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BEHAVIOURAL, CHROMOSOMAL ABERRATIONS AND HEMATOLOGICAL CHANGES ASSOCIATED WITH ACUTE NITRITE TOXICITY IN AFRICAN CATFISH "*CLARIAS GARIEPINUS*"

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SUMMARY

This investigation presents the results of acute toxicity in *Clarias gariepinus* (*C. gariepinus*) exposed to median lethal dose of nitrite (58 mg/L) for 1, 2 and 4 days. The 96 hr LC_{50} of nitrite for *C. gariepinus* was determined. Behavioural changes, as well as the developing lesions in nitrite-treated fish were recorded. Hematological changes and chromosomal aberrations induced were also observed. The results revealed that, the nitrite-treated fish developed a significant decrease in erythrocyte population, total leukocyte count, hematocrite value and hemoglobin content. The percent of methemoglobin was highly significant increased in all nitrite-treated groups compared with controls. Serum cortisol was significantly higher in nitrite-treated fish for 4 days compared with control. Different types of chromosomal aberrations were observed as break, fragement, deletion, end to end, endomitosis and centromeric attenuation. The total aberrations were significantly increased in all nitrite-treated fish compared with controls. Addition of sodium chloride (25 mg sod. chloride / 1 mg nitrite) or transferring of fish to freshwater after 24 hr of exposure treat methemoglobinemia. Hemoglobin content and methemoglobin percent of recovered fish in both treatments returned nearly to the normal level within 24 hr post treatment.

INTRODUCTION

Nitrite toxicity became widely recognized as a common problem in pond rearing of salmonids (Lewis and Morris, 1986) and of catfish (Tucker et al., 1989). Nitrite is an intermediate product of nitrification, denitrification and transformation of

ammonium salts to nitrite and nitrate (Arrignon, 1999) and is considered one of the most important inorganic nutrients often recorded at higher level in intensively rearing facilities. The toxic level of nitrite developed in cultured ponds with excess fertilization (Hargreaves, 1998); sudden massive death of planktons (Soderberg, 1995); insufficient, inefficient and malfunctioning biofiltration system (Michael et al., 1999) and high stocking and feeding rates (Schwedler and Johnson, 2000). Toxicity is primarily due to nitrite ability to oxidize the iron in the hemoglobin from a ferrous to a ferric state forming methemoglobin (Grant et al., 1987) which is not capable of carrying oxygen leading to functional hypoxia (Tomasso, 1994 a). Methemoglobinemia (brown blood disease) is considered an important signs of nitrite poisoning and has been observed in Clarias lazera (Hilmy et al., 1987 and Mohamed and Saleh, 1996), Ictalurus furcatus (Schoore et al., 1995); Anguilla anguilla (Kamestra et al., 1996) and Oreochromis nloticus (Galal, 1999 and Atwood et al., 2001 a). There is no or even very little available literature about genotoxic effect of nitrite in fish. Sodium nitrite has been shown to be mutagenic and induces chromosomal aberrations in bone marrow of adult albino rats and in liver cells of transplacentally exposed embryos (El-Nahas et al., 1984). Also, invitro studies revealed that nitrite has a mutagenic effect in hamster cells (Tsuda and Kato, 1977) and peripheral blood lymphocytes in children (Tsezou et al., 1996). The present work was planned to determine the 96 hour LC_{50} of nitrite for *C. gariepinus* as well as to high lights its adverse impact on fish behaviour, some hematological parameters, serum cotrisol and frequency of chromosomal aberrations caused by acute nitrite toxicity. Also, application of sodium chloride and freshwater as a treatment trials for methemoglobinemia have been taken in consideration.

