



Immunosuppressive action of nuvan (dichlorovos) in the banded pearl spot, *Etroplus suratensis* - evaluation of effect on haematology and humoral immune response

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Abstract

Experiments were conducted to elucidate the effect of the organophosphorus pesticide, nuvan (dichlorovos) on some of the haematological parameters and humoral immune response in the pearl spot, *Etroplus suratensis*. The 96 hour LC_{50} of nuvan for juveniles of this fish, was determined as 0.09 mg l^{-1} . Juveniles were exposed to a sub-lethal dose of nuvan i.e., 0.01 mg l^{-1} (a value close to $1/10^{\text{th}}$ of the 96 hour LC_{50} value) for a period of 4 weeks by bath exposure. Blood samples were analyzed for hematological parameters such as total erythrocyte count (TEC), total leucocyte count (TLC), hemoglobin content (Hb), packed cell volume (PCV), erythrocyte sedimentation rate (ESR) and serum protein content at fortnightly intervals. There was significant ($P < 0.05$) reduction in hemoglobin content and total serum protein in the test groups compared to the control fish. The TEC, PCV and ESR values were lower in the test groups compared to the controls but the differences were not statistically significant ($P < 0.05$). The total leucocyte counts were significantly higher ($P < 0.05$) in nuvan treated groups compared to control fish. There was also significant ($P < 0.05$) reduction in serum agglutinating antibody production to sheep red blood cells (SRBC) in the fish exposed to nuvan, as compared to the control group.

Keywords: Immunosuppression, Nuvan, haematology, pearl spot, *Etroplus suratensis*

Introduction

Due to the rapid industrialization and use of innovative agricultural practices, pollution of water bodies, particularly the estuaries and near shore waters has increased. Pesticides, which drain into inland and coastal water bodies through run off from agricultural fields, sewage disposal and effluents of industries can adversely affect food chains and food webs. Pesticides with run off from agricultural farms into aquaculture systems can potentially trigger the outbreak of diseases, as they cause deterioration of the ecosystem, as well as suppress the fish immune system.

Insecticides such as organochlorines and organophosphates are widely used world wide which has resulted in a high contamination risk to aquatic environment (Wagner, 1981). Most of these insecticides are known stress factors and immunotoxins in mammals; however little information is available on the immunotoxic response of fish to these pollutants. There is a great deal of interest in establishing sub-lethal responses which occur at lower pollutant concentrations.

In the present study the effect of nuvan on selected haematological parameters and specific immune response was evaluated in the banded pearl spot, *Etroplus*

suratensis. This species is an excellent food fish, distributed in the brackish waters of the coastal regions of Kerala, Tamil Nadu, Pondicherry and Orissa (Talwar and Jingran, 1991). It is highly relished by consumers, especially in states like Kerala and is cultured on a small scale in the traditional *Pokkali* paddy fields of Kerala and *Khazan* lands of Goa.

Materials and methods

Experimental fish: *E. suratensis* (average weight $29.44 \pm 4.38 \text{ g}$; average length $10.31 \pm 3.54 \text{ cm}$) procured from the brackish water fish farm of Matsyafed, Narakkal, Kerala were stocked in circular fibre glass tanks holding 300 litres of filtered, well aerated and dechlorinated sea water (20‰). The fishes were acclimatized in the experimental tanks for two weeks prior to starting the experiments. The water temperature ranged between 26 to 30 °C during the experimental period. Fish received a practical diet at the rate of 5% of the body weight/day during this period. The water in the tanks was static and 25% of the same was replaced daily throughout the experimental period after siphoning out the leftover feed and fecal matter.

Nuvan and treatment concentrations: Commercial grade Nuvan® of Syngenta India Ltd., containing dichlorovos (0,0-dimethyl-2,2-dichlorovinyl phosphite) 76% EC was

used for the experiment. One ml of nuvan was dissolved in 100 ml distilled water to make a stock solution of 10 mg insecticide/ml. The desired concentration of the test media was obtained by diluting the stock solution in distilled water.

The LC_{50} value of nuvan for *E. suratensis* was determined in the laboratory as per the methods of Reish and Oshida (1987). Short term static bioassays were conducted starting with range finding tests leading to estimation of LC_{50} for pearl spot juveniles exposed to nuvan for 96 hours. The 96 h LC_{50} value was estimated as 0.09 mg l^{-1} . A value very close to the one-tenth concentration of the 96 h LC_{50} value i.e., 0.01 mg l^{-1} (very close to 0.009 mg l^{-1}) was selected for the sub-lethal test trials.

