.2: Micronucleus and nuclear abnormalities of *O. niloticus*: A and B – Notched nuclei; C- Lobed nuclei; D- Lobed nuclei (large arrow) and blebbed nuclei (small arrow); E- Broken eggs (large arrow) and blebbed nuclei (small arrow); F- Micronucleus. (Silvia M. et al., 2006).

3.3.2 Comet Assay

The comet assay procedures performed generally based on some modification of Speit and Hartmann (1999). Three fishes were used for each treatment. After 96 h of exposure, a heparinized syringe was used to take the blood from the caudal vein and diluted 1:10 in phosphate buffer saline (PBS). 1% normal melting agarose was prepared and placed on the slides. 120 µl LMP agarose (0.7%) was mixed with a 10 µl aliquot that has been taken from each of diluted sample. The mixture was then put that have been already layered with 1% normal melting agarose. The cover slip was then was then used to cover the slides and the slides were conditioned in the refrigerator at 4ºC for 5 minutes to crystallize the low melting agarose. After 5 minutes, the cover slips were carefully removed and the gel was coated again as the third layer with 200 µl of 0.5% low melting agarose during the lysing process to avoid the nuclear DNA from escaping.

The slides were left incubated at 4°C for one hour in alkaline lysis buffer. The slides were horizontally arranged in electrophoresis buffer in the tank. The slides were left for 20 min in electrophoresis tank for denaturation of DNA, and then electrophoresed at 25 V (~1.5 V/cm) and 300mA. After the process of electrophoresis, neutralizing buffer of Tris-HCL (pH 7.5) was used to neutralize the slides for three times and five minutes. Finally, ethidium bromide (20 µg/mL) was used to stain the samples and before being examined by 590 nm filters of fluorescence microscopy, the samples were left overnight at 4°C.

The triplicate slides for each treatment were analyzed. 50 cells were counted for each slide depending on the DNA breakage from the nucleus. The cell conditions were recorded as 0, 1, 2, 3, and 4 which are from normal to the permanent DNA damage. The total DNA damage was counted by using the following formula;.

$$\%DNA_T = (I_T \div I_c) \times 100 \quad (3.2)$$

where I_T is the intensity of the total number of pixels in the tail of the comet, and I_c represents the total pixel intensity of the comet as a whole.

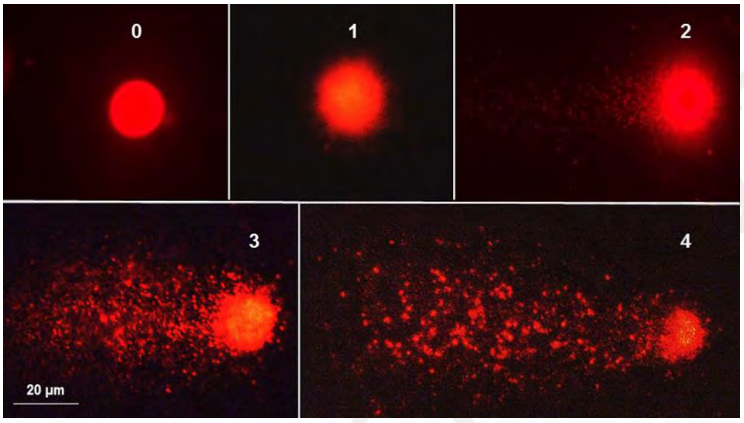

Figure 3.3: Comet scoring (Collins, 2004).

Based on figure 3.3, the cells were accessed visually and received class 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail.

Table 3.2: Cell condition against the scoring of alkaline comet assay.

Comet scoring	Cell condition
0	Healthy
1	Reversible DNA damage
2	Reversible DNA damage
3	Permanent DNA damage
4	Permanent DNA damage

3.4 Hematology studies

3.4.1 Complete Blood Count (CBC)

After 96 h of exposure, blood samples from the caudal vein were taken out by heparinized syringe and transferred to 1 ml of purple vacutainer tubes immediately. These tubes contained EDTA and helped keeping the blood from clotting. The blood samples were then submitted to Division of Laboratory Medicine, University Malaya Medical Centre to analyze. The parameters measured in this study were HGB, HCT, RBC, MCV, MCH, MCHC, WBC and platelet. The methods used to derive CBC parameters are based on the Beckman Coulter method of counting and sizing, in combination with an automatic diluting and mixing device for sample processing, and a single beam photometer for hemoglobinometry.