MATERIALS AND METHODS

Fish:

A total number of 131 clinically normal males *C. gariepinus* weighted 160 ± 10 g were obtained from a private fish farm at Kaluobia Governorate. The fish were transported in large tanks to the Wetlab at Fac. of Vet. Med., Moshtohor where they were kept in glass aquaria for 7 days at 24 ± 1°C to be acclimated to lab conditions prior to the experiment. They received commercial pellets according to Eurell et al. (1978). The fish were divided into 18 groups, the first nine groups each of 7 fish, while the second 6 groups each of 6 fish and the last four groups each of 8 fish. Fish excreta and uneaten food were aspirated regularly. The fish were fasted two days prior and during the period of experiment to avoid the undesirable effect of excreta and feed (Halte, 1986).

Aquaria used:

Clean glass aquaria were used for holding fish during the experimental period. Each aquarium measured 1 x 0.5 x 0.5 m, supplied with dechlori-

nated tap water (Innes, 1966) and sufficient aerators (Rena, Italy) to maintain an adequate water aeration.

Chemicals used:

Sodium nitrite (Technolab Lab Chemicals) is used to produce the required concentration of nitrite.

Sodium chloride (Almania Company for Chemicals) is used for treatment of nitrite toxicity.

Experimental design:

(1) Determination of 96 hr LC_{50} of nitrite:

Nine groups of fish each of 7 fish were used, the first 8 groups were exposed to 10, 20, 30, 40, 50, 60, 70 and 80 mg/L nitrite respectively, while the remaining 9th group was left as non-exposed control. The number of dead fish in each group was counted at 96 hr post exposure. Daily water change and reconstitution of nitrite levels were carried out. The 96 hr LC_{50} value of nitrite was estimated as described by Sprague (1969).

(2) Acute toxicity:

For studying the acute nitrite toxicity, four groups of fish were used each of 6 fish. The fish were exposed to the 96 hr LC_{50} of nitrite (Espey, 2001). The first 3 group were exposed to 58 mg/L nitrite for 1, 2 and 4 days respectively, while the fish in the 4th group was used as control. A constant nitrite concentration was maintained through daily water change and reconstitution of nitrite levels.

Clinical examination:

The exposed and control fish were subjected to clinical examination as described by Amlacker (1970). The behavioural changes as well as lesions observed in nitrite-treated fish were recorded.

Hematological changes:

For hematological examination, blood samples were taken in heparinized tubes from caudal veins of 6 fish per each group pre-exposure and on days 1, 2 and 4 post exposure to nitrite as described by McKnight (1966). The number of erythrocytes and leukocytes were counted according to Lehmann and Sturenberg (1974). The hemoglobin content and hematocrit value were determined as mentioned by Michael (1993). The methemoglobin as a percent of total hemoglobin was determined by reading at 630 mμ on scanning spectrophotometery according to Lewis et al. (2001). Moreover, serum cortisol was also estimated by using immuno radiometric assay (IRMA) according to Wilson and Miles (1977).

Cytogenetic examination:

For studying the developed chromosomal aberrations, all fish in each groups were sacrificed pre- and on 1st, 2nd and 4th days post exposure. Three hours before sacrificing, the fish were injected intraperitonialy with 0.05% colchicines in a dose of 1 ml per 100 g of fish. Squash techniques from kidneys tissue were used for preparations of metaphases spread as described by

Al-Sabti et al. (1983). For every fish at least 50 metaphase spreads were examined and the chromosomal aberrations were recorded.

(3) Treatment trials of methemoglobinemia:

For studying the effectiveness of sodium chloride in treatment of nitrite toxicity four groups each of 8 fish were used. The fish in the first group were used as a control. The fish in the second group were exposed to 58 mg/L nitrite along the time of experiment. The fish in the 3rd group were exposed to nitrite (58 mg/L) for 24 hr then received sodium chloride in a rate of 25 mg to each 1 mg nitrite (Welborn and Schwedler, 1980). While, the fish of the 4th group were exposed to 58 mg / L nitrite for 24 hr then were transferred into nitrite free freshwater (Huey et al.. 1980). All treated and control groups were observed for 7 days. Behavioural changes and mortality rate were recorded. Blood samples were taken from control as well as treated groups after exposure for 24 hr to nitrite only, nitrite sodium chloride mixture and fresh water for estimation of hematocrite %, RBCs and WBCs counts, hemoglobin and methemoglobin % as previously described. Statistical analysis of data was followed using students "t" test according to Snedecor and Cochran (1967).

