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HAEMATOLOGICAL AND IONIC ALTERATIONS IN *OREOCHROMIS MOSSAMBICUS* (PETERS) INDUCED BY PHENOLIC COMPOUNDS

Remya Varadarajan, Jisha Jose, Hari Sankar, H.S and Babu Philip*

Department of Marine Biology, Microbiology & Biochemistry, Cochin University of Science & Technology, Fine Arts Avenue, Cochin – 16, Kerala, India

**Email: drbabuphilip@gmail.com*

Abstract: Phenolic compounds are a group of wide-spread aquatic pollutants. *Oroechromis mossambicus* were exposed to sub lethal (1/10th of 96 hr LC50) concentration of phenol (3.12 mg l-1) and m-cresol (2.2 mg l-1) for a period of three weeks by semi-static test system. Important haematological parameters and serum ions were experimented after the exposure period. Significantly ($P<0.05$) increased level of haemoglobin, red blood cell count and haematocrit were observed. Also significant ($P<0.05$) decrease in serum sodium ions and an increase in potassium ions suggest that phenolic compounds can influence osmoregulation. The blood responses apparently indicate adaptation to hypoxic conditions arising from prooxidants, gill degradation and perhaps oxygen - level fluctuations.

Key words: Phenolic compounds, toxicity, haemoglobin, haematocrit, RBC count

INTRODUCTION

The existence of phenols and their derivatives in the aquatic environment is undesirable because of their toxicity to aquatic organisms. Phenolic compounds are common constituents of aqueous effluents from the industrial processes such as resin production, oil refining and cooking plants Kibret *et al.,* 2000). Among the different phenolic compounds, phenols and cresols are widely used organic solvents. These compounds have been identified in water- soluble fractions of oil since they are potential degradation products of aromatic hydrocarbon metabolism. Phenol is among the first compound described as toxic by the Environmental Protection Agency - United States and due to its relevance as an ecotoxin it has been maintained in the priority list (USEPA, 1977). Phenol is a metabolite of a widely used organic solvent-benzene. Creosol is classified as a hazardous substance for occupational exposure (ChemWatch, 2006). Crude cresol (commercial grade) contains approximately 20% o-cresol, 40% m-cresol, and 30% p-cresol. m-cresol is used to produce certain herbicides, as a precursor to the pyrethroid insecticides, to produce antioxidants, and to manufacture the explosive, 2,4,6 trinitro-m-cresol (ATSDR,1990).

Blood is a pathophysiological reflector of the whole body and therefore, blood parameters are important in diagnosing the structural and functional status of fish exposed to toxicants (Adhikari *et al.,* 2004). Hematological parameters can provide satisfactory information on the physiological response of fish to environmental stressors for two major reasons, namely, the close association of the circulatory system with the external environment and the ease of availability of fish blood (Cazenave *et al.,* 2005; Houston, 1997; Lohner *et al.,* 2001).

Electrolytes of body fluids have various functions - the most important of which are to contribute a majority of the osmotically

active particles, to provide buffer systems and mechanisms for the regulations of pH (acid–base balance). In addition, they provide proper ionic balance for normal neuromuscular irritability and tissue functions. In the regulation of osmolarity of a system, sodium, potassium and calcium ions play significant roles to keep the hyper osmotic properties of freshwater fishes. Potassium is the main cation of the intracellular fluid and it is an important constituent of the extracellular fluid. Ion uptake from water is required to maintain internal acid–base balance and ionic equilibrium between blood and tissues for those ions that are continuously lost by diffusion across permeable parts of the external body surface (Burton, 1973).

The use of primary haematological indices such as haemoglobin (Hb), packed cell volume (PCV) and red blood count (RBC count) in assessing sub lethal concentrations of two different phenolic compounds are considered. As indicators of ion regulation, serum Na^+ and K^+ ion levels were also measured. These studies could be used to indicate the health status of fish as well as water quality.

MATERIALS AND METHODS

Chemicals

Analar monohydric phenol and m-cresol purchased from Sisco Research Laboratories (SRL), India were used. For all the experiments, desired concentrations of phenolic compounds were prepared from fresh stock solutions. All other chemicals used were analytical grade reagent purchased from Merck and Himedia (India).

Fish and maintenance conditions

The investigations were carried out in a fresh water fish *Oreochromis mossambicus* (15±3g) procured from the culture ponds of Kerala Agricultural University (Puduvypu), India. The water in the aquarium was renewed daily and was aerated mechanically. The average values of water quality parameters were as following: dissolved oxygen concentration of 7.8 ± 0.06 ppm, hardness below detectable amounts, pH 7.0 ± 0.37, temperature 26 ± 30C and salinity 0 ppt (parts per thousand) (APHA, 2005). The fishes were fed on a commercial diet ad libitum and were maintained in tanks for a month before the experiment in order to acclimate to the experimental system.

