UNIVERSITI PUTRA MALAYSIA

TOXICITY AND IMMUNOSUPPRESSIVE EFFECTS OF DIAZINON IN GRASS CARP, CTENOPHARYNGODON IDELLA (CUVIER AND VALENCIENNES)

REZA POORGHOLAM

FPV 2005 6
TOXICITY AND IMMUNOSUPPRESSIVE EFFECTS OF DIAZINON IN
GRASS CARP, *CTENOPHARYNGODON IDELLA*
(CUVIER AND VALENCIENNES)

By

REZA POORGHOLAM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in
Fulfilment of Requirements for the Degree of Doctor of Philosophy

May 2005
DEDICATION

WITH LOVE AND APPRECIATION TO:

My parent: Rajabali Pourgholam and Sedigheh Sajoodi

My wife: Sekineh Dashti

My sons: Hamzeh, Mohebali and Mohammadali
Grass carp is one of the valuable warm water fish species that is currently being cultured in polyculture system in Iran. Despite of large scale grass carp farming in the East Asian countries, only minimum data is available concerning the fish immune system and the effect of organophosphate chemicals on the fish immune response. Diazinon is one of the major organophosphate pesticides currently used in Northern and Southern part of Iran. Unfortunately, these areas are also the main regions for grass carp culture and there are regular reports of the disease outbreaks particularly in the provinces of Gilan and Khozestan. In addition, previous studies has indicated that Aeromonad septicemias was one of main factor in the high mortality occurrences in the grass carp, in particular whenever the fish immune system seems to be suppressed by some toxicants. The specific objectives of this study were: (i) determination of 96-h LC50 diazinon in grass carp; (ii) purification and partial characterization of grass
carp IgM; (iii) assessment of some humoral and cellular immunoresponses of non-immunized and immunized grass carp, following exposure to diazinon.

In this study, LC$_{50}$ of diazinon at 96 hour in grass carp was determined to be 15.13 mg/L. The examination of hematological and tissue enzyme parameters indicated that diazinon at sublethal levels had caused an effect similar to anemia. In addition, a significant decrease of lymphocytes values and significant increase of PMNs values were observed. There were also significant and insignificant changes in some blood parameters such as monocytes and myelocytes counts, MCH, MCHC, AST, ALP, ALT and LDH values at different days of post exposure of diazinon. Such fluctuations indicated that fish hematopoietic tissues were in stress and were in constant struggles to maintain normal condition.

The pathological effects of diazinon on the liver, kidney, spleen, gills, and nostrils of grass carp examined under light and electron microscope, showed that diazinon caused severe damage to the cell structure such as congestion of blood vessels, haemorrhage, cellular infiltration, pyknosis of cells nuclei, vacuolar degeneration and general necrosis in the tissues of kidney, spleen and liver. There were also degenerative changes of interstitial tissue, detachment of tubular basement membrane in kidney. In the gills, hyperplasia and fusion of secondary lamellae, separation and sloughing of epithelium from the underlying basement membrane were also observed. In the lysozyme study it was indicated that grass carp reacted to diazinon by raising the level of lysozyme in tissues of spleen and kidney and also in
serum of Aeromonas-immunized fish exposed to diazinon and control positive (immunized only), as compared to control negative (non-immunized and not exposed to diazinon). However, the level of lysozyme in immunized fish exposed to diazinon is lower than control positive that indicated the depressive effect of toxicant on fish immune system. Analysis on data of leucocytes chemiluminescent response indicated that cellular factors of fish immunity following immunization with A. hydrophila and also under influence of diazinon, responded by changing its functional activities, as evidenced by a high chemiluminescent response in both of immunized fish exposed to diazinon and control positive groups as compared to the control negative group. However, the level of chemiluminescent response in immunized and exposed group was insignificantly lower than control positive group that indicated the negative effect of diazinon on fish immune system. In summary, all of the above findings proved the immunosuppressive effect of diazinon on nonspecific immune system of grass carp.

The level of IgM in serum of normal grass carp was found to range from 3 to 4 mg/ml. Estimation of molecular weight of grass carp IgM was performed using three different methods. Affinity chromatography method gave the approximate values of about 480 and 640 KDa in SDS-PAGE, while gel chromatography and ion-exchange chromatography methods showed an identical molecular weight with an approximate value of 490 KDa.

In antibody study, the titers of immunized fish were significantly higher than immunized fish exposed to diazinon. In addition, a strong positive correlation
was also demonstrated between the results of ELISA and agglutination titers. These observed results confirmed the immunosuppressive effect of diazinon on specific immune system of grass carp.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

Ketoksidan dan Kesan Imunotindas Diazinon pada Ikan Kap Rumput, *Ctenopharyngodon idella* (Cuvier and Valenciennes)

Oleh

REZA POORGHOLAM

Mei 2005

Pengerusi: Profesor Madya Hassan Hj. Mohd Daud, Ph D

Fakulti : Perubatan Veterinar


Objektif spesifik kajian ini adalah : (i) penentuan LC$_{50}$ diazinon pada 96-jam pada ikan kap rumput ; (ii) permurnian dan pencirian separa IgM kap rumput;
(iii) penilaian beberapa ciri ransangan imun humoral dan selular ikan kap yang diimun dan tak diimun, selepas didedahkan kepada diazinon.

