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Effect of Unionized Ammonia (UIA) on Virulence of *Clostridium perfringens* in *Oreochromis niloticus*

Zeinab M. EL-Bouhy, Gamal El-Nobi, Mohammed E. Hassanin and Afaf N. Abd EL-Rahman*
Fish Diseases and Management Department, Faculty of Veterinary Medicine,
Zagazig University, 44511, Egypt

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Abstract

The present work was performed to study the effect of unionized ammonia (UIA) on mortality rate, clinical signs, postmortem findings, some biochemical and immunological parameters and histopathological findings of *Oreochromis niloticus* challenged with *Clostridium perfringens* type A. The experiment was carried out on 160 *Oreochromis niloticus* with average body weight 45±5 gram which were divided into 8 equal groups. Groups (1-4) were inoculated intraperitoneally with 0.2 ml/ fish of sterile cooked meat broth, while, groups (5-8) were inoculated intraperitoneally with 0.2 ml/fish of 24 hours old culture of *Clostridium perfringens* type A ($0.5x10^7$ CFU) on cooked meat broth. The groups (1 and 5) were used as a control (not subjected to UIA) but the groups (2 and 6), (3 and 7) and (4 and 8) were subjected to 0.53, 0.265 and 0.132 mg/L of UIA. The results revealed that the highest mortality rate was 80% and 70% in infected fish exposed to 0.53 and 0.265 mg/L of UIA, respectively. The fish showed nervous manifestation, effort to swallow air from the water surface and mortality occurred with open mouth with dark body coloration, hemorrhages on body and increased mucus secretion on skin and gills. The levels of serum alanine aminotransferase (ALT), creatinine and cortisol were significantly increased, while, the level of serum immunoglubulin M (IgM) was significantly decreased. Also, some histopathological changes were recorded in liver, kidney, gills and intestine. In conclusion, the elevated levels of UIA increased the virulence of *Clostridium perfringens* type A infection in *Oreochromis niloticus.*

Keywords: Unionized ammonia, *Oreochromis niloticus, Clostridium perfringens* type A, Virulence

Introduction

The rapid development in aquaculture has led to the appearance of diseases, which are the main causes of economic losses [1]. Bacterial infections that are found in fish environment cause diseases under stress conditions [2,3]. Clostridium infection is an example to such pathogens; addition of animal manure and poultry droppings as organic fertilizers in the earthen ponds of aquaculture provides an ideal source of *Clostridium perfringens* infection for fishes. *Clostridium perfringens* can affect fishes through the production of toxins and causes high mortalities with decreasing the productivity of farms [4,5]. Environmental factors act as stressors that may display bacterial infection and disease outbreaks in fishes [4]. Poor water quality (such as high ammonia), low dissolved oxygen concentration and high salinity are involved in environmental factors of bad effect [6].

Intensification of aquaculture increased the exposure of fish to high levels of nitrogenous wastes such as ammonia. The exposure to sub lethal concentrations of unionized ammonia (UIA) causes behavioral, physiological and histological alterations in fish and makes fish susceptible to bacterial infections due to impairment of the immune response [7]. The increased levels of ammonia lead to fish mortality [8].

***Corresponding author e-mail: (afne55@yahoo.com), Fish Diseases and Management Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt.**

To the best of our knowledge, no literatures about the effect of UIA on the virulence of *Clostridium perfringens* are available. Therefore, the aim of the present study was to evaluate the effect of different levels of UIA on the mortality rate, clinical signs, postmortem findings, some biochemical and immunological parameters and histopathological findings of *Oreochromis niloticus* challenged with *Clostridium perfringens* type A.

Material and Methods

A total number of 160 apparently healthy a live *Oreochromis niloticus* with average body weight (45±5 gram) were obtained from Abbassa fish hatchery at Sharkia Governorate, Egypt. Fish were transported to the lab of Fish Diseases and Management Department at the Faculty of Veterinary Medicine, Zagazig University, Egypt.

