EFFECT OF DIETARY ZEOLITE ON AMMONIA TOXICITY AND ON SOME OF BLOOD PARAMETERS IN COMMON CARP *CYPRINUS CARPIO*

Alaa A. Abdulathem   **Abdulmotalib J. Al-Rudainy**
Researcher   Prof.
Dept. Pathol. and Poultry Dis.;Coll. Vet. Med.; University of Baghdad
alrudainy612003@yahoo.com

ABSTRACT
The present study aimed to evaluate the effect of dietary zeolite on ammonia toxicity and in some of blood parameters in *Cyprinus carpio*. Fish were fed either a control diets (0% zeolite) or control diet supplemented with 2, 4 and 6% (T1, T2 and T3) respectively for 28 days. Results showed that the mean value of ammonia concentrations ranged between 0.10 mg/l in T1 and T3 in the first week to 1.85 mg/l in control in fourth week, which could be noticed a gradually increased in the values of ammonia in the end of the experiment. Results showed that the ammonia concentration has a significant (p≤ 0.05) increased in control treatment for third and fourth week compared with other treatments. For blood parameters, results showed high fluctuation in the mean values of RBC count, the mean of WBC count recorded the highest value in control treatment, and significantly decreased in T1,T2 and T3. PCV value and Hb content recorded the lowest values in control treatment and the highest values in T1,T2 and T3 respectively. It can be concluded that T3 have reached the saturation point for lower values in the first three weeks of ammonia , then the active of zeolite decreased resulted increasing ammonia in fourth week for all treatments.
Key words: common carp, clinoptilolite, hematology,minerals, mortalities

تاثير الزيولايت في العليقة على سمية الامونيا وبعض المعايير الدمية في اسماك الكارب الشائع *Cyprinus carpio*

الاء عدنان عبدالعظيم   عبدالمطلب جاسم الرديني
باحث   استاذ
قسم الامراض وامراض الدواجن ،كلية الطب البيطري ، جامعة بغداد

المستخلص

هدفت الدراسة الحالية لتقييم تاثير الغذاء الحاوي على الزيولايت على سمية الامونيا وبعض المعايير الدمية في سمكة الكارب الشائع *Cyprinus carpio*. تم تغذية الأسماك بعليقة السيطرة (0 ٪ زيوليت) أو عليقة السيطرة مضاف اليها 2 و 4 و 6 ٪ (T1و T2 و T3) على التوالي لمدة 28 يومًا..اشارت النتائج الى ان معدل قيم تراكيز الامونيا تراوحت بين 0،10ملغم/لتر في المعاملتين الاولى والثالثة في الاسبوع الاول الى 1،85 ملغم/لتر في معاملة السيطرة في الاسبوع الرابع، اذ يلاحظ زيادة تدريجية في معدلات قيم الامونيا في نهاية التجربة.اشارت النتائج الى وجود زيادة معنوية (0.05 ≥P) في قيم الامونيا للاسبوعين الثالث والرابع مقارنة ببقية المعاملات. اشارت النتائج الى وجود تذبذب عالي في عدد كريات الدم الحمر ، بينما سجلت كريات الدم البيض اعلى عدد لها في معاملة السيطرة ، بعدها انخفضت في المعاملة الاولى والثانية والثالثة. اشارت نتائج الدراسة الحالية بان قيم حجم خلايا الدم المرصوصة والهيموغلوبين سجلت ادنى القيم في معاملة السيطرة واعلاها في المعاملات الاولى والثانية والثالثة. يمكن ان نستنتج بان المعاملة الثالثة وصلت الى نقطة التشبع للامونيا الى ادنى قيمة لها في الاسابيع الثلاثة الاولى

