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The Acute Effects of Un-ionized Ammonia on Zebrafish (*Danio rerio*)

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Abstract

Zebrafish is a commonly used species in toxicology studies investigations such as molecular genetics, histopathology and developmental biology. A toxicity test was conducted to investigate the effects of toxic ammonia (NH_3) in relation to time and concentration by establishing a measurable endpoints for chemical exposure and monitoring the biological responses on zebrafish exposed to high levels of toxic ammonia concentration using ammonium chloride (NH_4Cl) as E1: 17.21 mg/l, E2: 24.51 mg/l, E3: 25.81 mg/l, E4: 32.11 mg/l and E5: 38.91 mg/l NH_3-N at 24 hr, 48 hrs and 72 hrs periods. Fish exposed to the high levels of toxic ammonia exhibited hematological, histopathological and molecular alterations to the gills, liver and kidney at all exposure periods 24 hrs, 48 hrs and 72 hrs. The interrelation between the hematological, histopathological and gene expression have proven to be time and dose dependent having a linear relation under the influence of high levels of toxic ammonia exposure. Such hematological and histopathological changes observed in the liver and kidney of zebrafish in the present study indicates that the fish were responding to the direct effects of the toxic ammonia and secondary effects caused by stress.

Keywords Toxic Ammonia; Histopathology; Hematology; Gene expression

Introduction

Water pollution is one of the most critical environmental threats facing man and his environment impacting various levels of biological organizations. The impacts of water pollution have become a matter of great concern not only because it threatens the public water supply but also causes damages to the aquatic life [1]. Aquatic systems are highly vulnerable due to their tendency to accumulate relatively high concentrations of chemicals entering from a variety of point and non-point to its water bodies such as rivers, streams, marine ecosystem and groundwater [2].

The increased socioeconomic activities result in introducing contaminations to the aquatic systems with toxic substances, which are harmful to their inhabitants. Human activities produce increasing amounts of nitrogenous material in a significant proportion being in the form of ammonia or compounds that can yield ammonia [3]. Natural aquatic systems can be introduced to sources of high concentrations of ammonia by direct means mainly of socioeconomic sources including industrial wastes, sewage effluents, agriculture inputs and animal feedlots and indirect means (natural biogenic) such as nitrogen fixation and ammonia excretion from animals leading to various impacts on the fish [4,5].

Socioeconomic effects may lead to severe consequences for aquatic populations or species occupying the area. However, consequences at the ecosystem level may display a long response time and when effects occur it may be too late to take countermeasures. Pollution exposure may lead to decreased growth rates and increased infection but even these responses are preceded in time effected at the molecular level. The effects on the aquatic systems can act as early warning indicators on human health and the environment due to their complex food chain structures. Biomarkers being either biochemical, physiological or histological are important to interpret the toxicity of the aquatic environmental systems [6].

In this study the main objective is to develop measurable biological endpoints that can aid in evaluating the health condition of the fish affected by toxic ammonia (NH_3) in relation to time and concentration by monitoring the biological responses of the effected target organs. Such a study can yield a better assessment of the overall fish health conditions in polluted aquatic environments affecting the fish community and their commercial values.

Materials and Methods

Test fish

Adult zebrafish with an average standard length of (3.87 ± 0.37 cm) and an average weight of (0.62 ± 0.18 g) were purchased locally. Upon arrival the fish were stocked (n=100) in a semi-static 57l aquarium tank system containing UV treated (REI-SEA UVF-600) de-chlorinated tap water with a pH 7.4 ± 0.04, and maintained at a temperature of 24°C (aquarium water heater Nisso type-180) and dissolved oxygen at 70%. Zebrafish were acclimatized to laboratory conditions at 12L: 12D photoperiod for 14 days with daily feeding (once per day) of commercial feed (Tetramin tropical flakes). Feeding was stopped 24 hrs prior to the actual experiment. During the acclimatization period commercial chemical kits were used to help monitor water quality to ensure that the levels of ammonia and nitrite were maintained below harmful levels.

Experimental procedures

Reagent grade ammonium chloride (NH4Cl MW=53.49, WAKO pure chemical LTD.) was mixed with dechlorinated tap water using a magnetic stirrer (IKA) to obtain the required ammonia concentration.

Preliminary tests using E5: 154 mg/l NH4Cl proved that this concentration had the most histological effects on zebrafish, with no mortalities at 72 hrs of exposure. From E5: 154 mg/l NH4Cl concentration four other lower concentrations (E=effect) in a geometric series were prepared with a factor of 1.24 (E1: 65.14 mg/l, E2: 80.8 mg/l, E3: 100.2 mg/l and E4: 124.2 mg/l). Diluted solutions of reagent grade 1 M hydrochloric acid (HCl) and sodium hydroxide (NaOH) were prepared and used to maintain the desired pH (7.4 ± 0.04) in the experimental beakers after adding NH_4Cl concentrations. The Salicylate method (Test 'N Tube Vials) certified ammonia standards (Hach company) was used as a precise indication of high levels of toxic ammonia ranging from 0-50 mg. Toxic un-ionized ammonia levels were determined respectively as E1: 17.21 mg/l, E2: 24.51 mg/l, E3: 25.81 mg/l , E4: 32.11 mg/l and E5: 38.91 mg/l NH_3-N. Experiment testing was conducted under laboratory conditions following the OECD [7].