After acclimation to the experimental conditions for 2 weeks, the fish were divided into 8 equal groups. Each group was kept in 2 glass aquaria (80×30×25cm) with dechlorinated tap water. Pathogenic and virulent strain of *Clostridium perfringens* type A was previously isolated and identified from naturally infected *Oreochromis niloticus* [9]. The bacterial isolate was reconstituted and aliquoted in 2 ml microfuge tubes containing glycerol - phosphate buffered saline (pH 7.4 - 1: 1 volume /volume) and stored at -80°C freezer till the onset of the experimental challenge. The stored bacterial suspensions were thawed at room temperature and agitated using electric vortex to ensure equal distribution of the bacteria.

Table 1: Mortalities of the *Oreochromis niloticus* challenged with *Clostridium perfringens* type A and exposed to different levels of UIA

Group No=20	Mortalities within						Total mortality	
	24 hours	48 hours	72 hours	96 hours	1 week	2 weeks	No	%
1*	0	0	0	0	0	0	0	0
2*	0	2	1	1	1	0	5	25
3*	0	0	1	1	0	0	2	10
4*	0	0	0	0	0	0	0	0
5**	0	2	2	3	4	1	12	60
6**	2	5	4	3	2	0	16	80
7**	1	3	4	3	3	0	14	70
8**	0	2	3	4	3	0	12	60

* Groups non-infected and exposed to 0, 0.53, 0.265, 0.132 mg/L of UIA respectively.

** Groups infected with *Clostridium perfringens* type A and exposed to 0, 0.53, 0.265, 0.132 mg/L of UIA, respectively.

Loopful from the bacterial isolate aliquot was inoculated into sterile cooked meat broth then incubated at 25°C under complete anaerobic condition for 24 hours. The groups (1-4) were inoculated intraperitoneally with 0.2 ml/ fish of sterile cooked meat broth. The groups (5-8) were inoculated intraperitoneally with 0.2 ml/fish of 24 hours old culture of *Clostridium perfringens* type A on cooked meat broth contained $0.5x10^7$ CFU. The groups (1 and 5) were used as a control (not subjected to UIA) but groups (2 and 6), (3 and 7) and (4 and 8) were subjected to 0.53, 0.265 and 0.132 mg/L of UIA which matched 1/5, 1/10 and 1/20 of 96 hours LC50 respectively [10]. Ammonium chloride stock solution was prepared and used to produce the required concentrations [11]. The total ammonia nitrogen (TAN) NH_3/ NH_4^+ was measured by using ammonia bottle kits. The amount of UIA was calculated by multiplying the TAN by the appropriate conversion factor according to the measured water temperature and pH [12]. The fish were observed for 15 days under continuous aeration. Dissolved oxygen, water salinity, water temperature, PH, UIA were daily recorded and adjusted till the end of the experiment [11]. The mortalities, clinical signs and postmortem findings were recorded [13]. Blood samples were collected into Eppendorf

tubes and centrifuged at 3000 rpm for 15 minutes for serum separation for the determination of serum alanine aminotransferase (ALT) [14], creatinine [15], cortisol [16] and serum immunoglubulin M (IgM) levels [17].Samples from liver, kidney, gills and intestine were taken for histopathological studies [18]. All data were analyzed using one-way analysis of variance (ANOVA) [19].

Table 2: Effect of different levels of UIA on some biochemical and immunological parameters of *Oreochromis niloticus* challenged with *Clostridium perfringens* type A