الكلمات المفتاحية: كارب شائع، كلينوبتيلولايت، دم، معادن، هلاكات

INTRODUCTION

Ammonia is toxic to aqueous life and reduces oxygen carriage capacity of hemoglobin. Furthermore, ammonia damages the gills and prevents fish to get oxygen (17,29). The nature and degree of ammonia toxicity depends on many factors, including the chemical form of ammonia, pH and water temperature, period of exposure, and the life stage of the exposed fish (30). Ammonia is toxic to fish and other aquatic organisms in very low concentration about 0.2 mg/l (1,21). In rainbow trout *Oncorhynchus mykiss*, sublethal concentrations of ammonia can cause hyperexcitability, resulting in fish crashing into the sides of the tank in response to any disturbance (21). Zeolites are porous, hydrated aluminosilicates, may be natural minerals or synthetic materials. The general chemical composition of a zeolite is: Mn x/nSi1-xAlxO2 · yH2O. Pellet zeolite can easily be spread to the ponds by the farmers without dust. Pellet stays rigid until it falls to the bottom of the pond, turns into powder form and expands its surface area. Powder zeolite absorbs the toxins and ammonia and heavy metals with a greater efficiency (12,23). The present study aimed to calculate the period in which zeolite affects ammonia and determine the best treatment of zeolite cause reduction of ammonia, as well as the effect of zeolite on hematological parameters (RBCs and WBCs count, Hb content and PCV value).

MATERIALS AND METHODS

Preparation of diets and zeolite

Four weeks of feeding trail were started using a commercial diet, with protein content of 28% total protein, the raw materials were grinded by using food grinder and weighed. Zeolite was grinded through a feed grinder to a very small particles to form powder, then added to the raw materials of pellets and mixed by hand until ingredients homogeneity. Each formulated diet was mixed to prepare each of the experimental diet. Warm water was added with continuous mixing. The paste like passed through meat mincer to produce pellets (2 mm in diameter) , then dried in room temperature. The diets collected and stocked in plastic containers and stored in refrigerator at 4 ºC during the experimental periods to avoid the deterioration of nutrients. The experimental diets are:

Cont.: Use the commercial basal diet (0% zeolite).

T1 : Commercial basal diet with 2% zeolite .

T2 : Commercial basal diet with 4% zeolite.

T3 : Commercial basal diet with 6% zeolite

Each of the five treatment fish fed twice daily at rate of 3% body weight during the f experimental trail .

Experimental design

The present study was conducted at the College of Veterinary Medicine, University of Baghdad, Fish Diseases Laboratory. A total of 200 fingerlings of common carp *Cyprinus carpio* with an average weight of 20g. were obtained from a commercial farm (Al-Messayyab, Babylon). Fish were briefly bathed in NaCl for 5 minutes to remove all of the external parasites if present. Two weeks of acclimation for fish before starting the experiment. During this time, they were stocked in two baths with a dimensions of 150 × 80 × 50 cm. Then healthy fish were randomly selected and distributed into 10 glass aquariums (dimensions; 60x40x40 cm) at rate of 6 fish per aquaria (two replicates / treatment) were maintained for each of the five treatments (T1, T2, T3 and control) in de-chlorinated water tap water filled the aquarium to reach 60L.Chemo-physical parameters of water such as temperature was recorded daily using thermometer, while pH, dissolved oxygen (DO) were measured twice weekly, using pH meter and digital DO respectively. Aquariums are ventilated with air pumps and heater device to maintain the ventilation and temperature.

Determination of Ammonia concentration

Total ammonia was measured using drops Kit: Hanna Ammonia test kit for fresh water : determines the ammonia concentration in water in several easy steps. The kit is portable and can be used in the field as well as in the laboratory.

Chemical reaction of kit : The ammonia level in mg/l , ammonia as nitrogen is determined by a colorimetric method . The nesssler reagent reacts with ammonia , under strong alkaline conditions, to form a yellow colored complex . an addition of reagent 1(EDTA solution) inhibits precipitation of calcium and

magnesium ions due to the presence of the alkaline nessler reagent..The color intensity of the solution determines the ammonia concentration

method of the kit: 1.Remove the cap from the plastic beaker . rinse the plastic beaker with water sample before filling it up to the 10 ml mark. Add 2 drops of ammonia reagent 1 for freshwater, replace the cap and mix by carefully swirling the beaker in tight circles. Add 8 drops of nessler reagent, replace the cap and mix by carefully swirling the beaker. Then remove the cap and transfer the solution into the color comparator cube, wait for 5 minutes to allow color to develop. Finally determine which color matches the solution in the cube, and record the results in mg/l NH3 -N. 6. it is better to match the color with a white sheet at about 10 cm behind the comparator.