At the end of the acclimatization period 72 fish were randomly collected for each experimental period (24 hrs, 48 hrs and 72 hrs) divided (n=12) and transferred into four 3 liter semi-static system test beakers supplied with aeration and water conditions matching that of the fish rearing tanks. The beakers were then arranged and labelled as group 1 (control) de-chlorinated tap water free of ammonia, group 2 (E1: 17.21 mg/l NH_3-N), group3 (E2: 24.51 mg/l NH_3-N), group 4 (E3: 25.81 mg/l NH_3-N), group 5 (E4: 32.11 mg/l) and group 6 (E5: 38.91 mg/l NH_3-N) respectively prepared from the ammonia stock solutions.

Blood collection and analysis

Fish samples (n=4) were collected randomly and anesthetized by placing them in a 100 ml beaker (one beaker for each group) containing ice cold water (5-7°C). A longitudinal incision was made by cutting off the tail. A blood drop from the caudal vein was collected on a clean microscopic glass slide were a smearing slide was used to create a thin film. Blood smears were prepared and stained according to May-GrUnwald- Giemsa technique and examined at 100x using a compound light microscope (Nikon Eclipse E600) equipped with a mounted digital camera (Nikon Ds-Fi1) connected to a computer with a high resolution computer screen.

Erythrocytes cell size (n=50) were measured using a computerized digital imagery software (AxioVision-Carl Zeiss) and the data are presented as mean ± standard deviation and data sets were represented as one. Erythrocyte were divided and identified according to the stage of maturation and general morphology as mature erythrocytes and immature erythrocytes according to Hibiya et al. [8-10]. The percentages of immature erythrocytes (per fish) were computed by randomly selecting 10 microscopic fields (1,000x) and relatively counting 1,000 erythrocytes [11-13].

Similar experimental procedures were repeated for the glucose test using the same quantity of fish. A FreeStyle-lite (Abbott) hand-held diabetic blood glucose measuring device was used to measure whole blood glucose levels in fish (n=3) with accordance with Eames et al. [14]. The resulting means ± standard deviation (SD) values of these standardized data sets is equal to one.

Histopathology

Histological examination was performed on randomly collected fish samples (n=4) from each control and exposure treatment groups. Fish samples were anesthetized in ice cold water and sacrificed by cervical decapitation. The abdomen was opened to improve the diffusion and penetration of the fixative into the targeted tissue [15,16] and immediately placed whole in 10% neutral-buffer formalin solution (NBF) in a refrigerator for 48hrs at 4°C to improve rapid fixation [17]. The internal viscera including the liver and kidney were then removed and placed into histological cassettes. Tissue samples were then dehydrated through five graded series of ethanol solution starting with 70% ethanol using dehydration machine (Sakura RH- 12DM), cleared in exylene, embedded in paraffin and sectioned at 5 µm.

The sections were stained with Hematoxylin and Eosin and examined with a light microscope (Nikon Eclipse E600) equipped with a mounted digital camera (Nikon Ds-Fi1) connected to a computer with a high resolution coloured computer screen. The images were captured and analysed from intermediate to high magnification objective lenses (20x, 40x and 100x) using a digital imagery software (Nikon: NIS-Element F). Cellular components and types for each target organ were identified. These cells were categorized to assess the degree of alteration observed from previous histological observation [18-24] The hepatocyte cell and nucleus (around 50 cells per slide) and kidney Bowman's capsule and mesangium (around 7- 10 glomerulus) surface areas (μm^2) were obtained in 10 microscopic fields (40x-100x) and measured using computerized digital imagery software (AxioVision-Carl Zeiss) for image analysis. Such study was conducted to monitor the changes in size when induced under the different toxic exposures levels. Means ± standard deviation (SD) values were calculated for each experimental group (n=4) and the resulting values of these standardized data sets is equal to one.

Histopathological assessment

Histopathological alterations (lesions) of the liver and kidney were evaluated for the treatment groups by the use of a modified method proposed by Bernet et al. [25]. Such a method classifies the histopathological lesions into five major reaction patterns: circulatory, regressive, progressive, inflammatory and neoplasm. An importance factor (w) ranging from 1 (minimal importance) to 3 (marked importance) is assigned to each alteration indicating the effects of organ function on fish health and survival. Score values (a) are evaluated for each sample for each lesion using a scoring range from 0 (unchanged) to 6 (sever occurrence). The liver and kidney indices (Il), (Ik) are calculated by the sum of the multiplied score values (a) and importance factors (w) of the alteration = alt of the corresponding reaction pattern. Liver and kidney indices (Il), (Ik) were calculated for each individual (n=4) using the following equation to indicate the severity of lesion in each group of each given period of exposure.

$$I(l,k) = \sum_{alt} (\text{a x w}) \text{.....(1)}$$

An integrated analysis (Ia) was calculated for each individual (n=4) from the multiplication of each period or time (t) and the toxic ammonia concentrations (c) of the exposure groups only. Such a study was assigned to indicate if time and concentrations had an effect on the fish liver and kidney lesions when correlated with the liver and kidney indices (Il), (Ik).

$$\text{Ia} = \text{t x c} \text{ .....(2)}$$

Statistical Analysis

Differences in the percentage of immature erythrocytes of the experimental groups for each period were presented as a means ± SE, analyzed with Kruskal-Wallis non parametric tests followed by post

hoc comparisons with a Mann Whitney-wilcoxon test. All tests were performed in Excel Microsoft software package with XLSTAT version 7.5.2 (addinsoft, USA). The statistical level was set as $P<0.05$.