Sub lethal toxicity studies

The acclimated fishes (15±3 g) were divided into three groups; Group 1 exposed to sub lethal phenol concentration (3.12 mg l-1 corresponding to 10% of the LC50-96h) for 21 days. Group 2 exposed to sub lethal m-cresol concentration (2.2 mg l-1 corresponding to 10% of the LC50-96h) for 21 days and, Group 3 control groups which were subjected to the same protocol, but without phenolic compounds. Triplicates were kept for both the treated groups and respective controls. The exposure media was replaced every 24h (semi-static –with the same concentrations of phenolic compounds) so as to maintain constant toxin concentration throughout the study period.

Collection of blood

Blood was drawn directly from the cardinal vein using 1 ml plastic syringe containing 0.2% EDTA as the anticoagulant.

Parameters investigated

Estimation of Haemoglobin (Cyanmethae moglobin method)

Blood was drawn from the common cardinal vein and used for the estimation. 0.2 ml of blood was mixed with 5 ml of Drabkin's diluent solution and allowed to stand for 5minutes for the formation of cyanmethaemoglobin. Absorbance was measured 540 nm against a reagent blank, which consisted of 5 ml of Drabkin's diluent solution (Cook, 1985). Using haemoglobin standard, a standard calibration curve was prepared, from which the values of haemoglobin was calculated as g/dl.

Determination of other hematological parameters

The blood was analyzed using cell counter automated analyser (Celltak marketed by Pan Company in India) for the hematological parameters such as PCV (packed cell volume) and RBC count.

Estimation of serum ions

Serum ions were estimated by flame photometry. Using the stock solution of sodium (1000 mEq/l) and potassium (100 mEq/l), working standards of sodium/ potassium (120/2.0 mEq/l), sodium/ potassium (140/4.0 mEq/l), Sodium/ potassium (160/6.0 mEq/l) were prepared. Serum and the three working standards were diluted (0.1:10) in separate beakers. Gas flame was adjusted until the flame is divided into five sharp cones at a compressor pressure 12 lb/sq.inch. Proper filters were selected for the simultaneous determination of sodium and potassium.

Statistical analysis

The statistical analysis was carried out using the software SPSS 13.0 package. One-way analysis of variance (ANOVA) was done followed by Tukey's test in order to determine the significant difference between different treatments. All the data were presented as mean ± SD. and the differences were regarded as statistically significant when $P<0.05$.

RESULTS

The results obtained are shown in Table 1 and Table 2. Comparison between different treatments revealed that there was significant increase ($P<0.05$) in haemoglobin content, packed cell volume and RBC count in both phenol and m-cresol treated groups compared to control. Statistical analysis also revealed that m-cresol treated group showed the highest level among the treated groups. Serum Na^+ ion levels decreased significantly ($P<0.05$) on exposure to the toxins when compared to their respective controls. The serum K^+ ion level was significantly elevated ($P<0.05$) in both m-cresol and phenol treated groups.

DISCUSSION

Increased red blood cell count and haematocrit was observed on exposure to sub lethal concentrations of phenol and m-cresol. Erythropoiesis, whereby the number of red blood cells in the circulation gets increased is in fact a mechanism through which fish might compensate for poor oxygen uptake in prevailing hypoxic conditions (Wepener *et al.,* 1992). Another mechanism by which fish might compensate for low oxygen uptake during hypoxic conditions is via the release of a large number of mature red blood cells in the general circulation. This is thought to be stimulated by β-adrenergic action on the haemopoietic tissues, which contract and release stored mature red cells

Table 1. Effect of different phenolic compounds on haematological parameters in O. mossambicus. Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA)

Parameters	Control	Phenol	m-Cresol
Haemoglobin (g/dl)	8.0±0.511a	9.3±0.321b	9.0±0.25b
Packed cell volume (%)	17.2±0.93a	21±0.7b	21.6±0.87b
Total RBC count (millions/ cu.mm of blood)	2.4±0.13a	3.6±0.11b	4.0±0.09c

Table 2. Effect of different phenolic compounds on serum sodium and potassium levels in O. mossambicus. Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA).