Hematological parameters
Blood samples were collected from four fish randomly selected from tank. Samples were taken from caudal vein puncture using 3 ml plastic syringe. Blood was transferred to eppendorf coated with lithium heparin that work as anticoagulant and used for measuring the hematological parameters. Dices fluid was used to dilute blood to count RBCs and WBCs by haemocytometer chamber. Pipette was filled it to the marker (0.5) and diluted with the Dacies fluid was filled to mark (9) to make the dilution range 1:20, mixing well and remain for 5 min. The counting chamber was filled and left for about 5 minutes, then the WBCs were counted in the four corner primary squares of the chamber and the RBCs are counted in five secondary squares of the center primary square. The following formulae were applied to calculate the WBCs, and RBCs (10):

Total WBCs count $= \frac{N \, x \, 20}{0.1}$ cell / µl
N: number of the cells in 1 large square ,20: dilution factor,0.1: volume factor = (width x length x height).

Total RBCs count $= \frac{N \, x \, 20 x 5}{0.1}$ cell / µl ,20: dilution factor,0.1: volume factor = (width x length x height).5: 5 squares equals
Concentration of Hb was determined by using the standard cyanomethemoglobin method described by Dacie and Lewis (9). Value of

PCV was determined according to the method described by Klontz (18).

Statistical analysis
The Statistical Analysis System- SAS (28) program was used to detect the effect of difference factors (Treatments and Period) in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means in this study.

RESULTS AND DISCUSSION
Water quality
Water temperature ranged between 17.4 – 19.0 ^{o}C for all experimental treatments , pH values ranged between 7.7 – 8.2 , while dissolved oxygen level ranged between 5.4 – 7.5 mg/l . Finally salinity in water ranged from 0.05 to 0.08 g/l. These values within the limiting of live for warm fish particularly common carp (22,27).

Ammonia concentration and mortality rate
Table 1 showed that the means of ammonia concentrations ranged between 0.10 mg/l in T1 and T3 to 1.85 mg/l in control, which could be noticed a gradually increased in the values of ammonia from 0.18 mg/l in first week to the highest value in the fourth week attained 1.85mg/l in control treatment. Mortality rate was increased reached to 100% in the fourth week (Tab.2).Mean values of ammonia have continuously increased in T1 , which ranged between 0.10 – 1.00mg/l in first and fourth week respectively with increasing of mortality rate reached up to 66.66% in fourth week (Tab.2), also observed in T2 and T3 which ranged between 0.14 mg/l in first week to 0.98mg/l in fourth week for T2; between 0.10 – 1.24 mg/l in first and fourth week respectively for T3 , and between 0.18 -0.70 mg/l in the same weeks for T4 . No significant different (p > 0.05) in T2 was observed within second and third weeks. On the contrary there are significant increased (p≤ 0.05) within previous week in T1 and T3. In fourth week the value of ammonia continued increased significantly (p≤ 0.05) in all treatments , so it can be concluded that T3 have reached the saturation point for lower values in the first three weeks of ammonia , then the effect of zeolite decreased resulted increasing ammonia in fourth week for all treatments . Tab. 1 showed that the ammonia concentration has a

significant increased (p≤ 0.05) in control treatment for third and fourth week compared with other treatments. Results of statistical analysis of ammonia concentration showed no significant difference (p > 0.05) among experimental treatments in first week . Generally results showed that ammonia and

mortality decreased with increased dietary zeolite . Mortality rate in the end of experiment reached 66.66% in T1 and T2 , while it was 50.0% in T3, it can be noticed increased of mortality rate in the fourth week especially in control treatment which reached up to 100% (Tab.2).

Table 1. Mean (± SD mg/l) values of ammonia in different treatments during experimental period

week	Mean ± SD of Ammonia (mg/l)				
	Cont.	**T1**	**T2**	**T3**	**LSD value**
1st	0.18 ± 0.07	0.10 ± 0.01	0.14 ± 0.03	0.10 ± 0.01	0.119 NS
	A c	A c	A c	A c	
2nd	0.59 ± 0.08	0.60 ± 0.08	0.50 ± 0.07	0.22 ± 0.05	0.207 *
	A b	A b	AB b	C c	
3rd	1.17 ± 0.12	0.70 ± 0.09	0.60 ± 0.08	0.44 ± 0.07	0.289 *
	A a	B ab	B b	B b	
4th	1.85 ± 0.14	1.00 ± 0.11	0.98 ± 0.09	1.24 ± 0.11	0.865 *
	A a	A a	A a	A a	
LSD value	0.613 *	0.326 *	0.318 *	0.224 *	---

Means with different small letters in the same column and capital letters in the same row are significantly different. (P≤0.05).