Analysis for hepatocytes and Bowman's capsule and mesangium areas alterations (μm^2) were calculated using computerized digital imagery software (AxioVision-Carl Zeiss) for image analysis. Means ± standard error (SE) values were calculated for each experimental group (n=4) and the resulting values of these standardized data sets is equal to one. Data was further analyzed by descriptive statistics, and normality was evaluated by kurtosis and skewness analysis. No transformation was required. A one-way ANOVA was performed for each time period to analyze the differences between the six groups (CTRL, E1, E2, E3, E4 and E5). *Post hoc* test Student-Newman-Keuls (SNK) was applied to the different groups to detect the differences in which significance was accepted with a values of $P<0.05$. A Pearson correlation test was conducted to indicate if time and concentration (integrated study) have a correlation on the liver and kidney indices. The integrated analysis was performed using all the periods and all the concentrations.

Real-time PCR

Liver and kidney tissue from control and exposed treatment groups (n=4) were removed and placed in a 1.5 ml micro eppendorf test tubes containing 1 ml of an RNA stabilization solution (RNAlater- Applied Biosystems), stored at 4°C overnight and then removed for prolonged storage at -20°C. Samples were thawed on ice for RNA extraction and then re-suspended in 1ml of Sepasol-RNA I Super G reagent (NACALAI TESQUE, INC.) according to the manufacture's protocol. cDNA synthesis quality was evaluated by amplifying zebrafish Beta-actin by PCR. The PCR cycling conditions were 95°C followed by 40 cycles of 3s at 95°C and 30s at 60°C. Gene expression levels were measured by the use of a Step One Plus real-time PCR with SYBR Green Master Mix. In this study the primer sets were chosen specifically to monitor the changes of Myelopoiesis, Erythropoiesis and Hematopoiesis under toxic ammonia exposure. Such genes were previously identified and reported in scientific literature [26,27] in zebrafish gene analysis which made them more desirable targeted genes for this study and are summarized in Table 1.

Gene	Forward	Reverse
Beta-actin	TC CCAAAG CCAACAGAGAGAA	CACCAGAGTCCATCACAATACCA
Csfla	ACTGTGCCCAGAG CAG CTTT	CCTCACAGTT CCAGTCCACAG A
Csf3	GTGTGCAGCGGATGCTCAT	CTGCGAGGTCGTTCAGTAGGTT
MPO	GCTGCTGTTGTGCTCTTTCA	TTGAGTGAGCAGGTTTGTGG
GA TA-1	CAGTTCAGCAGCGCTCTATTCA	AGCCTCAGGTGGCGAAAGT
GA TA-2	CCACTGCAAGAATGGACGAA	GCCAAGCTTCCCCGAAGA

Table 1: List of Primer sequence used for RT- PCR amplification. All primers are listed from 5' to 3'.

Results

Peripheral blood smears

The mean mature erythrocyte volume showed a variation between both the control and treatment groups after exposure toxic ammonia (Figure 1). The typical average sizes of the control mature erythrocytes were approximately 55.89 ± 4.33 μm^2. As a result, two types of erythrocyte cells were distinguished in the zebrafish blood as mature and immature erythrocytes [10,13,28-32]. The mean percentage analysis of immature erythrocytes varied widely between control groups and exposed treatment groups (Figure 2). Control immature erythrocyte frequency had an average percentage ranging between 0.9 to 2.9%. Immature erythrocytes, on the other hand exposure treatment groups ranged between 9% to 49% at different exposure periods and concentration.

Blood glucose

The average blood glucose levels of zebrafish in the rearing tank under normal conditions were calculated as 69.2 ± 13.6 mg/dl (n=10). Such levels of resting zebrafish blood glucose values were in close range when compared with other studies conducted on zebrafish [33,14]. Blood glucose levels of the control fish throughout the experimental periods were observed to decline in values reaching around 23-27 mg/dl. Such reduction is normal and was due to the 24 hrs no feeding period prior to the actual experimental test. Difference in blood glucose levels were noticed between fish from the control groups and ammonia exposure treatment groups being higher in values when compared with the control group (Figure 3). Exposed groups showed elevated glucose levels at highest concentrations at E4 and E5: 55.3 ± 21.7 and 88.6 ± 54 mg/dl respectively.

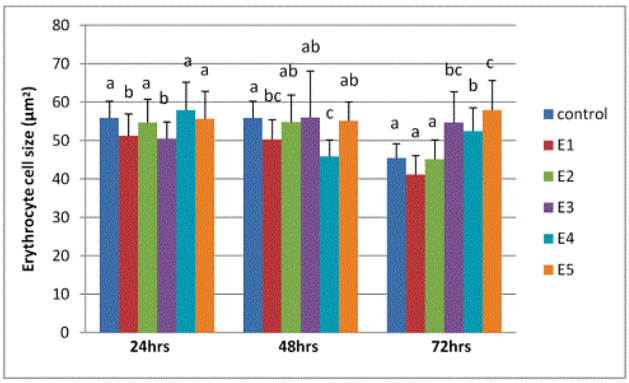

Figure 1: Erythrocyte size (n=50) variations for each exposure treatment group at 24 hr, 48 hr and 72 hrs.