RESULTS AND DISCUSSIONS

Determination of 96 hr LC_{50}:

In the present study, the 96 hr LC_{50} of nitrite for *C. gariepinus* was 58 mg/L nitrite. These observations were nearly similar to those recorded by Galal (1999) who found that 96 hr LC_{50} for nitrite in *O. niloticus* was 60 mg/L. The obtained level was higher than that observed by Hilmy et al. (1987) who recorded that 96 hr median tolerance limit of nitrite was 28 and 32 mg/L for 65 and 166 g *Clarias lazera*. On the other hand, the 96 hr LC_{50} value of nitrite for green sunfish and blue gill bass were 160.4 and 9.9 mg/L, respectively (Tomasso, 1986); yellow perch, *Perca flavescens* was 12.8 ± 1.6 mg/L (Espey, 2001), *O. niloticus* was 81 mg/L (Atwood et al., 2001a) and Sunonthern flounder was 72.5 mg/L (Atwood et al., 2001b). The difference in LC_{50} value may be attributed to fish species, rate of nitrite uptake and activity of methemoglobin reductase enzyme (Stoskopf, 1993) as well as size variance (Bartlett and Neumann, 1998).

Acute toxicity of nitrite:

Behavioural abnormalities:

The results revealed that, the fish exposed to the median lethal dose (58 mg/L) of nitrite showed abnormal swimming behaviour as surface swimming, gasping and hanging vertically in water then become less active and of pale gray colour. Finally the fish became motionless and lied on the bottom. These findings are more or less simi-

lar to those observed by Hilmy et al. (1987) and Mohamed and Saleh (1996). In the same respect similar findings were also recorded by Almendras (1987), Robert et al. (1997) and Pullium et al. (1999). Signs of asphyxia observed on exposed fish may be attributed to reduction of the oxygen carrying capacity of the blood by oxidizing the hemoglobin and reduction of the oxygen affinity induced by nitrite (Williams et al., 1993). Lesions developed were clear in 96 hr treated group including, excess mucus allover the skin, eroded fins, wide open mouth in one dead fish, dark brown blood and brown coloration of gills and all internal organs. These observations were partially in agreement with that observed by Hilmy et al. (1987) and Mohamed and Saleh (1996) in C. lazera and by Galal (1999) in *O. niloticus*. Moreover, the lesions observed in this study were also more or less similar to those reported by Stoskopf (1993); Noga (1996) and Williams et al. (1997). Brown coloration of the internal organs and blood may be due to conversion of hemoglobin to methemoglobin causing hemolytic anemia that acquire a reddish brown color to plasma (Williams and Eddy, 1988).

Hematological changes:

As shown in Table (1), the present investigation revealed a significant decrease in erythrocytes number (RBCs) in treated fish particularly at 4 days post-exposure. The total leucocytic count (WBCs) was significantly decrease in exposed fish particularly at 2nd day of exposure. Hematocrit value in all treated groups was significantly decreased than in control fish. These observations were nearly agreed with that reported by Badawi (1995), Mohamed and Saleh (1995), Galal (1999) and Pratap et al. (2004). On the other hand, Woo and Chiu (1995) recorded non significant changes in hematocrit value and red blood cells count in sea bass exposed to high nitrite concentration.