Experimental design: *E. suratensis* were stocked in six fibre glass tanks at a uniform density of 10 fish/tank. Two groups of fishes were maintained in triplicate. One group served as control and the other group was exposed to nuvan at the sub-lethal concentration of 0.01 mg l^{-1} for a period of 4 weeks. One fish per tank i.e. 3 fish per group were sampled at fortnightly intervals. Samples of blood were collected for haematological investigations from the treatment and control groups on 15th and 30th days of nuvan exposure by puncturing the caudal vein using heparinised 26-gauge needle, attached to sterile 2 ml syringe. Fishes were anaesthetized using benzocaine (10 ppm) prior to bleeding.

On termination of the experiment, 10 fish per treatment were immunized with sheep red blood cells (SRBC), which is commonly used as a standard model antigen for evaluating humoral immune response. Sheep red blood cells (SRBC) procured from Veterinary College, Mannuthy, in sterile Alsever's solution (dextrose 2.05 g; sodium citrate 0.80 g; sodium chloride 0.42 g; distilled water 100 ml; pH adjusted to 6.1 using 10% citric acid) was harvested by centrifugation at 3500 g for 10 minutes at 4 °C. The SRBC were then washed three times in sterile phosphate buffered saline (PBS, pH 7.2). The cells were then resuspended in sterile PBS (pH 7.2) at a cell density of 10^7 cells/ml. The cells were counted using a haemocytometer and the required dilution was made shortly before the injection. Fishes were injected (i.m) at the dorsal region of the body with 0.1 ml of the SRBC suspension. The non-immunised control group received 0.1 ml sterile PBS (pH 7.2)/fish. The fish were released back to the experimental tanks after injection.

Two weeks after immunization, blood samples were collected from the immunized groups and the non-immunized controls for estimation of antibody agglutination titre. Blood was allowed to clot at room temperature,

stored overnight at 4 °C and serum separated by centrifugation at 3500 g for 10 minutes at 4 °C. Pooled sera from the treatment and control groups were analyzed in triplicate for antibody agglutination titre.

Haematology: For total leukocyte and erythrocyte counts, the blood was diluted with Dacie's fluid in the ratio 1:4, as per Blaxhall and Daisley (1973). TEC and TLC were estimated in haemocytometer under a microscope. One hundred micro-litre of the diluted blood prepared as above was loaded into the counting chamber and total erythrocyte and leukocyte count per micro-litre of blood was determined. Pooled samples from three fish were counted in triplicates.

The packed cell volume (PCV) was estimated using heparinised capillary tubes as per the method described by Schaperclaus (1986). The capillary tubes filled with blood were centrifuged in a haematocrit centrifuge (Remi - T 12) at 3000 rpm for 3 minutes. The packed cell volume was read using microhaematocrit reader.

The erythrocyte sedimentation rate (ESR) was determined following the method of Westergreen with modifications (Schaperclaus, 1986). The blood with anticoagulant was filled in the heparinised Wintrobe's tubes up to the 0- mark and kept vertically. The sedimentation rate was read after one hour.

Haemoglobin percentage was determined following the cyanmethemoglobin method (Van kampen *et al.*, 1961). Twenty micro-litre of blood was added to 5 ml of Drabkin's reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate. This was allowed to stand for 4 minutes and read against a water blank at 546 nm. The absorbance of cyanmethemoglobin standard was taken in a similar manner. The haemoglobin content was expressed as g%.

For the estimation of serum protein, blood was collected in vials without anticoagulant. The blood samples were allowed to clot at room temperature for 1h, kept at 4 °C in refrigerator and then centrifuged at 3500 g for 10 minutes at 4 °C. The supernatant was separated, collected in eppendorf vials and stored at -20 °C until analysis. The serum samples were analyzed in triplicates for protein concentration using the method of Lowry (Lowry *et al.*, 1951). The optical density was measured at 630 nm in a spectrophotometer. The standard graph was drawn using Bovine Serum Albumin as standard protein.