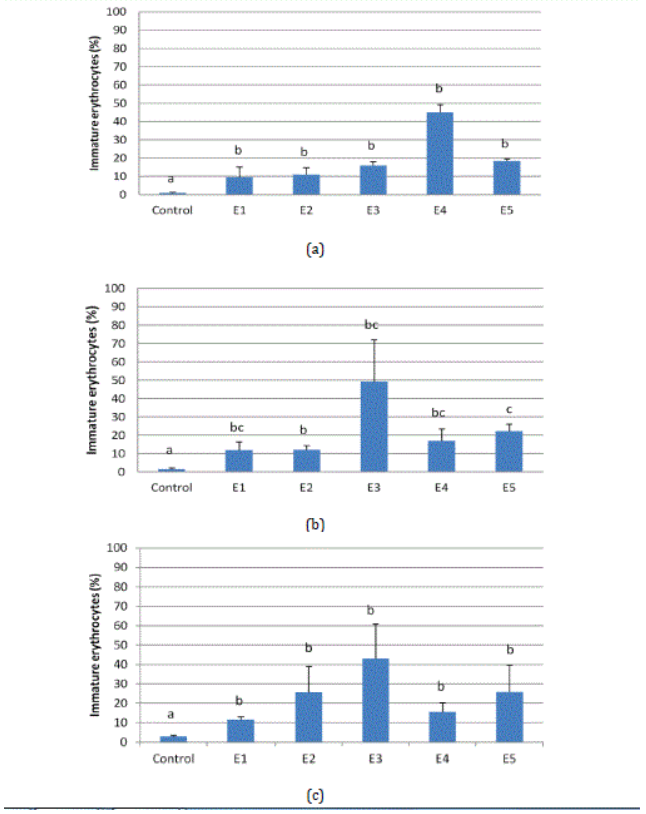

Figure 2: Immature erythrocyte percentage (%IE) (n=1000) between control and the ammonia treatment groups at a) 24 hrs b) 48 hrs and c) 72 hrs.

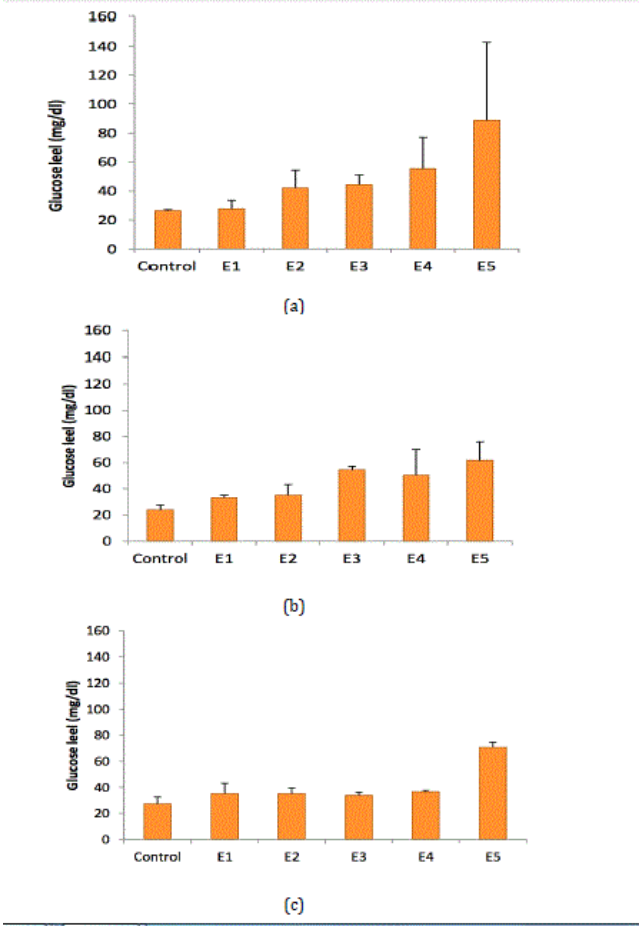

Figure 3: Zebrafish blood glucose values after toxic ammonia exposure periods (n=4). A) 24 hrs exposure period. B) 48 hrs exposure period and C) 72 hrs exposure period.

Histopathological assessment of the liver and kidney

A histopathological assessment was conducted to estimate and confirm if the degree of lesion observed in this study increased in severity in relation to the concentrations levels and periods of exposure. The histopathological lesion observed were slightly modified and classified according to the most frequently observed lesion and morphological and cellular alterations for each target organ listed in Tables 2 and 3.

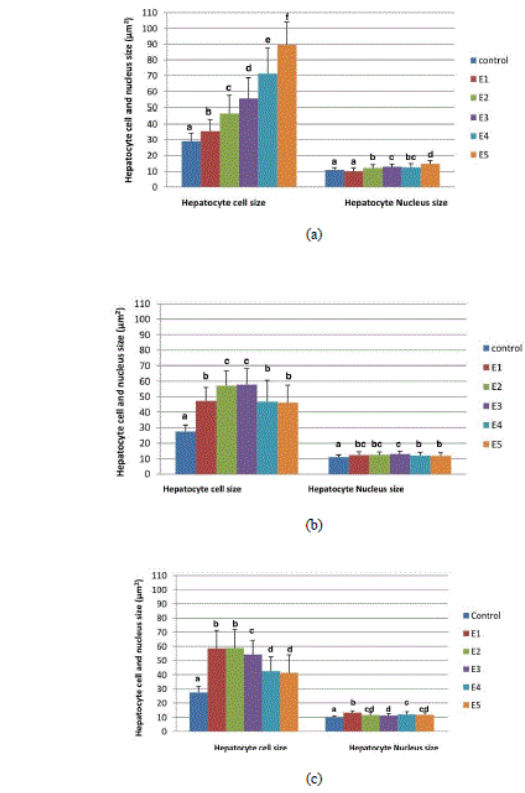

Figure 4: Liver hepatocytic cellular and nuclus size altration at ammonia treatment groups A) 24 hrs B) 48 hrs and C) 72 hrs.