Hemoglobin concentration was significantly decreased in treated fish compared with control group. Methemoglobin as a percent of total hemoglobin was significantly increased in treated fish and the maximum level (78.52 ± 1.94) was recorded at 4 day post exposure. These results were similar with that recorded by Hilmy et al. (1987) and Mohamed and Saleh (1996) in C. lazera, by Badawi (1995) and Atwood et al. (2001a) in *O. niloticus*, by Duncan et al. (1999) in Amazonian catfish, by Grosell and Jensen (2000) in *Platichthys flesus* and by Huertas et al. (2002) in Siberian sturgeon Acipenser baeri. The percent of methemoglobin recorded in this investigation was lower than that detected by Wellborn and Schwedlar (1980) and Huertas et al. (2002) and higher than that observed by Duncan et al. (1999) and Grosell and Jensen (2000). This may be due to difference in fish size (Barteltt and Neumann, 1998) and fish species (Schoore et al., 1995). The control fish were found to have a measurable percent of methemoglobin (11.31 ± 0.66%) this due to normal autoxidation of oxyhemolobin to methemoglobin (Jensen, 2001).

Serum cortisol was significantly increased in fish exposed to nitrite for 4 days (16.22 ± 0.3 ug/dL) than in control group (8.36 ± 0.3 ug/dl). This finding came in accordance with that recorded by Tomasso (1994b) and Carballo et al. (1995) who described that exposure of fish to high nitrite level stimulate the release of cortisol into the blood circulation. The higher level of cortisol induced during acute nitrite toxicity may disturb the immune defense mechanism. This opinion was supported by the findings of Noga (1996), Robert et al. (1998) and Pullium et al. (1999) who revealed that fish exposed to high concentration of nitrites developed outbreak of columnaris disease and streptococcosis.

Cytogentic changes:

As presented in Table (2), examination of metaphase spread from the anterior kidney cells of *C. gariepinus* revealed that nitrite toxicity induced different types of chromosomal aberration such as break, fragment, deletion, end to end, endomitosis and centromeric attenuations. The total aberrations were significantly increased in all

Table (1): Selected hematological parameters and serum cortisol of *C. gariepinus* exposed to 58 mg/L nitrite.

Parameters	Control M ± SE	Days post-exposure		
		M ± SE One day	M ± SE 2 days	M ± SE 4 days
Erythrocytes (10^6/mm^3)	2.57 ± 0.035	1.76 ± 0.036***	1.65 ± 0.35***	1.63 ± 0.038***
Total leukocytes (10^4/mm^3)	1.34 ± 0.02	0.99 ± 0.04***	0.89 ± 0.02***	0.92 ± 0.03***
Hemoglobin (mg/dL)	6.75 ± 0.17	5.02 ± 0.16*	4.75 ± 0.19***	4.50 ± 0.19***
Hematocrit (%)	32.17 ± 0.55	23.42 ± 0.43***	20.42 ± 0.18***	20.5 ± 0.46***
Methemoglobin (%)	11.31 ± 0.66	68.77 ± 3.75***	70.76 ± 0.94***	78.52 ± 1.94***
Serum cortisol (ug/dL)	8.36 ± 0.30	--	--	16.22 ± 0.3****

* Significant at P > 0.05 ** Significant at P > 0.01 *** Significant at P > 0.001

nitrite-treated fish particularly at 2nd and 4th days post-exposure compared with control group. Fragments (Fig. 1Bf) were non significantly increased at 1st and 2nd days post-exposure, but significantly increased in fish exposed for 4 days. Deletions (Fig. 1Bd) were significantly increased in all exposed fish particularly at 4 day of exposure. Centromeric attenuations (Fig. 1Bc) were non significantly increased in all exposed groups compared with control. End to end associations (Fig. 1C) were significantly increased at first day and highly significant increase at 4 days post-exposure. Endomitosis type of aberrations (Fig. 2E) were significantly increased at 1st day and very highly significant increase in fish exposed for 4 days. Breaks (Fig. 2D) were non significantly increased in nitrite-treated group at 1st and 2nd day, but were highly significant increased in fish at 4 day post-exposure. These observation were supported by the findings of El-Nahas et al. (1984) who found that sodium nitrite resulted in chromatid break gab, centric fusion and dicentric types of aberrations in bone marrow cells of adult female rats and liver cells of transplacentally exposed embryo. Moreover, the results were in consistent with in vivo studies indicating that nitrite induced chromosomal aberrations in male rat, mice and rabbit (Luca et al., 1987) and in germ cells of male mice (Alavantic et al. 1988). The recorded higher frequency of total chromosomal aberrations in the exposed group may attributed to the direct exposure to nitrite.