The WBC differential uses VCS technology. Analysis and classification of WBCs use three simultaneous measurements of individual cell volume (V), high frequency conductivity (C), and laser light scatter (S). The scatter gram plots the cells based upon the measurements of these three parameters. The results tests were then analyzed by using SPSS software.

3.5 Data Analysis

The data collected from these three methods were analyzed by using SPSS software. All data were expressed as mean±SD. The one-way ANOVA, followed by Tukey Multiple Comparisons test was employed to compare mean differences in frequency between the control and different exposure groups and time.

For the micronucleus test, 500 of total of erythrocytes for each slide were counted. For the scoring of micronuclei, the diameter of the micronucleus should be separated, that is less than $1/5^{th}$ of the main nucleus and should have similar staining properties with the main nucleus. Erythrocytes with nuclear abnormalities such as binucleated cells, blebbed nuclei, lobed nuclei and notched nuclei of tilapia will be observed.

For the comet assay evaluation, all the slides were independently coded and scored by a single observer. Under a fluorescence microscope (magnification of 100 X), first a squared perimeter was delimited in a random location within the field of vision of the slide, and within each square, fifty cells were analyzed and counted for each fish. The patterns of DNA migration, determined visually (Matsumoto et al., 2006; Villela et al., 2006), were used to categorize four different damage classes in the cells, as follows: class 0 (no damage), class 1 (little damage), class 2 (medium damage), class 3 (extensive damage) and class 4 (permanent damage).

Complete blood count test method run the samples in triplicate and average the data. The differences between triplicate values for all the parameters were calculated and evaluated to determine if the differences are within the following CDC established precision limits.

CHAPTER 4: RESULTS

4.1 Genotoxicity and haematology studies

To investigate the potential of genotoxicity of Al, Pb and the mixture Al+Pb, the MN test and comet assay were used while to determine the potential of haematology of each heavy metals, the CBC test was performed. These tests are complementary to each other as they are not indicative of the same damages. MN test detected chromosomal aberrations whereas CA detected DNA single and double strand breaks measurement which could be found under alkaline conditions. Besides, CBC test detected a wide range of disorders and measures several components and features of the blood.

4.2 Micronuclei and nuclear abnormalities detection using micronucleus test

The occurrences of MN observed in erythrocytes of *Oreochromis sp.* after 96 h exposed to $1/4^{th}$ of the Lc_{50} value are summarized in Table 4.1. Results of triplicate samples were recorded as mean±SD. The data showed that MN induction in all treatment groups had increased significantly when ($p<0.05$) compared with the control groups. MN induction in single exposure of Al was significantly different with binary mixtures of Al+Pb. Meanwhile, there was no significant difference between Pb single exposure and the binary mixtures. Among all the groups treated, Al (1.09±0.20) treated group showed as the highest frequency of MN while the binary mixtures (0.64±0.08) treated groups emerged the lowest frequency of MN.

Table 4.1: Frequencies (%) of MN and cell of NA in *Oreochromis sp.* with different exposure of heavy metals to 96 h (n=3).

Micronuclei and Nuclear Abnormalities	Total of erythrocytes	Frequencies (mean ±SD)			
		Control	Al	Pb	Al + Pb
Micronuclei	1500	0.16±0.08	1.09 ± 0.20^{ab}	0.93 ± 0.13^{a}	0.64 ± 0.08^{a}
Binucleated cells	1500	0.09±0.04	0.38 ± 0.10^{ab}	0.66 ± 0.07^{ab}	0.69 ± 0.08^{ab}
Blebbed nuclei	1500	0.11±0.08	0.22 ± 0.08^{ab}	0.71 ± 0.08^{ab}	0.40 ± 0.18^{ab}
Lobed nuclei	1500	0.11±0.04	0.31 ± 0.14^{ab}	0.82 ± 0.21^{ab}	0.40 ± 0.07^{ab}
Notched nuclei	1500	0.13±0.07	0.29 ± 0.10^{ab}	0.78 ± 0.10^{ab}	0.56 ± 0.19^{ab}

a $p<0.05$; represent values significantly different from the controls.

b $p<0.05$; represent values significantly different when compared with other treated groups (Al, Pb and Al+Pb).