Serum Na^+ (mEq/L)	161.25±2.11c	148±2.04b	134±2.97a
Serum K^+ (mEq/L)	3.5±0.34a	4.53±0.73c	4.13±0.53b

(Wepener *et al.,* 1992). This mechanism might, however, compensate for short-term variations in oxygen concentration in blood or water (Nespolo and Rosenmann, 2002). It has been found that the erythrocytes number and haemoglobin levels may vary with oxygen requirement (Hubrec *et al.,* 2000; Tavares *et al.,* 2004). Therefore, the increase of packed cell volume in *O. mossambicus* is likely to be due to either increased metabolic demand or gill damages resulting in impairment of oxygen transport, or both.

Two reasons can be attributed to the increased level of haemoglobin in both the groups exposed to phenolic compounds. One is, the presence of phenolics in water creates a high oxygen demand and the compensation by the organism for low dissolved oxygen content is by synthesizing more haemoglobin for binding more oxygen. Another reason that can be attributed is phenolic compounds or their toxic metabolites are oxidized to free radicals within erythrocytes and induce haemolysis of the erythrocyte membrane. As a consequence, haemoglobin is released which was shown as increased haemoglobin level. The haemoglobin released induces a multitude of toxic effects, summarized by Everse and Hsia (1997). The effects include nephrotoxicity through the formation of Hb dimers, formation of cross-linked haemoglobin, which induce heme oxygenase and liberation of the heme moiety. Other effects such as, cell damage, peroxidation, induction of phagocytosis and liberation of iron may also occur. The iron liberated itself serves as a prooxidant and increases the risk of bacterial infections (Nohl and Stolze, 1998).

Iron liberation from oxidatively modified haemoglobin or myoglobin was reported by Gutteridge (1986), Harel *et al.* (1988) and Rice-Evans *et al.* (1993) when drastic oxidizing conditions were applied such as hydrogen peroxide or lipid hydroperoxides. Since iron ions play an important role as redox catalysts (Fenton reaction, Haber-Weiss), iron liberation from erythrocytes will increase the total prooxidant effect of the xenobiotic.

The physiological function of haemoglobin is to transport oxygen to the tissues; this process depends on the ability of the ferrous form ($Hb2^{+}$) of reversible binding of molecular oxygen. However in the presence of xenobiotics, oxyhaemoglobin (oxy-Hb) is able to turn to methaemoglobin (met-Hb) (the $Hb3^{+}$ form), which is unable to transport oxygen. This conversion is associated with superoxide anion production (Misra and Fridovich, 1972) and hence with products such as hydrogen peroxide or hydroxyl radicals, which may be derived from superoxide anion itself.

Eyer *et al.* (1975) and Riley (1984) reported that xenobiotics such as phenolic compounds are able to oxidize oxy-Hb to met-Hb in a so-called co-oxidation reaction in which the haem oxygen serves as the active oxidant that oxidizes both the ferrous haem centre of haemoglobin and the reducing xenobiotic (R–H):

$$Hb^{2}+O_{2} + R–H \rightarrow [Met–Hb^{3}+–O–O^{2}–] + H+ + R\bullet$$

The unstable $Met–Hb_{3}+–O–O^{2}–$ complex (Gasyna, 1979) immediately stabilizes to secondary products that are dependent on the nature of the respective xenobiotic R–H: in the case of phenolic compounds (Stolze and Nohl, 1991, 1992), the transient formation of a compound l type ferryl haemoglobin has been postulated.

$$[TyrH\ Met–Hb^{3}+–O–O^{2}–] + H^{+} \rightarrow [Tyr\bullet\ Hb^{4+}=O^{2-}] + H_{2}O$$

The highly reactive free radical intermediates were found to attack SH groups on the haemoglobin molecule, at position β-93 (Maples *et al.,* 1990) or on the constituents of the erythrocyte membrane. (Feix and Butterfield, 1980; Wyse *et al.,* 1989a, 1989b; Hensley *et al.,* 1993; Butterfield *et al.,* 1994). Apart from the decrease in oxygen binding

capacity, the Met-Hb generators irreversibly destroy the haem proteins with which they were interacting, thereby releasing metabolites that may affect thiol-dependent bioactivities and functional membrane processes.

Also, a decrease in serum sodium ion levels and an increase in potassium ion levels in the groups exposed to low dose suggest that phenolic compounds can affect osmoregulation. A reduction in the major electrolyte sodium may be due to disturbances in the membrane permeability due to toxicity of phenolics which could have impaired the flux of ions. Freshwater fish tends to have a passive efflux of ions (loss) and a passive influx of water through the gill epithelium (McDonald and Milligan, 1997). To cope with the change in blood osmolarity, they have two main strategies: active uptake of ions through the gill using the Na^+K^+-ATPase and production of large volumes of diluted urine in the kidney, which can also actively uptake ions (Eddy, 1981; Marshall and Grosell, 2005; Iwama *et al.,* 2005). However, in the present investigation branchial Na^+K^+-ATPase activity was impaired which may have affected the ionic homeostasis. de la Torre *et al.* (1999) have shown that the inhibition of this enzyme by monocrotophos prevents the buildup of high ion concentrations in the extracellular spaces resulting in a blockage of the movement of internal harmful extra ions towards the external medium via the leakage junctions.