Table (2): Chromosomal aberrations in *C. gariepinus* exposed to 58 mg/L nitrite.

Time of exposure	No. of fish per group	No. of examined cells	Chromosomal aberrations						
			Fragment M ± SE	Deletion M ± SE	Centromeric attenuation M ± SE	End to end M ± SE	Endomitosis M ± SE	Break M ± SE	Total chromosomal aberrations M ± SE
Pre-exposure "control"	6	300	00.50± 0.31	0.33± 0.19	1.33± 0.45	0.00± 0.00	0.17± 0.15	1.33± 0.38	3.67± 0.87
One day Post-exposure	6	300	00.50± 0.20	2.0± 0.53*	2.17± 0.44	1.17± 0.44*	1.00± 0.33*	1.33± 0.38	8.33± 0.99**
2 days post-exposure	6	300	001.33± 0.45	3.00± 0.75**	1.50± 0.39	1.67± 0.38**	2.83± 0.44***	2.17± 0.44	12.50± 1.02***
4 days post-exposure	6	300	3.17± 0.96*	4.33 ± 0.56***	2.67± 0.65	3.5± 0.57***	3.67± 0.45***	4.17± 0.86**	21.67± 1.61****

* Significant at P > 0.05 ** Significant at P > 0.01 *** Significant at P > 0.001

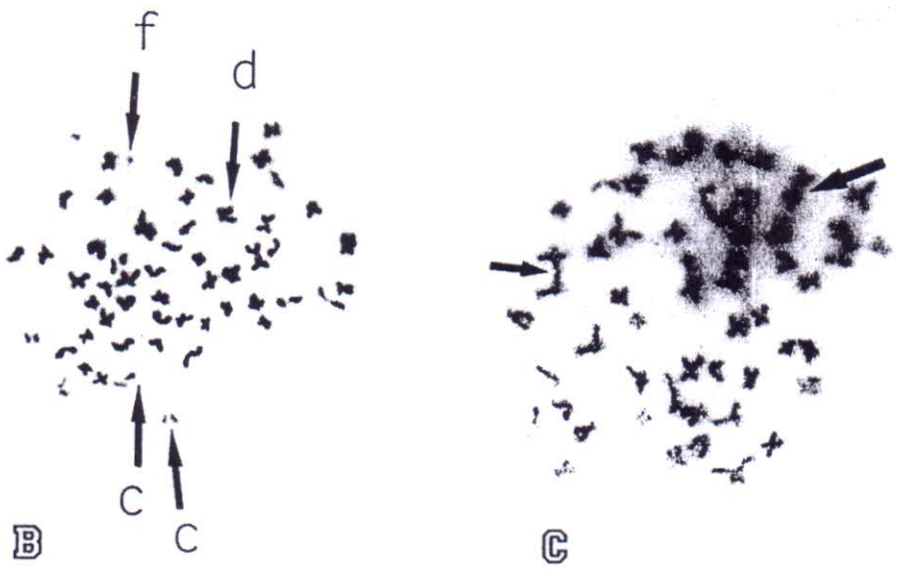

Fig. (1): Metaphase spread from anterior kidney cells of *C. gariepinus* exposed to nitrite showing, fragment (B f) deletion (B d) and centromeric attenuation (B c) and end to end (C) (arrow).