Such lesions were classified into main groups which included: regressive changes, progressive changes and inflammation for the liver, and into: circulatory changes, regressive changes, progressive and inflammation for the kidney. The increase in both liver indices (IL) and kidney indices (IK) due to lesion severity at the optimum exposure period 72 hrs at highest concentrations at E4 and E5 included a reduction in hepatocytes cell volume and an increase in bowman's capsule volume (Figure 4 and 5). The associated cellular lesion at optimum exposure periods at highest concentrations resulted in the reduction of melanomacrophage infiltration and an increase in cellular degeneration resulting in a marked focal necrosis and cellular regeneration in the nephron renal corpuscles and tubules. Histopathological indices were different in the degree of alterations between the different toxic ammonia groups exhibiting a tendency to increase dependent on time of exposure and intensity of toxic ammonia concentrations. This was also true for the total organ indices (liver and kidney) which were calculated to estimate the overall the health conditions of the effected fish. The integrated analysis values against each of the liver and kidney and total organ indices assessment vales were found to be highly correlated (Figure 6). Such a result indicates that time and concentration have a strong linear progressive effect on liver indices, kidney indices and total organ indices ($r=0.95$; $P<0.00001$) (Figure 7).

Such a result indicates that time and concentration have a strong linear progressive effect on liver indices, kidney indices and total organ indices ($r=0.95$; $P<0.00001$) (Figure 7).

Reaction Pattern	Alterations
Regressive changes	Architectural and structural alterations
	Plasma alteration (Vaculation)
	Changes in cellular cytoplasm (esoinophilic)
	Hepatocytic necrosis (single cell or necrotic foci)
	Hepatocyte nuclear pleomorphism (picnotic or hypertrophy)
	Distended sinusoids
	Atrophy
Progressive changes	Hepatocyte cellular hypertrophy
Inflammation	Profusion and dilation of blood vessels

Table 2: Histopathological assessment lesions observation of the liver.

Reaction Pattern	Alterations
Circulatory disturbance	Hemorrhage in Bowman's Space
Regressive changes	Narrow of tubular lumen
	Dilation of glomerulus capillaries
	Dilation of tubular lumen
	Reduction in Bowman's space
	Hylaine droplet degeneration
	Enlargement of glomerulus
	Tubular necrosis
	Tubular regeneration
	Tubular degeneration
	Nuclear degeneration (Tubule)
	Altered architecture of glomeruli
	Glomerular atrophy
Progressive changes	Tubular hypertrophy
	Tubular hyperplasia
	Glomerular hypertrophy
	Glomerular hyperplasia
Inflammation	Leukocyte infiltration (Macrophage)

Table 3: Histopathological assessment lesions observation of the kidney.

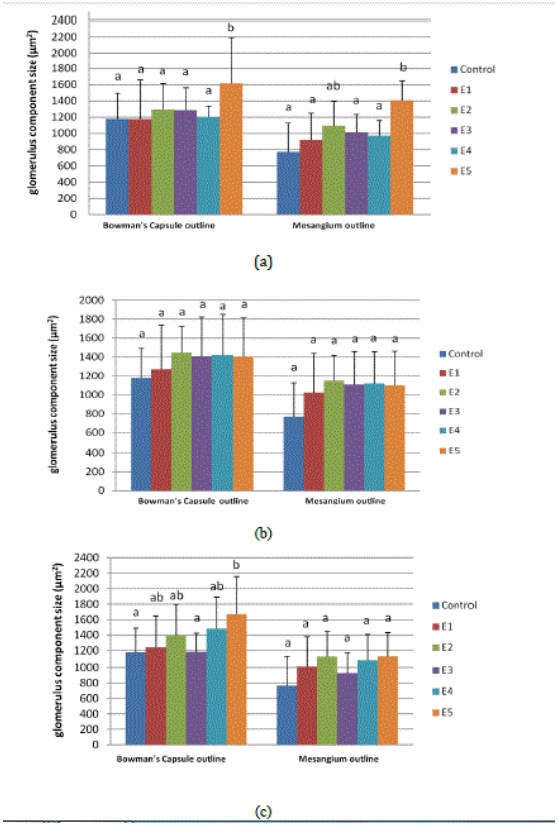

Figure 5: Bowman's capsule and mesangium volume outline measurements (n=10).

Liver gene expression

The liver mRNA expression folds were observed to be altered for all the toxic ammonia treatment groups at all the exposure periods. At 24 hrs mRNA levels were observed with an increase in expression folds at Gata-1(E1: 2.52-fold, E2: 1.47-fold, E4: 1.72-fold and E5: 1.45-fold), Gata-2 (E1: 9.93-fold, E2: 7.17-fold, E3: 3.18-fold, E4: 2.6-fold and E5: 3.50-fold), Csf3 (E1: 6.37-fold, E2: 8.65-fold, E4: 10.40-fold and E5: 5.05) and MPO (E1: 1.18-fold, E2: 2.21 -fold, E4: 4.68-fold and E5: 6.77) respectively (Figure 8A and 8B). E3 expression folds down regulated for Gata-1, Csf1a, MPO E3: 0.47-fold, E3: 1.2-fold, E3: 0.33-fold. Moreover, Csf1a showed down-regulation for all its exposure groups relative to the control level.