4.2.1 Induction of nuclear abnormalities (NA)

After 96 h exposure to Al, Pb and the binary mixtures of Al+Pb, many types of NA such as binucleated cells, lobed nuclei cells, blebbed nuclei cells and notched nuclei cells were induced. All treatment groups demonstrated a significant difference in NA compared to control group. The statistical analysis had also showed NA induction in both single exposure of Al and Pb was significantly different with binary mixtures of Al+Pb (Table 4.1).

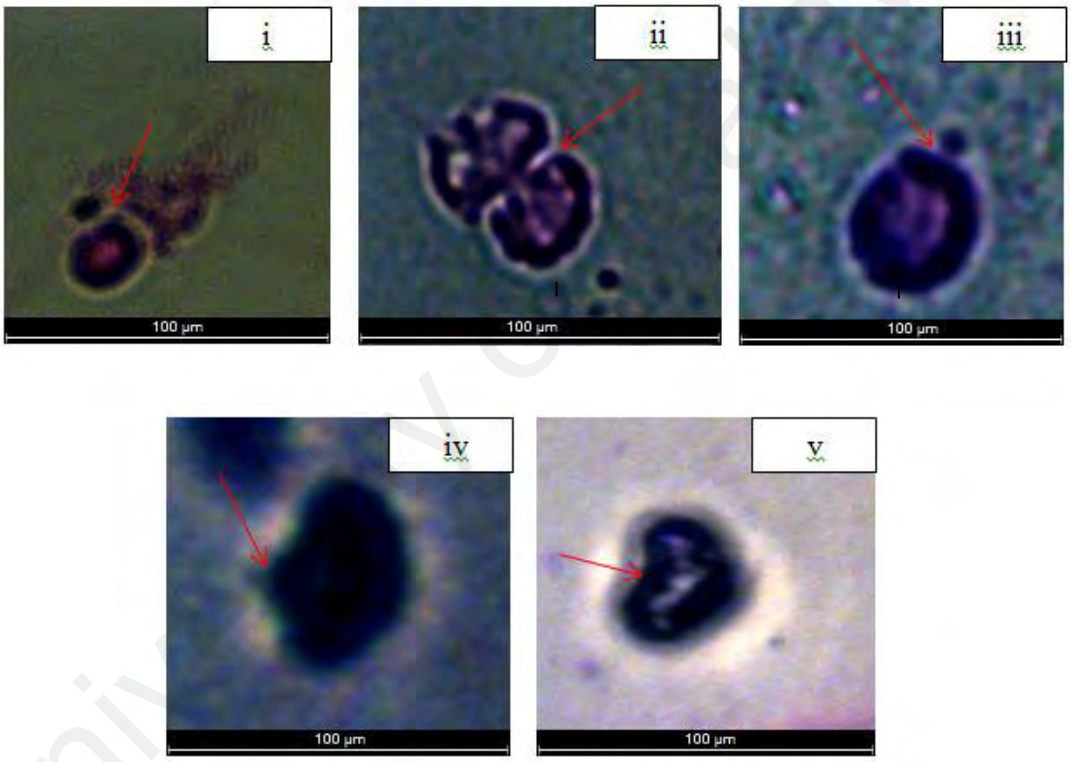

Figure 4.1: MN and types of NA, (i) micronucleus, ii) binuclear, (iii) notched nuclei, (iv) lobed nuclei and (v) blebbed nuclei.

4.3 Detection of DNA damage using alkaline comet assay

The DNA damage scores in the erythrocytes of exposed groups with Al, Pb and Al+Pb and control of tilapia fish by using comet assay is demonstrated in Table 4.2. According to the data presented, there was a significant difference ($p<0.05$) between the groups of tilapia fish treated with the heavy metals and the group of untreated fish. The total DNA damage (TDD) score of the control group showed frequencies of 10% erythrocytes with damaged DNA and 90% erythrocytes without damaged DNA as presented in Table 4.2 and Table 4.3. Total DNA damage that was observed in the control groups mostly belonged to class 1.