Antibody agglutination titre: The sera were inactivated at 50 °C for 30 minutes in a water bath. Pooled sera from the treatment and control groups were analyzed in triplicate for agglutination titre by standard method using

96 well microtitre plates (Sundick and Rose, 1980). Agglutination titres for each sample were expressed as \log_2 values based on visual observation of the highest serum dilution showing clear agglutination.

The data from different assays and tests were analysed statistically using ANOVA (SYSTAT version 7.0.1, 1997)

Results

The data pertaining to total erythrocyte counts in the treatment and control groups are presented in Figure 1. Total erythrocyte count (TEC) in the nuvan exposed animals were lower than control groups on both the sampling days. However, the difference was statistically not significant. Figure 2 depicts the data on total leucocyte counts in the experimental groups. Though there was not much difference in TLC values between the treatment and control groups on the 15th day, TLC in the nuvan exposed fish were significantly ($P < 0.05$) higher on the 30th day of exposure to sub-lethal dose of nuvan compared to the control group.

There was significant reduction ($P < 0.05$) in haemoglobin content in the Nuvan treated group (Fig. 3) compared to control on both the sampling days. Though there was slight reduction in the ESR and PCV values in the nuvan treated animals on both the sampling days (Figs. 4 and 5) the differences were not statistically significant.

There was reduction in serum protein in the treatment group on both sampling days (Fig. 6). The serum protein level in nuvan treated fish were significantly ($P < 0.05$) less compared to the control group on the 30th day, whereas on the 15th day the difference was not statistically significant.

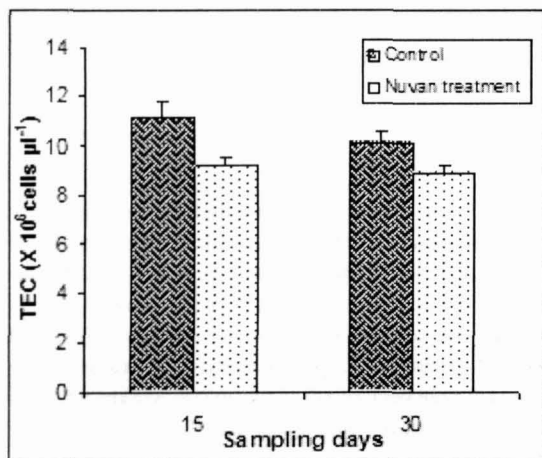


Fig. 1. Total erythrocyte counts (TEC) in treatment and control groups of *E. suratensis* at 15th and 30th days of nuvan exposure.

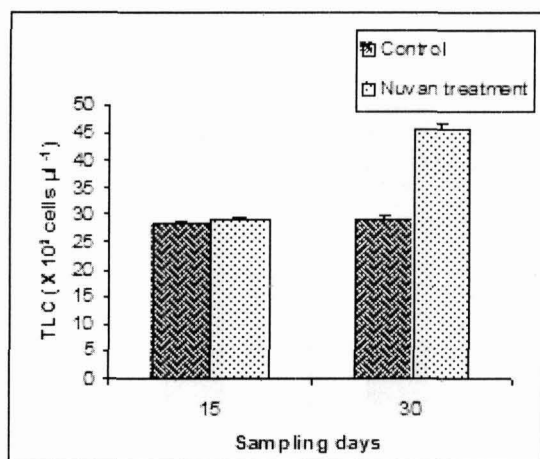


Fig. 2. Total leukocyte counts (TLC) in treatment and control groups of *E. suratensis* at 15th and 30th days of nuvan exposure.

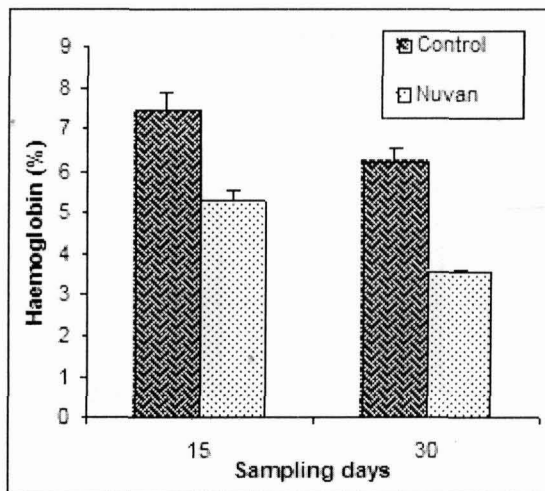


Fig. 3. Haemoglobin (%) in treatment and control groups of *E. suratensis* at 15th and 30th days of nuvan exposure.