Shifts in the hydromineral balance may be a consequence of the action of pollutants on organs involved in osmoregulation, on the endocrine system, on metabolism or on active transport processes (Martinez and C´olus, 2002). Usually, after exposure to a single stressor, freshwater fish respond by increasing the efflux of ions through the gills (McDonald and Milligan, 1997). Since freshwater fish take up most of the ions necessary for homeostasis from the water via their gills, an increased efflux of ions apparently causes the drop of plasma electrolytes across these organs and an impairment of active ion uptake by the chloride cells of the gill (Wendelaar- Bonga and Lock, 1992). A reduction in the plasma electrolyte level has two important causes. First, there is an elevated passive efflux of ions across the gills due to more or less non-selective branchial permeability to water and ions. This may lead to haemodilution by enhanced osmotic uptake of water across the gills and to passive diffusional ion loss.

The blood responses seemingly indicate adaptation to hypoxic conditions arising from prooxidants, gill degradation and perhaps oxygen-level fluctuations. The results also show that ionic homeostasis in *O. mossambicus* was affected.

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REFERENCES

Adhikari, S., Sarkar, B., Chatterjee, A., Mahapatra, C.T. and Ayyappan, S., 2004, Effects of cypermethrin and carbofuran on certain haematological parameters and prediction of their recovery in a freshwater teleost, *Labeo rohita* (Hamilton). *Ecotox. Environ. Saf.* 58, 220–226.

Agency for Toxic Substances and Disease Registry (ATSDR). 1990. Toxicological Profile for Cresols. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.

APHA, 2005, *Standard Methods for The Examination of Water and Wastewater.* 21st Edn. Washington DC, USA.

Chemwatch, 2006. Creosote oil acenaphthene fraction. Chemwatch, *MSDS Data Sheet.* pp. 1–19.

Burton, R.F. 1973. The significance of ionic concentration in the internal media of animal, *Biol. Rev.,* 48: 195-231.

Butterfield, D.A., Sun, B., Bellary, S., Arden, W. A. and Anderson, K.W. 1994, Effect of endotoxin on lipid order and motion in erythrocyte membranes. *Biochem. Biophys. Acta.,* 1225: 231–234.

Cazenave, J., Wunderlin, D.A., Hued, A.C. and de los Angeles-Bistoni, M. 2005. Haematological parameters in a neotropical fish, *Corydoras paleatus* (Jenyns, 1842) (Pisces, Callichthyidae), captured from pristine and polluted water. *Hydrobiology,* 537: 25–33.

Cook, J.D. 1985. Measurement of iron status. A report of the International Nutritional Anaemia Consultive Group (INACG). New York, Washington DC, Ch.II: pp 4.

de La Torre, F.R., Salibian, A. and Ferrari L. 1999. Enzyme activities as biomarkers of freshwater pollution: Responses of fish branchial Na+K+-ATPase and liver transaminases. *Environ. Toxicol.,* 14: 313–319.

Eddy, F.B. 1981. Effects of stress on osmotic and ionic regulation in fish. In: Pickering, A.D. (ed.), *Stress in Fish.* Academic Press, New York, pp. 77–95.

Everse, J. and Hsia, N. 1997. The toxicities of native and modified haemoglobins. *Free Radical Biol. Med.,* 22: 1075–1099.

Eyer, P., Hertle, H., Kiese, M. and Klein, G. 1975. Kinetics of ferrihaemoglobin formation by some reducing agents and the role of hydrogen peroxide. *Mol. Pharmacol.,* 11: 326–334.

Feix, J.B. and Butterfield, D.A. 1980. Selective spin labeling of sialic acid residues of glycoproteins and glycolipids in erythrocyte membrane. *FEBS Lett.,* 115: 185–188.

Gasyna, Z. 1979. Intermediate spin-states in one-electron reduction of oxygen hemoprotein complexes at low temperature. *FEBS Lett.,* 106: 213– 218.

Gutteridge, J. M. C. 1986. Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Lett.,* 201: 291–295.