The total cells analysed was 50 cells per slide. The groups of fish treated with Pb showed the highest DNA damage compared to Al treated groups and their binary mixtures Al+Pb treated groups as it demonstrated the mean score of damage, 62.33±10.6, doubled the value determined in the binary mixtures (33.33±10.5) and Al (36.33±4.16) (Table 4.2 and Table 4.3). The statistical analysis had also observed that the score in groups of Pb was significantly greater than Al and their binary mixtures when compared to the group of untreated fish.

Table 4.2: Analysis of DNA damage in erythrocytes of *Oreochromis sp.* treated with Al, Pb and Al+Pb (n=3).

Groups	Total cells analyzed	Total cells with comet	Classes					Score/ TDD
			0	1	2	3	4	
Control	50	6	44	6	0	0	0	6
	50	4	46	4	0	0	0	4
	50	5	45	5	0	0	0	5
Total	150	15	135±1.0	15±1.0	0	0	0	5±1.0
Al	50	15	35	6	2	3	4	35
	50	19	31	8	4	3	4	41
	50	17	33	7	5	4	1	33
Total	150	51	99 ± 2.0^{a}	21±1.0	11 ± 1.5^{a}	10 ± 0.6^{a}	9±1.7	36.3±4.2
Pb	50	35	15	13	10	9	3	72
	50	24	26	7	9	6	2	51
	50	29	21	7	13	5	4	64
Total	150	88	62 ± 5.5^{ab}	27±3.5	32 ± 2.1^{ab}	20 ± 2.1^{ab}	9±1.7	62.3±10.6
Al+Pb	50	17	33	8	4	3	2	33
	50	19	31	6	5	4	4	44
	50	13	37	7	3	2	1	23
Total	150	49	101 ± 3.1^{a}	21±1.0	12 ± 1.0^{a}	9±1.0	7±1.5	33.3±10.5

a $p<0.05$; represent values significantly different from controls (Tukey multiple comparison of means).

b $p<0.05$; represent values significantly different when compared with other treated groups (Al, Pb and Al+Pb).

The percentages of DNA damage with different classes in erythrocytes of *Oreochromis sp.* after all the treatments were summarized in Table 4.3 and Figure 4.2. The comet was recorded separately between classes and it demonstrated that there were significant differences in the number of erythrocytes with damage in class 0, 2, and 3 among the treated groups with control group. According to the data presented, Pb has higher total DNA damage than control group, single Al group and the binary mixtures of Al+Pb group. The statistical analysis had also demonstrated that Pb has significant differences when compared to the control groups, Al single groups and binary mixtures.

Table 4.3: Total DNA damage (TDD) scores in erythrocytes of *Oreochromis sp.* treated with Al, Pb and Al+Pb (n=3).

Groups	Percentage of score (%)					Total DNA Damage (TDD)	Total cell analyzed
	Classes						
	0	1	2	3	4		
Control	90.00	10.00	0.00	0.00	0.00	10.00	150
Al	66.00^{a}	14.00	7.33^{a}	6.67^{a}	6.00	34.00	150
Pb	41.33^{ab}	18.00	21.33^{ab}	13.33^{ab}	6.00	58.67	150
Al+Pb	67.33^{a}	14.00	8.00^{a}	6.00	4.67	32.67	150

a $p<0.05$; represent values significantly different from controls (Tukey multiple comparison of means)

b $p<0.05$; represent values significantly different when compared with other treated groups (Al, Pb and Al+Pb)