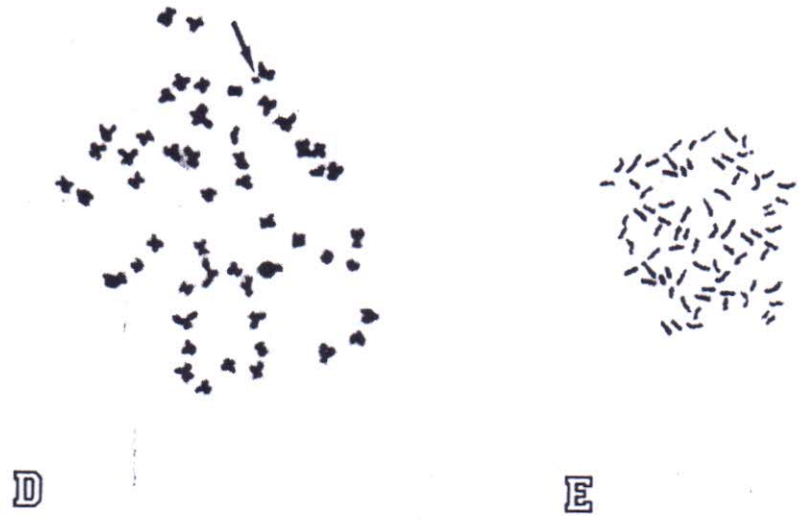

Fig. (2): Metaphase spread from anterior kidney cells of *C. garipeinus* exposed to nitrite showing: break (D) (arrow) and endomitosis (E).

Treatment trials of methemoglobinemia:

The present investigation revealed that, addition of 25 mg NaCl for each 1 mg nitrite after 24 hr from start of exposure to 58 mg/L nitrite has proven to be an effective method for treatment of methemoglobinemia and could prevent mortalities. Hemoglobin and methemoglobin % (6.37 ± 0.09 mg/dL and 11.5 ± 0.83%, respectively) were returned nearly to the normal levels after 24 hr from beginning of treatment. Moreover, hematocrite % (28.83 ± 1.4%), RBCs (2.12 ± 0.08 x 10^6/mm^3), WBCs (1.25 ± 0.04% x 10^4 /mm^3) of the sodium chloride-treated group were also improved. While, fish exposed for 58 mg/L nitrite allover the experimental period showed typical signs of methemolobinemia with mortality rate of 87.5%. These observations nearly agreed with that recorded by Wellborn and Schwedler (1980), Hilmy et al. (1987), Tuker et al. (1989), Mohamed and Saleh (1996) and Atwood et al. (2001a). These findings may be attributed to that the chloride ions competitively inhibits the nitrite uptake across the gills thus allowing the enzyme reductase system in RBCs to convert methemoglobin back to hemoglobin (Hilmy et al., 1987). Also, the increase in the number of RBCs after addition of NaCl in this study elevated the total hemoglobin content. The addition of chloride to freshwater reduced the accumulation of nitrite in plasma (Weirich et al., 1993) and the loss of plasma nitrite is closely followed by disappearance of methemoglobin but plasma nitrate level decrease more quickly than methemoglobin (Schoore et al., 1995). In addition, the results of the present study showed that, fish transferred after 24 hr of exposure to freshwater had normal behaviour, and their hemoglobin and methemoglobin % (6.3 ± 0.06 mg/dl and 11.4 ± 0.18%, respectively) nearly return to the normal level. Moreover, hematocrite % (26.98 ± 0.12%), RBCs (2.01 ± 0.06 x 10^6/mm^3) and WBCs (1.09 ± 0.025 x 10^4/mm^3) of the freshwater-treated fish were elevated. The obtained results were similar with that recorded by Huey et al. (1980); Almendras (1987), Mohamed and Saleh (1996) and Galal (1999) they showed, recovery of nitrite poisoned fish and their methemoglobin returned near the normal level within 24 hr after transferring of fish to nitrite free water.

In conclusion, nitrite is a potential mutagenic chemical agent as indicated by high frequency of total chromosomal aberrations and it may have immunosuppresive effect through elevation of serum cortisol. Also, it induced methemoglobinemia and changes in almost all the hematological parameters. Therefore, special arrangement must be needed for the management of this in organic nutrient in fish rearing facilities especially with intensive culturing system.

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