Group No=20	ALT(µg / dL)	Creatinine (mg / dL)	Cortisol (µg / dL)	IgM (g / L)
1*	11.33 ± 0.882^{e}	0.37 ±0.012^{e}	2.17 ±0.028^{f}	2.30 ±0.115^{a}
2*	30.00 ±1.00^{d}	0.84 ±0.009^{d}	4.61 ±0.093^{d}	0.86 ±0.018^{b}
3*	13.67 ±0.882^{e}	0.36 ±0.012^{e}	3.54 ±0.072^{e}	2.25 ±0.088^{a}
4*	11.67±0.333^{e}	0.38 ±0.009^{e}	2.25 ±0.075^{f}	2.29 ±0.116^{a}
5**	42.33 ±1.453^{c}	1.12±0.009^{c}	5.47 ±0.120^{c}	0.45 ±0.115^{c}
6**	78.67±0.882^{a}	3.30±0.115^{a}	7.93±0.088^{a}	0.13±0.009^{e}
7**	64.00 ±1.154^{b}	2.67 ±0.088^{b}	6.40±0.153^{b}	0.28 ±0.009^{d}
8**	46.00 ±1.154^{c}	1.16±0.067^{c}	6.37±0.088^{b}	0.43 ±0.012^{c}

Means within the same column carrying different superscripts are significant at p value p≤ 0.05.
* Groups non-infected and exposed to 0, 0.53, 0.265, 0.132 mg/L of UIA respectively.
** Groups infected with *Clostridium perfringens* type A and exposed to 0, 0.53, 0.265, 0.132 mg/L of UIA, respectively.

Results and Discussion

Mortality rate

The highest mortality rate was 80% in group (6) followed by 70% in group (7) and 60% in groups (5 and 8) (Table 1). These results might be due to the elevated UIA concentrations which resulted in increased susceptibility of fish to bacterial infection.

The results were nearly similar to those reported in wild Klunzingeri mullet (*Liza klunzingeri*) where massive mortalities and increased susceptibility to *Streptococcus agalactiae* infection were resulted from exposure of wild Klunzingeri mullet to high ammonium concentrations [20]. Also, ammonia caused mortality of Taiwan abalone (*Haliotis diversicolor supertexta*) due to *Vibrio parahaemolyticus* infection [21]. However, the results were dissimilar to those showed in Lost River suckers (*Deltistes luxatus*) where increasing UIA concentrations caused increasing survival of Lost River suckers (*Deltistes luxatus*) exposed to *Flexibacter columnare* [22]. Also, Farmer *et al.* [23] reported that high ammonia concentrations lowered the impact of bacterial infection. The mortality rates were 25% and 10% in groups (2 and 3), respectively, while no mortalities were observed in groups (1 and 4) (Table 1).

These results could be attributed to the rapid penetration of UIA molecules to gill and tissue membranes, resulting in mortalities [24]. Our results were nearly similar to those mentioned by Voslářová *et al.* [25] who reported that accumulation of organic matter led to the release of ammonia into the water causing diseases and mortality in fish. In addition, Francis-Floyd *et al.* [26] reported that if ammonia presented at elevated concentrations, it was a killer.