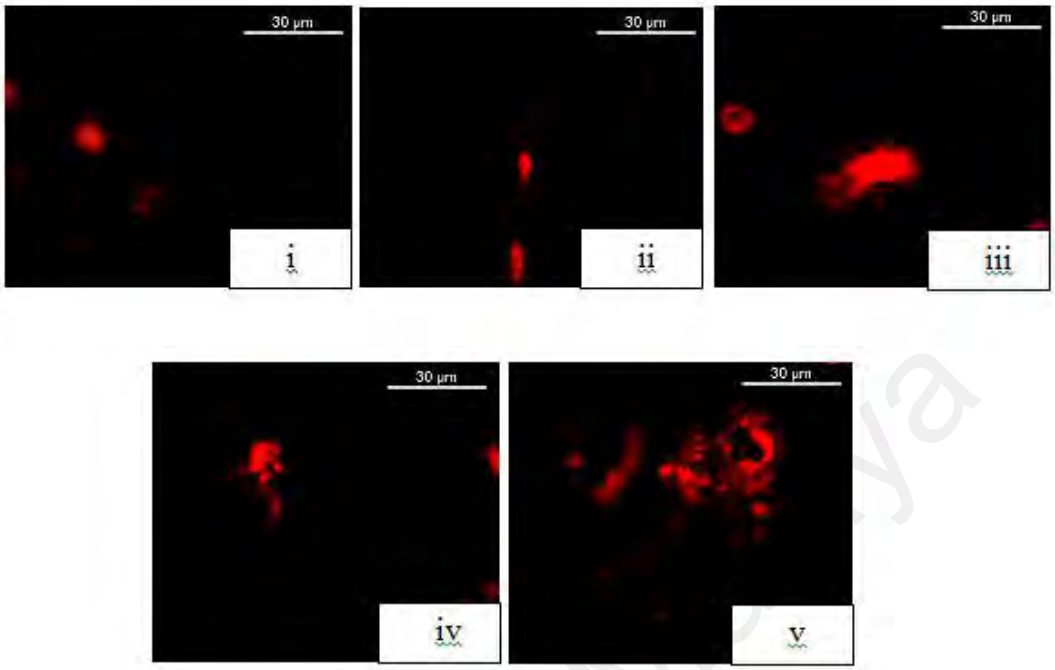

Figure 4.2: Comets appearances in the erythrocytes of *Oreochromis sp.*; (i) class 0 (undamaged); (ii) class 1; (iii) class 2; (iv) class 3 and (v) class 4.

4.4 Hematology Parameters using Complete Blood Count Test

The hematology parameters of *Oreochromis sp.* treated with Al, Pb and Al+Pb by using complete blood count test were summarized in Table 4.4. The triplicate samples were recorded as mean±SD. The parameters that have been demonstrated in this study were red blood cell (RBC), hematocrit (HCT), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet and white blood cell (WBC).

Hemoglobin levels of Al, Pb and Al+Pb in fish were decreased up to 96h exposure when comparing with control. Pb has the lowest hemoglobin levels compared to the control and the other treatment groups. Exposure to Pb treatment groups increased the level of HCT, RBC, MCV, MCH, MCHC, WBC and platelet when compared to control whereas the binary mixtures of Al+Pb has the lowest frequency. For the statistical analysis, only in white blood cells parameter, all the treatment has significantly different when compared to control group. The data showed HGB, RBC and MCH were no significant different when compared to control. However, in HCT parameter, the binary mixtures of Al+Pb has significant different when compared to control and single treatment of Al and single treatment of Pb.

Table 4.4: Hematology parameters of *Oreochromis sp.* treated with Al, Pb and Al+Pb. (n=3).

Hematology Parameters	Chemicals Exposure			
	Control	Al	Pb	Al+Pb
HGB (g/L)	35±14	33.33±2.89	28.67±1.15	31±3.61
HCT (L/L)	0.18±0.03	0.19±0.02	0.21±0.02	0.12 ± 0.02^{ab}
RBC (L)	1.29±0.18	1.31±0.02	1.41±0.03	1.07±0.02
MCV (fl)	140.33±2.08	141±2.65	146.33±2.31	129.33 ± 2.31^{ab}
MCH (pg)	28.7±5.72	26.33±0.4	31.33±3.15	22.97±2.3
MCHC (g/L)	194.67±16.04	228.67 ± 8.02^{a}	232 ± 10.44^{a}	215±6.08
WBC (L)	144.53±4.83	242.97 ± 46.39^{a}	226.13 ± 23.36^{a}	67.07 ± 5.91^{a}
Platelet (L)	9.67±2.52	20.67 ± 1.53^{a}	24.33 ± 3.79^{a}	14.67±3.06

a $p<0.05$; represent values significantly different when compared with controls (Tukey multiple comparison of means).

b $p<0.05$; represent values significantly different from other treated groups (Al, Pb and Al+Pb).