Table 2. Mortality rate in different treatments during experimental period

Treatment	No. of dead fish (week)				Mortality rate
	1st	**2nd**	**3rd**	**4th**	**%**
Cont.	0	1	2	3	100.00
T1	0	1	1	2	66.66
T2	0	1	1	2	66.66
T3	0	0	1	2	50.00

Different fish species have different sensitivity to different concentrations of the ammonia. For example, Salmonids (. *Salmo salar*) are more sensitive than the other fish species, due to their high oxygen need (2). Brinkmans (7) reported that chronic exposure in early life stages to ammonia concentrations as low of unionized ammonia nitrogen (UIAN) caused significant reductions in survival rate. Results of the present study are similar to Farhangi and Hajimoradloo (13) who indicated that this ability to absorb ammonia by the zeolite after some time depends on the amount of zeolite that used for removal ammonia compounds and with decreasing ammonia, the zeolites reached the saturation point and not being able to continue the absorbing of this compounds, the levels of ammonia from 0.01 to 1.0mg /l reduced growth of freshwater fish. In general, 0.02 mg/l is considered as limit for ammonia in aquaculture (30).Furthermore, when the zeolite is saturated, it can be reused after treatment with salt water. Muir (22) reported that effects of ammonia (0.130 mg /l) on growth for Tilapia, by using zeolite, removing of ammonia molecules was faster with

increasing of the zeolite concentrations in different treatments until zeolite reached the saturation point, hereby a particular amount of zeolite needed to be added as an active absorbance to remove the ammonia from the water solution. Ammonia excretion rates were investigated as a function of zeolite dosage in rainbow trout dietary, a diet with 2.5% natural zeolite decreased ammonia discharge by 24% compared to the control group of trout (12). Moreover, to improve water quality in aquaponic production of red tilapia *Oreochromis* sp. and lettuce *Lactuca sativa* . longifolia, natural zeolite has been reported to be very effective (26). Fish fed with zeolite-supplemented diets in different levels, ammonia and ammonium were found relatively to be lower than in control group (1,12). Obradović et al. (24) revealed that the diet containing zeolite decreased the release of ammonia. An important attribute of the zeolite is its capacity to effectively bind ammonia and, as a result, to reduce ammonia concentration by release into the environment (27). Zeolite, as feed additive, can contribute removal of excess ammonia in the digestive

system (4,27). However, it should be considered that some other factors, such as energy level or amino acid balance, as well as the growth rate, fish size or physiological state, feed intake and some various environmental and cultural conditions, might also affect the postprandial ammonia excretion in fish (19). Diet quality and feed ingredients are also important factors affecting ammonia excretion, (i.e. a better diet utilization may result in a lower ammonia excretion by fish). In contrary to present study , Oz et al.(25) stated that ammonia concentrations reached the highest level in the control group on 9th day of the study (1.6 mg/l), and on the same day it was 0.9 mg/l and 0.2 mg/l respectively in groups comprising natural zeolite, so it was found that the effect of natural zeolite had a positive effect on aquarium water conditions in terms of reducing the increasing ammonia concentrations levels. Öz et al. (25) detected that zeolite that was put inside a net bag reduced ammonia content in fish tanks 72%, while zeolite that was put in aquarium directly reduced ammonia by 33% compared with control. However, use of zeolite in the aquaculture a net bag inside will have advantages such as higher ammonia adsorption levels, prevention of aquarium water turbidity, and easy removal from production area for conditioning process, therefore, use of zeolite inside a net bag can be recommended (25). As aquarium fish are in a limited capacity of water, so keeping water conditions within the ideal range is one of the most important activities to be performed. The most important factors affecting water conditions are metabolic waste of live animals and unconsumed feed (5). According to the report of Farhangi et al. (14), adding 10 g/l zeolite was an optimum dose for improvement of water quality in angel fish culture, natural zeolite was applied for reduction of ammonia lethal concentration in rainbow trout culture (13). Zeolites has the ability to reduce the content of ammonia in both fresh and marine waters with efficiency exceeded 83% when the

initial content of ammonia was≤1.0ppm (3,6). Aly et al.(3) concluded that ammonia removal efficiency in freshwater is better than marine water. Zeolite has great potential for removing ammonia from water, especially at salinities lower than 10 g/l , but the main obstacle in using zeolite is its uselessness after a few hours (9). Farhangi and Hajimoradloo (11) reported that the use of 15 g /l powder zeolite can prevent mortality rate of rainbow trout fish. However the complete removal of ammonia was not achieved even when zeolite was applied at 12 g /l concentration.