Humoral antibody titres (\log_2 values) in the immunized treatment and control groups of *E. suratensis* on the 2nd week of immunization with SRBC are presented in Fig. 7. Two-way analysis of variance employing SYSTAT version 7.0.1 showed that the agglutination titres in the nuvan exposed fish were significantly ($P < 0.05$) lower than that of control groups. The non-immunised control group had only negligible titre.

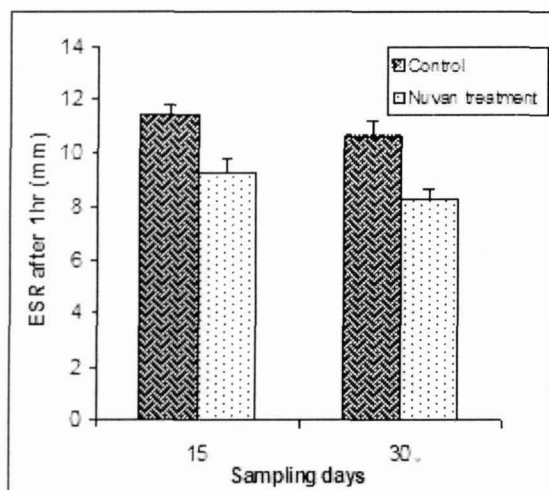


Fig. 4. Erythrocyte Sedimentation Rate (ESR) in treatment and control groups of *E. suratensis* at 15th and 30th days of nuvan exposure.

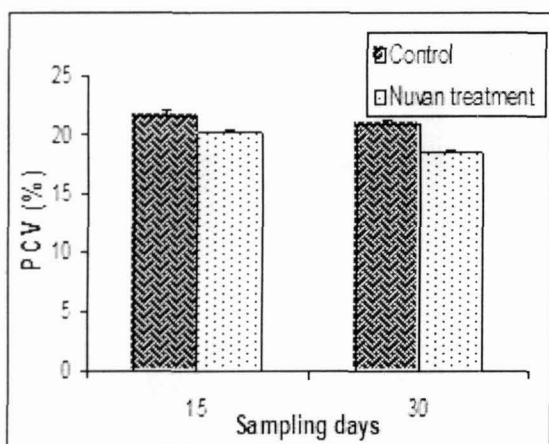


Fig. 5. Packed Cell Volume (PCV) in treatment and control groups of *E. suratensis* 15th and 30th days of nuvan exposure.

Discussion

The study indicates that exposure to nuvan in the laboratory does produce significant treatment effects in *E. suratensis*. The LC_{50} value obtained during the present study falls within the recommended treatment dose i.e., 0.05 to 0.1 mg l⁻¹ for ectoparasites in freshwater aquaculture (Rao *et al.*, 1992).

Mohapatra and Noble (1991) reported the 96 h LC_{50} value for *Liza parsia* as 0.482 mg l⁻¹. In a static lab bioassay, Verschueren (1983) recorded the 96 h LC_{50}

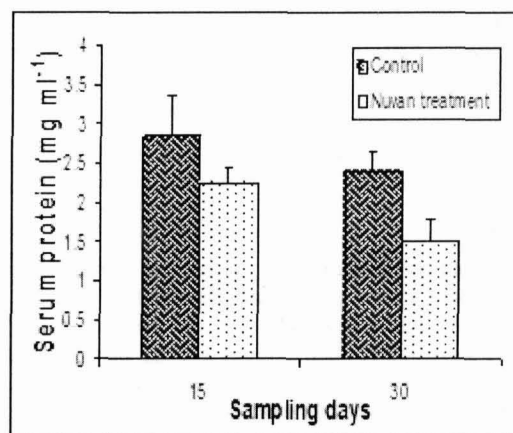


Fig. 6. Serum protein in treatment and control groups of *E. suratensis* at 15th and 30th days of nuvan exposure.