CHAPTER 5: DISCUSSION

5.1 Erythrocytes cells of *Oreochromis sp.* in the presence of micronuclei and types of nuclear abnormalities

During cellular division, micronuclei are formed and reflected the cytogenetic effects which are the rearrangement of chromosome and the number of chromosome changes; loss of the fragment of chromosomal or the whole chromosomes which are not involved in the nucleus of the parent during anaphase (Muranli & Guner, 2011). The occurrence of micronuclei has helped for over 40 years as an index of mitotic spindle apparatus dysfunction and chromosomal breaks based on the first study by Boiler & Schmid (1970) on mouse. MN test is then broadly used for the study of genotoxicity and some modifications were made by Hooftman & de Raat (1982). It has been used as the potential of genotoxicity for various chemicals and industrial waste by using different organisms (Nwani et al., 2011; Bucker et al., 2012; Nwani et al., 2013). Because of its sensitivity and reliability in detecting any nuclear lesions because of living organisms' exposure, this assay is applicable for environmental biomonitoring.

Earlier studies showed that some parameters of nuclear abnormalities were 'lobed', 'binucleated cells' "blebbed', and 'notched nuclei' as likely parameters of genotoxicity ((Muranli & Guner, 2011; Nehls & Segner, 2004). Therefore, it can correlate with the scoring of micronuclei in routine genotoxicity surveys. The cells with 'broken eggs', 'lobed' and 'blebbed' seemingly happened because of irregularities happened during the cell division, whereas 'notched' nuclei probably occurred when the nuclear membrane was disintegrated. In addition, due to the complication in the mitotic fuse formation caused by the aneugenic action of the heavy metals, the creation of binucleated cells may have surfaced (Fernandez et al., 2007).

According to this study, MN induction in all treatment groups had significantly increased ($p<0.05$) compared with the control groups. This shows that heavy metals can induce the micronuclei within 96 hours of exposure. This is followed by the previous studies that showed heavy metals can cause a significant induction of micronuclei. Research that has been carried out previously showed that heavy metal of arsenic induces micronuclei into human (Martinez et al., 2005) and copper has a capability to induce instability of genomic in mammals (Linder, 2011). Previous study by Yadav & Trivedi (2009) showed fish exposed to sub lethal concentration of Mercury (II) able to induce micronuclei for five treatment periods significantly. Similarly, sub lethal exposure of heavy metals to *Channa punctate* showed a significant induction of micronucleus (Yadav & Trivedi, 2009).

Among all groups, Al showed the highest frequency of MN and the mixture of Al+Pb showed the lowest frequency of MN. Single Al exposed to *Oreochromis sp.* alone demonstrated an increase in inductions of MN and NA significantly compared to the group of control (Table 1 and Table 2).

Frequencies of MN in Pb groups were also increased in this study. This were already observed and presented in the Red-tailed tinfoil barb (*Puntius altus*), Butterfish *(Poronotus triacanthus)* and also in the erythrocytes of *O. niloticus* (Wannee Jiraungkoorskul et al., 2007). Frequencies of MN and erythrocytes with NA in these three fish species were significantly increased for the period between 24 and 96. Hence, it was proved that the exposure periods were sufficient to induce MN and NA in erythrocytes and 96 h of exposure demonstrated that it can be a sensitive biomarker for genotoxicity studies.

5.2 Evaluation of DNA damage by comet assay

Usually a blood in fish was selected to carry out the comet assay, because it is not difficult and simple to obtain as it is does not need to dissociate the cellular (Kilemade et al., 2004). Generally, the comet assay has broadly used in toxicological biomonitoring of aquatic environments in genetic studies (Lee & Steinert, 2003) and in measuring the damage in the DNA molecule and aquatic organisms exposed to toxic pollutants.