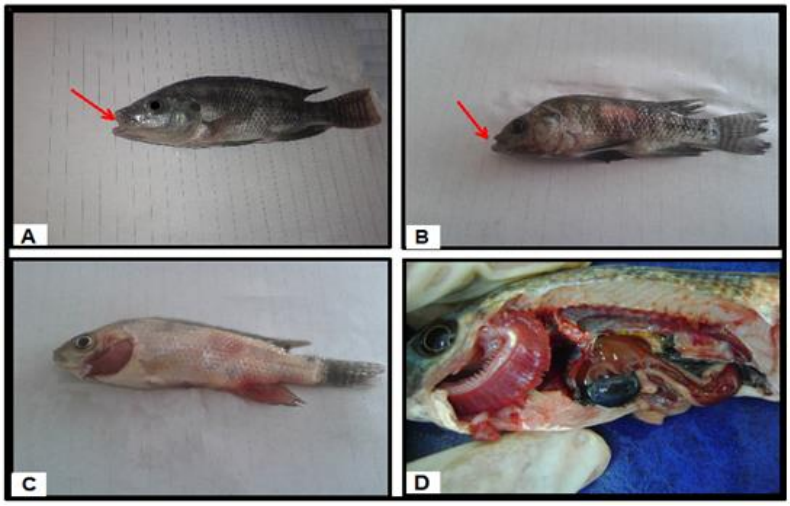

Figure 1: (A) *Oreochromis niloticus* of group (2) exposed only to 0.53 mg/L of UIA showed body darkeness and died with open mouth. (B-C) *Oreochromis niloticus* of groups (6 and 7) challenged with *Clostridium perfringens* type A and exposed to 0.53 and 0.265 mg/L of UIA respectively showed dark body coloration, fin rot, hemorrhages on skin and died with open mouth (B) with increasing of mucus secretion at skin and gills (C). (D) Postmortem findings of groups (6 and 7) showed congestion of the all internal organs including gills and liver appeared friable with enlarged gall bladder with bile.

Clinical signs and postmortem findings

Group (2) showed loss of equilibrium, nervous manifestation, body darkness and fish mortality occurred with open mouth (Figure 1A). These signs might be due to that UIA molecule was toxic, rapidly penetrated gill and tissue membranes and impeded central nervous system function, leading to nervous signs [24]. These results were nearly similar to those reported by Harper and Wolf [27] and EL-Shebly and Gad [28].

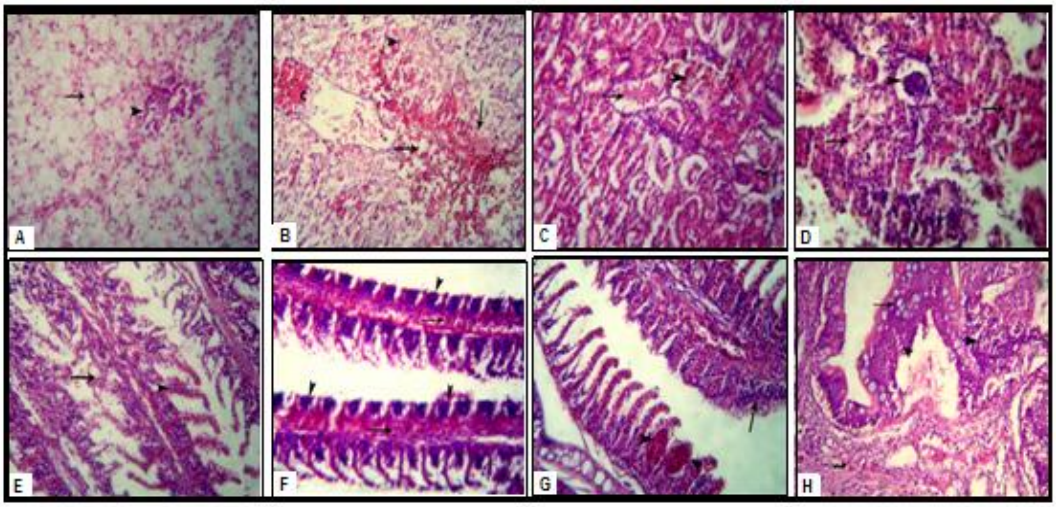

Figure 2: (A) Liver of group (2) exposed only to 0.53 mg/L of UIA showed diffuse vacuolation (arrow) and area of necrosis in the hepatocytes (arrowhead).(B) Liver of group (6) challenged with *Clostridium perfringens* type A and exposed to 0.53 mg/L of UIA showed coagulative necrosis (arrowhead), edema and hemorrhage around the portal area (arrows) (H&E x 400).(C) Kidney of group (2) exposed only to 0.53 mg/L of UIA showed mild coagulative necrosis (arrow) surrounded by melanomacrophages (arrowhead). (D) Kidney of group (6) challenged with *Clostridium perfringens* type A and exposed to 0.53 mg/L of UIA showed coagulative necrosis in the renal tubules (arrow) and severe shrunken glomerular tufts (arrowhead) (H&E x 400). (E) Gills of group (2) exposed only to 0.53 mg/L of UIA showed congestion of lamellar capillaries (arrowhead) and severe desquamation of lamellar epithelium (arrow). (F) Gills of group (6) challenged with *Clostridium perfringens* type A and exposed to 0.53 mg/L of UIA showed severe necrosis in the lining epithelium (arrowheads) and hemorrhage (arrow). (G) Gills of group (7) challenged with *Clostridium perfringens* type A and exposed to 0.265 mg/L of UIA showed severe congestion of lamellar capillaries and aneurysm (arrowheads) (H&E x 200) (H) Intestine of group (6) challenged with *Clostridium perfringens* type A and exposed to 0.53 mg/L of UIA showed catarrhal enteritis with severe mucinous degeneration in the lining epithelium (arrow) and lymphocytes infiltration in sub mucosa (arrowheads) (H&E x 400).