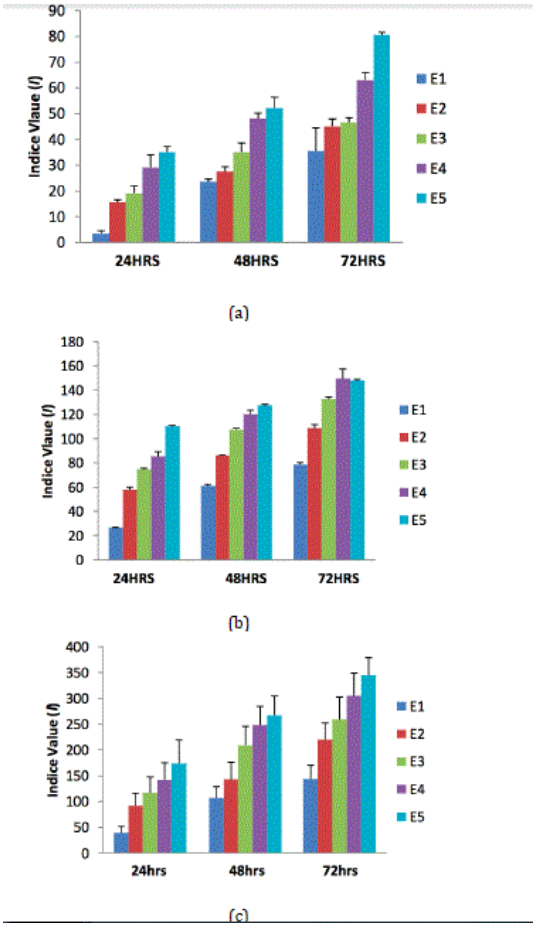

Figure 6: Histopathology lesion indices represented as mean ± SD values measured for the exposure treatments and periods (n=4). A) Liver indices (IL). B) Kidney indices (IK). D) Total organ indices (IT).

Gata-1 Gata-2 mRNA expression levels at 48hrs exposure period for all treatment groups were down-regulated (Figure 8). An increase in mRNA up regulation at E3 for Csf3 and Csf1a as 24.29-fold and 1.98-fold respectively (Figure 8C and 8D). MPO on the other hand, showed a linear down regulation with increase ammonia concentration being highest at E1: 4.60-fold. Interestingly, Gata-1, Gata-2 and Csf1a at 72 hrs did not show any marked increase in expression folds for all the treatment groups (Figure 8A, 8B and 8D). Csf3 expression folds down-regulated at E3 concentration to 2.17-fold with further reduction in E4 and E5 treatment groups (Figure 8C). An up-regulation of MPO expression folds was observed at E1: 15.68-fold, E2: 20.99-fold and E3: 18.07-fold respectively with a reduction in for both E4: 0.69-fold and E5: 0.47-fold respectively (Figure 8E).

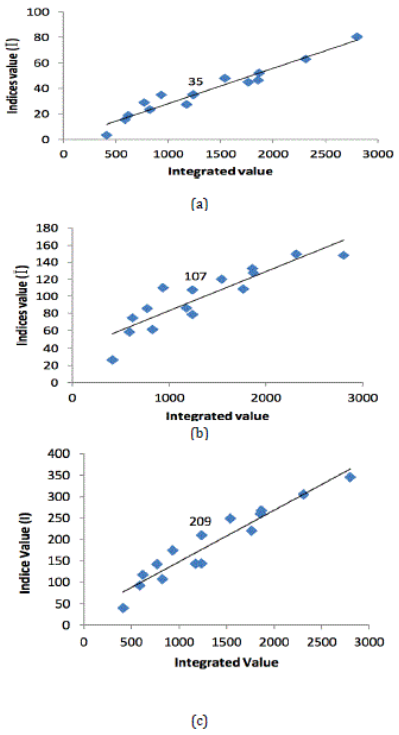

Figure 7: Integrated analysis test against the indices assessment for all the exposure periods and concentration treatment groups A) Liver integrated analysis. B) Kidney integrated analysis. C) Total organ integrated analysis. (r=0.95; p<0.00001).

Kidney gene expression

The mRNA expression folds unlike the liver were observed with a dramatic increase in its expression values. Kidney Gata-1, Gata-2, Csf1a and Csf3 mRNA levels were altered at 24 hrs of exposure to the 5 treatments of toxic ammonia concentrations resulting in an increase in expression fold (up-regulation).

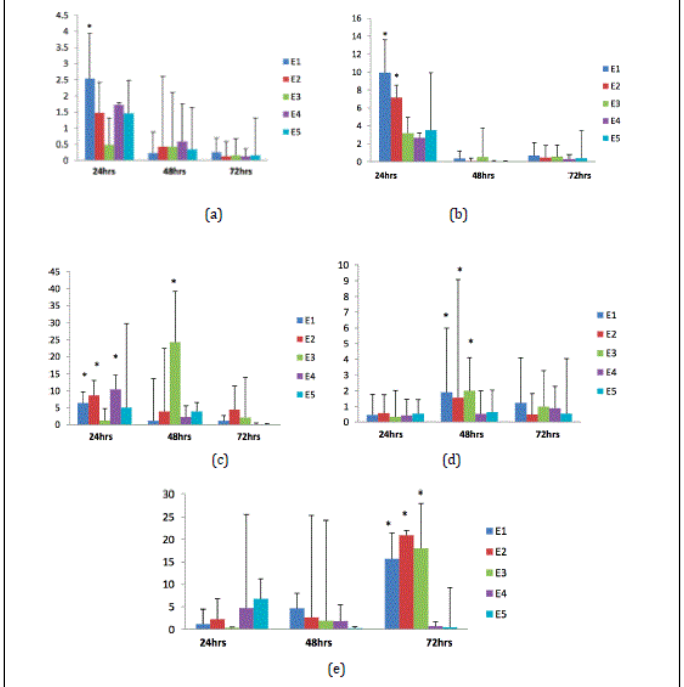

Figure 8: Liver relative mRNA expression levels of A) Gata-1 B) Gata-2 C) Csf3 D) Csf1a and E) MPO genes exposed to E1: 17.21 mg/l, E2: 24.51 mg/l, E3: 25.81 mg/l, E4: 32.11 mg/l, E5: 38.91mg/l NH_3-N at 24 hrs, 48 hrs and 72 hrs toxic ammonia levels (control=1). *indicating an increase in expression fold.