In the present study, Pb has the highest total DNA damage when compared to the control group and other treated groups. This has been proven with previous studies by Wang & Jia (2009) based on their research tested in the frog *Rana nigromaculata* that lead has the capability in inducing oxidative stress and damaging the DNA even at very low concentrations (0.2 mg Pb L^{-1}). Besides, previous studies by Zhang et al., (2008) proved that the aquatic animals were affected to low levels of Pb as their study on *Misgurnusanguilli caudatus* was affected by 0.5 mg Pb which caused DNA in hepatopancreas cells of loach damaged. These data demonstrated that aquatic animals were sensitive to very low levels of Pb.

Based on some research studies, the lead toxicity effects on aquatic organisms are related with oxidative stress (Zhang et al., 2007). Fish exposed to heavy metals can cause reactive oxygen species (ROS) increases which lead to dysfunctions of osmoregulatory related with activity of ATPase and oxidative stress to inhibit and damage of tissue (Atli & Canli, 2007). Emerging evidence proposed that the intoxication of lead was associated with production of reactive oxygen species (ROS) which resulted in DNA damage and defense systems of cell antioxidant to reduce (Shalan et al., 2005; Farmand et al., 2005; El-Ashmawy et al., 2006). Covalent binding

of Pb^{2+} to DNA was found by Hong et al., (2007) which is the direct interaction between lead and DNA.

ROS can generate peroxidation of lipid, reacting on the plasma membrane or can react on the molecule of DNA directly, which lead to damage (Ahmad et al., 2006). If the production of ROS' rate surpass the defence mechanisms' capacity, the DNA and cells may affect and injury can happen (Cadet et al., 2003), their bases can damage, causing the DNA strand to break (Reinecke, 2004) although organisms can defence with antioxidant against oxidative damage to protect the tissues.

5.3 Hematology parameters

Hemodilution which increasing the plasma volume and reducing red blood cells concentration in blood can occur because of the osmotic changes of metals in hematology parameters while hemoconcentrations can occur when the blood cells concentration increase due to the water loss or plasma loss from the bloodstream (Tort T & Torres P, 1988). In the present study, Pb has the lowest hemoglobin levels compared to the control and binary mixtures. This was similar by the previous study by Christensen et al. (1977) on *Savelinus fontinalis* in Brook trout which reported a significant decrease in the Hb level when exposed to lead in 60 days. Mustafa (2012) reported that, on the system of enzyme, the inhibitory effect of toxic substance was associated for Hb synthesis as the content of Hb can reduce when fish exposed to toxicants. To determine the content of Hb and erythrocytes, a small rising in values of mean was demonstrated during the study, suggesting a slight increase in volume of erythrocytes and the content of hemoglobin.

Abnormal erythrocytes were discovered over the entire experimental period. According to Alves & Wood (2006), lead demonstrates a high affinity for erythrocytes. Johansson Sjobeck & Larsson (1979) reported that HGB and RBC accompanied by a compensatory response (increase hematopoietic rate) in lead-intoxicated rainbow trout were reduced. 47.4 µg/l of lead exposed to *Barbus conchonius* resulted a severe microcytic anemic (decrease in HCT, RBC, HGB and MCV) (Tewari et al., 1987). Previous study by Ghazaly (1991) presented that RBC and HGB decreased, but not HCT in *Tilapia zilli* that was exposed to 8.3 mg/l of Pb. Allen (1993) reported that 10 mg/l of Pb exposed to *Oreochromis aureus* resulting in RBC, HGB and HCT to decrease.

However in this present study showed an increased level of RBC when compared other treatments' groups and control. Hg dissolved in water exposed to fish can cause hypoxia which resulting cellular hyperplasia in the secondary lamellae of the gills as it can reduce the surface area for the gas exchanges based on Oliveira-Ribeiro et al., (2000). Because of the difficulty in respiratory, the organism needs to enhance oxygen transfer by stimulating an increase in Hb, RBCs and MCHC (Affonso et al., 2002). A second mechanism stated in the general circulation via the large number of mature RBCs was released during hypoxic conditions by fish because the uptake of oxygen was poor. However, the mechanism compensate the concentration of oxygen variations for short-term in blood or water (Nespolo & Rosenmann, 2002).