Groups (6 and 7) showed restlessness, rapid movement, loss of equilibrium, nervous manifestation, effort to swallow air from the water surface and mortality occurred with open mouth. In addition, the fish showed dark body coloration, fin rot and hemorrhages on body with increased mucus secretion in the skin and gills (Figure 1B and C).

The postmortem findings showed congestion of all internal organs including gills. The liver appeared friable with enlarged gall bladder with bile (Figure 1D). These signs could be attributed to the exposure of fish to ammonia resulting in more susceptibility of the fish to bacterial infections, which in turn triggered the bacteria to produce the toxins and cause these signs [26]. The results were nearly similar to those observed in white shrimp (*Litopenaeus vannamei*) that were exposed to ambient ammonia-N at 11.13 mg/L where its susceptibility to *Vibro alginolyticus* was increased [29].

Biochemical and immunological aspects

The levels of serum ALT and creatinine were significantly increased in group (2) compared with groups (1, 3 and 4). Also, there were significant increases in groups (6 and 7) compared with groups (5 and 8) (Table 2). These results might be due to the destruction of liver and kidneys, resulted from elevated levels of UIA and production of toxins by bacteria [7]. The results were nearly similar to those mentioned by El-Sherif and EL-Feky [30] who reported that UIA was toxic to fishes and led to liver and kidney damage.

There was a significant increase in serum cortisol level in groups (2 and 3) compared with groups (1 and 4). Also, there was a significant increase in groups (6, 7 and 8) compared with group (5) (Table 2). The increase in cortisol level might be due to stress from high levels of UIA and *Clostridium perfringens* infection.

The results were nearly similar to those observed by Harper and Wolf [27] who reported that acute ammonia toxicity resulted in elevations of plasma cortisol.

Serum IgM was significantly decreased in group (2) compared with groups (1, 3 and 4). Also, there was a significant decrease in groups (6 and 7) compared with groups (5 and 8) (Table 2). These could be attributed to high UIA concentrations, which in turn, increased the susceptibility to bacterial infection and resulted in impairment of the immune mechanism [7].

Our results were nearly similar to those reported in Taiwan abalone (*Haliotis diversicolor supertexta)* infected with *Vibro parahaemolyticus* where the depression in immune parameters was resulted from the exposure to ammonia [21].

Histopathological findings

The histopathological findings in the liver of group (2) showed diffuse vacuolation and area of necrosis in the hepatocytes (Figure 2A). While the liver of group (6) showed coagulative necrosis associated with edema, hemorrhage and intense leukocytes infiltrations (Figure 2B), however, the liver of group (7) showed severe individualization and perivascular coagulative necrosis in the hepatocytes.

The kidney of group (2) showed mild coagulative necrosis surrounded by melanomacrophages and vacuolation in the renal tubular epithelium (Figure 2C). While the kidney of group (6) revealed coagulative necrosis in the renal tubules and severe shrunken glomerular tufts (Figure 2D).

The gills of group (2) showed congestion of lamellar capillaries and marked desquamation of lamellar epithelium (Figure 2E). While the gills of group (6) showed proliferation and massive necrosis in covering epithelium with hemorrhage and round cells infiltrations (Figure 2F) but the gills of group (7) showed severe congestion of lamellar capillaries and aneurysm (Figure2G).