Interestingly, such an increase was noticed with a correlation for the Gata-1, Gata-2, Csf3, Csf1a and MPO being of highest expression folds at E3: 26.26-fold, 48.61-fold, 27.98-fold, 64.51-fold and 2.92-fold and at E4: 20.28-fold, 39.71-fold, 8.60-fold, 70.32-fold and 2.60-fold respectively relative to the control level (Figure 9). Expression folds at 48hrs period were reduced at E3 for both Gata-1 and Csf3 5.038-fold and 17.96-fold respectively. Such expression folds at 48hrs increased additionally at E4 and E5 Gata-1 and Csf3: 68.47-fold, 92.91-fold and 17.76-fold, 20.73-fold respectively. The expression of Csf1a gene was highest E5 at 65.71- fold. However, MPO mRNA levels at E3 reduced from 2.92-fold at 24 hrs to 0.036-fold at 48 hrs. Moreover, mRNA expression folds at 72 hrs of exposure for all the previous genes showed a further down-regulation for all the exposure treatment groups (Figure 9).

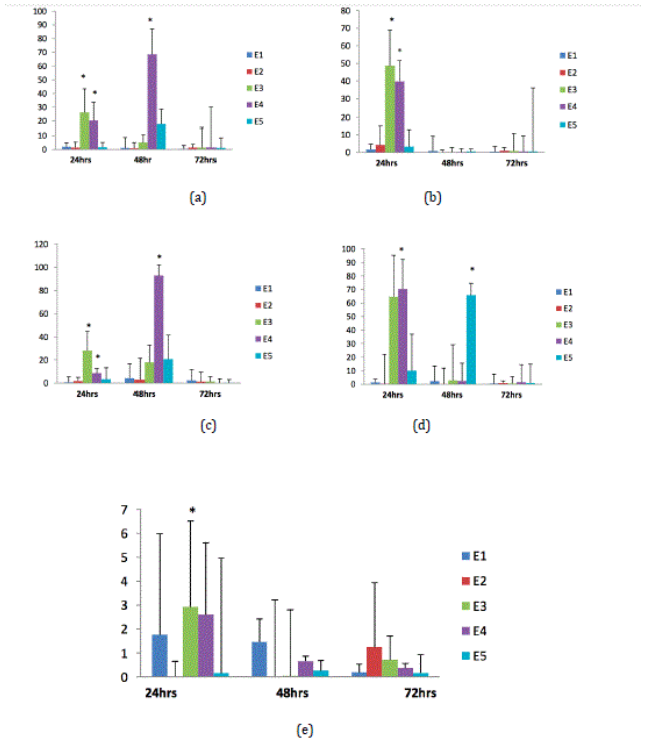

Figure 9: Kidney relative mRNA expression levels of A) Gata-1 B) Gata2 C) Csf3 D) Csf1a and F) MPO genes exposed to E1: 17.21 mg/l, E2: 24.51 mg/l, E3: 25.81 mg/l, E4: 32.11 mg/l, E5: 38.91 mg/l NH_3-N at 24 hrs, 48 hrs and 72 hrs toxic ammonia levels (control=1). * indicating an increase in expression fold.

Discussion

Such sub-lethal toxicological effects do not have endpoints like mortality and can have slight to no noticeable effects on behavioral changes or external damage to fish resulting in organ to tissue level alterations and biochemical disturbance [4].

Hematological indices such as immature erythrocyte percentage (%), hematocrit and hemoglobin concentrations, glucose, plasma ammonia and osmolality have been used as markers to evaluate fish health under various toxic exposures [11,12,31,34-40]. The increased immature erythrocytes and hematocrit seems to be attributed as a stress mediated release into the circulation from erythropoietic tissue (spleen) in response to hypoxia induced acute stress events [11,29]. The direct proportion between the numbers of mature to immature erythrocytes can be used as indicators of erythropoietic activity [41]. Erythropoiesis is activated in fish when the ratio between oxygen demand and oxygen supply increases (effected by anemia, temperature, seasonality and bleeding) influencing the number of the immature erythrocytes [13,30,41]. The reduction of immature erythrocyte however, is suggested to be associated with a reduction in erythropoiesis activity which is mainly due to the accumulation of toxicants. Under toxic exposure specific organs such as the liver and kidney result in the suppression of hematopoietic activities [37]. Such results, agree with the current finding were at E4 immature erythrocyte percentages values increased to 45% at 24 hrs and followed by a reduction to 16.8% and 15.4% at 48 hrs and 72 hrs respectively. Such change in values indicates the difference in erythropoietic activity due to the degree of hypoxia and stressful condition under the influence of toxic ammonia exposure.

An increase in blood glucose (hyperglycemia) concentrations is regarded as a physiological stress [1,13,34,35,42-45]. Hyperglycemia in the circulation is due to the direct activation of glycogenolysis and gluconeogenesis and via indirect mechanisms involving changes in hepatic blood flow or in the levels of other circulating hormones such as glucagon, insulin and cortisol [46]. High values than normal values of circulating glucose are indicators of a greater energy requirement of the fish under the influence of toxic ammonia. The reduction, however, is attributed to the progressive decrease in hepatic glucose production [47].