WBC levels in the group of the *Oreochromis sp.* treated with Pb showed the highest when compared to other exposure groups and control. For the statistical analysis, only in white blood cells parameter, all the treatment has significantly different when compared to control group. The WBCs count increase significantly could be because of an increase in production of antibody which aids in fish recovery and fish survival when heavy metals were exposed (Joshi & Deep, 2002). Based on the results reported by Fink and Salibian (2005), leukocytes showed an increase might be because of an induced proliferation as a result of the toxicity by chemical, of pluripotential hematopoietic cells that, in turn, may be a consequence of a depletion circulating differentiated. These results are supported by Salman (2014) who study the effects of histopathological and haematological of cadmium chloride on *M.sharpeyi* and Mustafa (2012) as he found leucocytes count was increased when they exposed fishes to heavy metal.

The results in this present study and the previous studies might be slightly different because of the species used was differ. Between the present data and previous studies, there is evident agreement with the previously published range, though some errors in

concentration of hemoglobin and hematocrit value variances for the same species can be noted. Such circumstances are common and might connect to different blood process techniques and different places and regions. However it was proved that even low toxicity of Pb can affect the immune system of animals including fish.

5.4 Interaction of lead and aluminium in *Oreochromis sp.*

Fundamentally, antagonism is the effects of toxicity because of the mixture significantly less than the sum of the toxic effects of the individual constituents while synergism is the toxic effects caused by the mixture that higher than the sum of effects of the individual constituents (Otitoloju, 2002). If the proportional independent contributions of each toxicant were added, the toxicity of mixture has commonly predicted additive effects. However, mixtures of heavy metals showed different type of interactions happening between the heavy metals based on previous studies of aquatic animals (Cooper et al., 2009; Lange et al., 2002; Obiakor et al., 2010).

Data collected from the MN test, CA and CBC test demonstrates that all groups treated by heavy metals were significantly toxic towards *Oreochromis sp.* when compared to the control group. For MN test, CA and hematology test by CBC test, the results obtained that the order of toxicity was Pb>Al>(Pb+Al). The DNA damage in erythrocytes showed that the binary mixtures were at the lowest value compared to the single exposure of Al and Pb.

The results revealed that the binary mixtures showed the lowest score of DNA damage and lowest frequency of MN and cell with NA compared to single Al and Pb exposure. Simultaneously, it was found that hematology parameters such as HCT, HGB and RBC were low when compared to single exposure to Al and Pb. The results in this study predicted antagonistic interactions occurred in the binary mixture of Al+Pb which presume that Al may decrease the toxic effects of Pb on *Oreochromis sp*.

Besides, the ionic mechanism of Pb toxicity appears mainly because of the capability of Pb metal ions in replacing other bivalent cations and monovalent cations which eventually disrupts the biological metabolism of the cell. According to Flora et al., (2008), Pb can replace calcium even in picomolar concentration affecting protein kinase C, which regulates neural excitation and memory stage. Most studies have been done on the single exposure of the Al and Pb alone, however lack of studies have been carried out on the binary mixtures of Al and Pb in *Oreochromis sp.*

CHAPTER 6: CONCLUSION

This study is focused on the genotoxicity and hematology of the tilapia fish to the exposure of heavy metals to Al and Pb for 96 hours. This study is also focused to compare the toxicity level between single and binary mixture of Al and Pb. MN test and CA were used to differentiate types of DNA damage while CBC test was used to evaluate the hematology parameters.

The results obtained from this study determined that Al and Pb had caused genotoxic effects and hematological effects in the *Oreochromis sp*. The LC_{50} values indicated that Pb was more toxic when compared to Al. This is according to their lower 50% lethal concentration value.

Overall results observed that the entire tests are supports each other relatively supports for the findings. It also demonstrates that single Pb gives a higher impact compared to Al and the binary mixtures of Al+Pb. The study contributes to raise the awareness about the toxicity of heavy metals in health of aquatic animal while act as an effective monitoring in aquatic surrounding. The data can be contributed to the environment health in Malaysia.

However, the simultaneous use of physiological and biochemical in toxicology also have certain disadvantages. The validity and accuracy of the results obtained from all different assays use are continuously questioned. There are many aspects that could be recommended for future research activities including *Oreochromis sp.* as well as Al and Pb and their binary mixtures.

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