Hematological parameters

Table 3. showed high fluctuations in the mean values of RBC , ranged between 1.99 – 2.55 $\times10^6$ /mm^3 in control and the T3 respectively, whereas the mean value of WBC recorded highest value in control treatment which reached 23.52 $\times10^3$/mm^3, then decreased to 20.13$\times10^3$/mm^3 in T1 with gradual decline in T2 and T3 attained 20.25 and 20.16 $\times10^6$/mm^3 respectively. Results of present study showed that the PCV values ranged between 24.0% in control treatment to 35.25% in T3, this result also recorded in Hb values which attained 6.90 in control treatment with gradually increase reached 11.70 g/dl in T3(Tab.3).Results of statistical analysis showed that RBC value with significant different (p ≤ 0.05) in T3 compared with control and T1, but no significant different (p > 0.05) compared with T2 . While the values of WBC showed significant different (p ≤ 0.05) in control treatment compared with other treatments .The values of PCV in T3 showed significant different (p ≤ 0.05) compared with other treatments, this corresponds completely to the Hb values. Results in Tab. (3) showed that the T3 can be considered as better treatment that contained zeolite(6%) in all values of the studied blood parameters followed by T2 (4% zeolite).

Table 3. Hematological parameters (means ± SD) of *C. carpio* at the end of experimental period

Treatment	RBC cells×10⁶/mm³	WBC cells×10³/mm³	PCV (%)	Hb g/dl
Cont.			24.00 ± 1.70 b	6.90 ± 0.52 c
	1.99 ± 0.25 b	23.52 ± 1.37 a		
T1			27.50 ± 2.08 b	9.24 ± 0.26 b
	2.05 ± 0.13 b	20.13 ± 1.20 b		
T2			28.75 ± 2.63 b	9.60 ± 0.96 b
	2.22 ± 0.22 a	20.25 ± 1.55 b		
T3			35.25 ± 1.70 a	11.7 ± 0.36 a
	2.55 ± 0.18 a	20.16 ± 1.03 b		

Means with different small letters in the same column are significantly different (p≤0.05)

Ammonia toxicant is a serious problem and can cause big mortalities in fish as showed in present study. On the contrary, there were no dramatically increased concentrations of ammonia in the fish blood (31). Highest values of RBC in present study were recorded in T3 are agreement with Güler and Uçar (16) who observed that the highest erythrocyte sedimentation rate value was in 3% zeolite additive feed and the lowest in 1%zeolite additive feed in *Oncorhynchus mykiss* .The sudden increase in erythrocytes can be explained by the stress-induced induction of catecholamine by contraction of the spleen and new erythrocytes participating in circulation (16). Results of RBC count in present study showed higher in treatments than control , a reduction in RBC numbers below the normal range in fish is assumed to be an indicator of anemia (20), which acts as an indicator with subsequent result of inhibition of erythropoietin in the haemopoietic organisms. When the effect of zeolite on the leucocytes count was examined, the highest value was measured in fish fed with 5% zeolite feed, the lowest value was obtained in fish fed 3% zeolite , it means that WBC increased with increased zeolite (16),the previous results are agreement with the present study that observed the highest value in control treatment and the lower value recorded in T1 using 2% zeolite, as well as the highest value in control treatment and lowest value in T1 (6 mg/l zeolite). WBC number generally increases after deteriorate of homeostasis due to an exposure to a stressful factor (8). Hb, WBC and RBC values obtained from the present study are agreement with the study of gilthead sea bream(15).In present study a reduction of hemoglobin in control treatment may be due to inhibition of aerobic glycolysis, prolonged reduction in hemoglobin content is deleterious to transport any blood dyspepsia and degenerated and could be ascribe as pathological conditions in fish exposed to toxicants(8). It can be concluded that T3 have reached the saturation point for lower values in the first three weeks of ammonia , then the active of zeolite decreased resulted increasing ammonia in fourth week .

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