Ammonia toxicity affects the cellular alteration of the liver and kidney of zebrafish under the different concentrations and periods of exposure. These alterations are attributed to the increase in plasma ammonia in the circulation which are transported and accumulated in the target organs affecting various cellular processes [48]. Such alterations are indicative changes to the overall homeostasis conditions of the fish induced by such lesions resulting in a weaker health condition. Ammonia is mainly produced in the fish hepatocytes and exits the mitochondrial matrix to be excreted through the gills [48]. The histopathological lesion observed from this study resulted in morphological changes in the hepatocyte and their nuclei size, which are sensitive indicators of response to toxic exposure. Such changes in hepatocytic size alteration were reported in several effects of stressors and contaminants on different fish species [21,49-52]. The enlarged hepatocyte cell size is attributed to severe hypertrophic hepatocytes prevalence from the proliferation of endoplasmic membranes of the hepatocytes, indicative of increased metabolic activities, increased glycogen deposits and enzyme function used to detoxify foreign compounds leading to an increased liver somatic index weight. Reduction in hepatocyte cell size however, is attributed to the loss of glycogen deposits and fat leading to cell death due to necrosis and cell atrophy indicating the reduction in metabolic activity of hepatocyte resulting to pyknotic nuclei. Fish exposed to high toxic ammonia levels showed a reduction in hepatic glycogen through rapid degeneration of tissue glycogen indicating stress generated by ammonia toxicity [53]. Increased concentration and periods of exposure to toxic ammonia were observed to reduce liver weight which is the expressed more frequently in stressed fish placing more energy demand on the energy stored in liver glycogen [42]. Degenerative changes in the renal tissue are irreparable lesions reflecting presence of toxic substances and poor water quality [54]. Such renal anomalies are accompanied by a dilation of tubular lumen and can result in the accumulation of certain glycoproteins leading to occlusion in the renal tubules, which are linked to the malfunction of the glomerulus [8]. Nephrotoxicant induced injury trigger the formation and development of new nephrons and is known to be a key mechanism to sustain physiological renal growth to repair the kidney after damage [34,54-58]. Renal regeneration identified by newly formed basophilic clusters was observed at the initial 24 hr exposure period at higher ammonia concentration treatment groups E4 and E5. Following the initial 24 hr exposure period such basophilic clusters were observed at lowest, medium and highest concentration at 48 hr and 72 hr exposure periods. Higher concentrations of toxic ammonia increased the kidney lesion severity triggering the start of renal regeneration process. Such changes have proven to be correlated with concentration and exposure periods.

The present results indicate that toxic ammonia concentrations induce alterations of hematological and histopathological indices resulting in cellular alterations triggered by changes in molecular gene expressions to the liver and kidney of zebrafish. The assessments of sub-lethal ammonia toxicity effects are important in terms of fish health as environmental endpoints. Such sub-lethal toxicological effects do not have endpoints like mortality and can have slight to no noticeable effects on behavioral changes or external damage to fish resulting in organ to tissue level alterations and biochemical disturbance [4].

The interrelation between the hematological, histopathological and gene expression have proven to be time and dose dependent under the influence of toxic ammonia exposure. A linear relationship with increase concentration and period of exposure between the indices and integrated analysis study was observed. An initial critical point from this study can be detected from such a point, and after such a point a deterioration of fish health quality is reached [59].

The critical point in the liver can be detected at E3 at 48 hrs. The integrated value at this point for the liver is at indices values around 35 and integrated value of 1238.8 respectively. Following such a point the glucose values fall at highest concentration associated with a reduction in the hepatic cell size at 48 hrs. At such a point the liver myeloid precursor gene expression increased in expression folds indicating the increase in granulocytes, neutrophils and macrophages activity, and a decline following the E3 48 hrs concentration indicates a reduction in myeloid precursors due to a weaker health state.

The critical point in the kidney can be also detected at E3 at 48 hrs. The integrated value at this point for the kidney is at indices values around 107 and integrated value of 1,238.8 respectively. The kidney Gata-1 and Gata-2 up-regulation at E3 24 hrs indicate increase activity of erythropoiesis and hematopoiesis process resulting in the elevation of IE% in the blood circulation observed at E3 48 hrs. The down-regulation of these genes is probably due to a reduction of hematopoitic cells and anaemia resulting from a high rate of erythrocyte destruction or insufficient generation of erythrocytes. Myeloid precursor (granulocyte, neutrophils and macrophage) precursor genes up-regulation in the kidney was highest at E3 24 hrs which later on down-regulated at 48 hrs and 72 hrs. Such high expression point to an increase activity and production of myeloid precursors in the kidney tissue at 24 hrs and released and detected in the circulation blood in high densities (data not shown) indicating their abundance at 48 hrs. As a result, the critical point indicating deterioration of fish health conditions and tissue alteration of the target organs occur following the E3 48 hrs concentration and increasing in severity leading to further complications. As a result, the total indices to estimating the critical points from the target organs (liver and kidney) at E3 is at indices values around 209 and integrated value of 1,238.8 respectively.

Such an approach conducted in this study applies hematology, histology and gene expression techniques for the assessment of the health status of the exposed fish. It has proved to be important to avoid possible misinterpretation of various other biomarkers responses. Such information confirms that the techniques used to detect the alterations are good biomarkers for assessment against toxic ammonia exposures and can be used to evaluate the early effects and responses to acute chemical exposure reflecting the stressful conditions in the environment, which can lead to fish health complication.

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