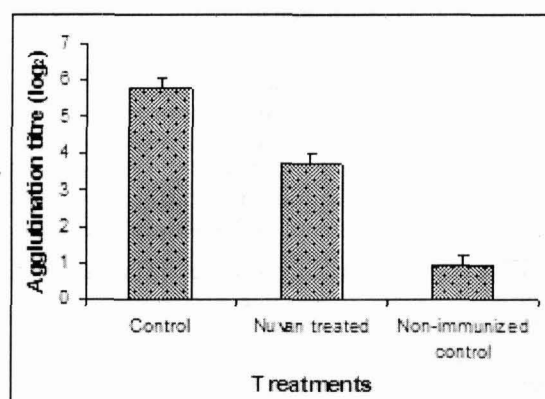


Fig. 7. Agglutination titres (log₂) in the treatment and control groups of *E. suratensis* at 2nd week post immunization with SRBC

value of nuvan (dichlorovos) in the striped mullet, *Mugil cephalus* as 0.2 mg l⁻¹.

Pearl spot exposed to sub-lethal concentrations of nuvan exhibited significant reduction in haemoglobin content, total serum protein and humoral immune response. Total leucocyte counts, however showed significant increase at 30 days of nuvan exposure. Elevated levels of peripheral blood leucocytes on exposure to pesticides have been reported by several workers (Bansal *et al.*, 1979; Agrawal and Srivastava, 1980). The elevated leucocyte levels can be attributed to several factors like increase in thrombocytes, lymphocytosis or due to squeezing of leucocytes in peripheral blood as a result of

pollutant induced tissue damage (Johansson-Sjoberg and Larsson, 1978).

Lethal or sub-lethal concentrations of pesticides affect the haematological parameters of fish (Mohapatra and Saha, 2000). Reduction in TEC, PCV, and Hb has been reported in *Heteropneustes fossilis* exposed to thiodan (Ramanujam and Mohanty, 1997). In *Notopterus notopterus* exposed to sub-lethal concentrations of chlordane and malathion, Gupta *et al.* (1995) have recorded elevated WBC and PCV and reduced clotting time. The Hb % was significantly reduced in fish exposed to nuvan in the present study and this may be attributed to the decrease in erythropoiesis and haemoglobin synthesis (Nayak *et al.*, 2004).

There was significant reduction in serum protein content in the nuvan exposed fish on the 30th day. Reduction of total serum protein is a strong indicator of an immunosuppressive effect of pesticide. Gill *et al.* (1991) have reported reduction in serum protein content in fish exposed to stress. Jyothi and Narayan (1997) recorded depletion of serum proteins in *Clarias batrachus* exposed to phorate. The reduction in serum protein might be due to the increased renal excretion of blood proteins leading to depletion of total protein in serum.

Sub-lethal doses of toxic agents can have effects upon immune structures and functions that may ultimately be almost as harmful as direct toxic doses. Holladay *et al.* (1996) reported lowering of pronephros cell counts and phagocytic functions in Nile tilapia on exposure to chlorpyrifos. Rainbow trout, *Oncorhynchus mykiss* exposed to TTDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) displayed a variety of lymphomyeloid effects, including thymic involution, splenic lymphocyte depletion, hypocellularity of pronephros and peripheral leucopenia (Spitsbergen *et al.*, 1988).

Channel catfish, *Ictalurus punctatus* exposed to malathion have displayed significantly inhibited humoral immune response (Plumb and Areechon, 1990). Areechon and Plumb (1990) have reported significantly depressed peripheral leucocyte counts in channel catfish exposed to malathion. However, in the present experiment the peripheral leucocyte counts were significantly higher in the fishes exposed to sub-lethal concentrations of nuvan for a period of 4 weeks as compared to the control group.

The role of stress hormone in immune suppression is well documented in fish (Bisset, 1949; Ellis, 1981). In fish corticosteroid receptors are expressed in leucocytes and hence they can affect any stage of the immune response like antigen uptake, processing and antibody production. Although a decrease in the number of circulating

leucocytes (leucopenia) is a frequent component of the stress response (Mc Leay, 1975; Iwama *et al.*, 1976), chronic elevation of plasma cortisol levels can apparently influence disease susceptibility without reducing leucocyte populations (Pickering and Pottinger, 1985). Hence, the much suppressed humoral immune response observed in pearl spot exposed to nuvan in the present study could be explained due to stress acting through the release of corticosteroids from the adrenal.

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