Harel, S., Salan, M.A. and Kanner, J., 1988. Iron release from methmyoglobin, methaemoglobin and cytochrome c by a system generating hydrogen peroxide. *Free Radical Res. Commun.,* 5: 11–19.

Hensley, K., Postlewaite, J., Dobbs, P. and Butterfield, D. A. 1993. Alteration of the erythrocyte membrane via enzymatic degradation of ankyrin (band 2.1): subcellular surgery characterized by EPR spectroscopy. *Biochim. Bio phys. Acta.,* 1145: 205–211.

Houston, A.H. 1997. Are the classical hematological variables acceptable indicators of fish health? *Trans. Am. Fish. Soc.,* 126: 879-893.

Hubrec, T.C. and Smith, S.A. 2000. Haematology of fish. In: Feldman, B.F., Zinkl, J.G., Jain, N.C. (eds.). *Schalm's Veterinary Hematology.* Williams & Wilkins, Philadelphia, pp. 1120–1125.

Iwama, G.K., Afonso, L.O.B. and Vijayan, M.M. 2005. Stress in fish. In: Evans, D.H., Claiborne, J.B. (eds.), *The Physiology of Fishes,* 3rd ed. CRC Press, Boca Raton, pp. 319–342.

Kibret, M., Somitsch, W. and Robra, K.H. 2000. Characterization of a phenol degrading mixed population by enzyme assay. *Water Res.,* 34: 1127-1134.

Lohner, T.W., Reash, R.J., Willet, V.E. and Rose, L.A. 2001. Assessment of tolerant sunfish populations *(Lepomis sp.)* inhabiting selenium-laden coal ash effluents. 1. Haematological and population level assessment. *Ecotoxicol. Environ. Saf.,* 50: 203–216.

Maples, K.R., Eyer, P. and Mason, R.P. 1990. Aniline-, henylhydroxylaminenitrosobenzene-, and nitrobenzene-induced hemoglobin thiyl free radical formation in vivo and in vitro. *Mol. Pharmac.,* 37: 311–318.

Marshall, W.S. and Grosell, M. 2005. Ion transport, osmoregulation and acid-base balance. In: Evans, D.H., Claiborne, J.B. (eds.), *The Physiology of Fishes,* 3rd ed. CRC press, Boca Raton, pp. 177–230.

McDonald, G. and Milligan, L., 1997, Ionic, osmotic and acid-base regulation in stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (eds.), *Fish Stress and Health in Aquaculture.* Cambridge University Press, Cambridge, pp. 119–140.

Misra, H.P. and Fridovich, K. 1972. The generation of superoxide radical during the autooxidation of haemoglobin. *J. Biol. Chem.,* 247: 6960–6962.

Nespolo, R.F. and Rosenmann, M. 2002. Intraspecific allometry of haematological parameters in *Basilichthys australis. J. Fish Biol.,* 60: 1358–1362.

Nohl, H. and Stolze, K. 1998, The effects of xenobiotics on erythrocytes. *Gen. Pharmacol.,* 31: 343–347.

Riley, P.A. 1984. Hydroxyanisole: the current status. In: Riley, P.A. (ed.), *Hydroxyanisole: Recent Advances in Anti-Melanoma Therapy,* IRL Press, Oxford, pp. 25–34.

Stolze K. and Nohl H. 1991. Formation of methemoglobin and phenoxyl radicals from p-hydroxyanisole and oxyhemoglobin. *Free Radical Res. Commun.,* 11: 321–327.

Stolze, K. and Nohl, H. 1992. Methemoglobin

formation from butylated hydroxyanisole and oxyhemoglobin: comparison with butylated hydroxytoluene and p-hydroxyanisole. Free *Radical Res. Commun.,* 16: 159-166.

US EPA 1977. US *Environmental Protection Agency. Sampling and Analysis Procedure for Screening of Industrial Effluents for Priority Pollutants.* Environment Monitoring and Support Laboratory: Cincinnati, OH, USA.

Wepener, V., Van Vuren, J.H.J. and Du Preez, H.H. 1992. The effect of hexavalent chromium at different pH values on the haematology of *Tilapia sparmani* (Cichlidae). *Comp. Biochem. Physiol.,* 101: 375–381.

Wendelaar-Bonga, S.E. and Lock, R.A.C. 1992. Toxicants and osmoregulation in fish. *Netherlands. J. Zool.,* 42: 478–493.

Wyse, J.W. and Butterfield, D.A. 1989. Interaction of heme with erythrocyte membranes: alterations in the physical state of the major sialoglycoprotein. *Biochim. Biophys. Acta,* 979: 121–126.