The intestine of group (2) was normal but there were few lymphocytes infiltration and edema in the lamina propria. While in group (6) catarrhal enteritis with severe mucinous degeneration in the lining epithelium and lymphocytes infiltration in the sub mucosa

were observed (Figure 2H). Also, few lymphocytes infiltration and edema in the lamina propria were recorded in the intestine of group (7). These lesions might be attributed to the high levels of UIA and toxins of *C. perfringens* type A which were motivated by high levels of UIA. Our results were nearly similar to that mentioned by EL-Shebly and Gad [28] and Hegazi [31].

Conclusion

In conclusion, the elevated levels of UIA increased the virulence of *Clostridium perfringens* type A infection in *Oreochromis niloticus* and affected the health and survival of *Oreochromis niloticus*. Therefore, measures should be taken to control ammonia in aquaculture systems to reduce the virulence of the pathogen and economic losses.

Conflict of interest

None of the authors have any conflict of interest to declare.

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الملخص العربى

تأثير الأمونيا الغير متأينة على القدرة المرضية للكلوستريديم بيرفرينجينس فى البلطي النيلي

زينب مصطفي البوهي، جمال النوبي احمد، محمد السيد حسانين، عفاف نورالدين عبدالرحمن

قسم أمراض ورعاية الاسماك، كلية الطب البيطري، جامعة الزقازيق

أجري هذا البحث لدراسة تأثيرالأمونيا الغير متأينة على معدل النفوق والأعراض المرضية والصفة التشريحية وبعض العوامل البيوكيميائية والمناعية والتغيرات الهستوباثولوجية لأسماك البلطى النيلي المعدية معمليا بميكروب الكلوستريديم بيرفرنجينس نوع (أ). وقد تم إستخدام ١٦٠ سمكة من أسماك البلطى النيلي بمتوسط وزن ٤٥ ± ٥ جرام مقسمة إلى ٨ مجموعات متساوية ، تم الحقن عن طريق التجويف البريتونى للمجموعات (١-٤) بجرعة ٠.٢ ملى لكل سمكة بالوسط المغذى المعقم والمجموعات (٥-٨) بجرعة ٠.٢ ملى لكل سمكة بميكروب الكلوستريديمبيرفرنجينس نوع (أ) ٠.٥ x ١٠^{٧} سى اف يو ثم تم إستخدام المجموعات (١و٥) كمجموعات ظابطة (غير معرضة للأمونيا الغير متأينة) في حين تم تعريض المجموعات (٢و٦) ،(٣و٧) ،(٤و٨) لتركيز ٠.٥٣ و ٠.٢٦٥ و٠.١٣٢ ملى جرام / لتر من الأمونيا الغير متأينة. وأوضحت النتائج أن بلغ أعلى معدل نفوق ٨٠% و٧٠% بين الأسماك المصابة والمعرضة لتركيز ٠.٥٣ و ٠.٢٦٥ ملى جرام / لتر على التوالى. وكان من أهم الأعراض الاكلينكية التى ظهرت على الأسماك هى ظهور بعض الأعراض العصبية وصعوبة فى التنفس وغالبا ما يحدث نفوق وهى فاتحة الفم. هذا بالاضافه إلى وجود دكانة وأنزفة دموية بالجسم ووجود كمية كثيفة من المخاط على الجلد والخياشيم. وقد أسفر إنزيم الكبد والكرياتينين وهرمون الكورتيزول عن زيادة معنوية بينما أسفرالأميونوجلوبيولين م عن إنخفاض معنوى كما سجلت التغيرات الهستوباثولوجية فى أنسجة الكبد والكلى والخياشيم والأمعاء . من ذلك إتضح أن المستويات المرتفعة من الأمونيا الغير متاينة أدت إلى زيادة القدرة المرضية للكلوستريديم بيرفرينجينس فى البلطى النيلي.