Di dalam kajian LC₅₀ diazinon pada 96-jam pada ikan kap rumput, nilainya ditentukan pada 15.13 mg/L. Pemeriksaan parameter hematologikal dan tisu enzim menunjukkan diazinon pada aras subletal telah menyebabkan kesan sama seperti anemia. Di samping itu terdapat penurunan bererti dalam nilai limfosit dan peningkatan bererti dalam nilai PMN. Disaksikan juga perubahan yang bererti dan tak bererti dalam nilai-nilai parameter darah yang lain seperti bilangan monosit dan mielosit dan nilai-nilai MCH, MCHC, ALP, ALT dan LDH. Perubahan-perubahan tersebut menunjukkan tisu hematopoietik adalah dalam situasi tindasan dan sentiasa berjuang untuk berada dalam keadaan normal.

Kesan patologikal diazinon pada hepar, ginjal, limfa, insang dan rongga nasal yang dilihat dibawah mikroskop cahaya dan elektron menunjukkan bahawa diazinon telah menyebabkan kecederaan teruk pada struktur tisu seperti kongesi saluran darah, hemoraj, penyusupan sel, piknosis nukleus sel, degenerasi perlompangan dan nekrosis am di dalam tisu ginjal, limfa dan hepar. Terdapat juga perubahan degeneratif pada tisu perantaraan dan perlu cutan tapak membran sel tubular ginjal. Pada insang, hiperplasia dan percantuman lamela skunder, perpisahan dan penghakisan epitelium daripada tapak membran juga dapat dilihat.
Di dalam kajian lisozim, ia menunjukkan bahawa kap rumput bertindak balas terhadap diazinon dengan meningkatkan aras dalam tisu limfa dan ginjal dan juga serum ikan yang diimunkan selepas pendedahan kepada diazinon dan dalam kumpulan kawalan positif (diimunkan), jika dibandingkan dengan kumpulan kawalan negatif (tidak diimunkan dan tidak didedahkan kepada diazinon). Walau bagaimanapun, aras lisisim dalam ikan terimun terdedah pada diazinon adalah lebih rendah dari kumpulan kawalan positif yang mana membuktikan bahawa kesan tindas toksikan terhadap sistem imun ikan. Analisis terhadap data ransangan pendaflorkimia leukosit, menunjukkan bahawa faktor selular imuniti ikan selepas imunisasi dengan A. hydrophila dan di bawah pengaruh diazinon, bertindak dengan menekan fungsi aktiviti yang mana dapat dilihat pada gerakbalas tinggi dalam pendarfluorkimia dalam ikan yang diimun dan terdedah kepada diazinon dan kumpulan kawalan positif dibanding dengan kumpulan kawalan negatif. Walau bagaimanapun, aras gerakbalas pendarfluorkimia di dalam kumpulan ujian adalah lebih rendah, walaupun tidak bererti, menunjukkan kesan negatif diazinon terhadap sistem imun ikan. Kesimpulannya, penemuan membuktikan kesan imunotindas diazinon terhadap sistem imun tak spesifik ikan kap rumput.

Manakala aras IgM dalam serum ikan kap rumput normal berada dalam renj 3-4 mg/L. Anggaran berat molekul IgM kap rumput telah dibuat menggunakan tiga metod yang berbeza. Metod kromatografi affiniti memberi nilai anggaran 480 dan 640 KDa dalam SDS-PAGE, sementara kromatografi
gel dan kromatografi tukaran-ion menunjukkan berat molekul yang identikal iaitu bernilai anggaran 490 KDa.

Dalam kajian antibodi, titer dalam ikan yang diimun adalah lebih tinggi dan bererti dari ikan yang diimun dan didedahkan kepada diazinon. Tambahan lagi, korelasi positif yang kuat telah ditunjukkan di antara keputusan ELISA dan titer agglutinasi. Penemuan membuktikan kesan imunosupresif diazinon terhadap sistem imun spesifik ikan kap rumput.
ACKNOWLEDGEMENTS

Praise is to the Almighty ALLAH. Lord of all creations, for his heavenly, luxurious blessings over me throughout my life and the period of this study.

I would like to express my heartfelt gratitude and appreciation to my Chief Supervisor, Associate Professor Dr. Hassan Hj. Mohd Daud, for his valuable guidance and constructive suggestions throughout the research program. I sincerely appreciate the innumerable hours he spent reading the draft and the suggestions made to improve the thesis.

I wish to express my deepest thankfulness to my co-supervisors: Professor Dato' Dr. Mohamed Shariff Mohamed Din, Associate Professor Dr. Abdul Rahman Omar and Professor Dr. Mehdi Soltani, for their valuable suggestions and kind assistance throughout this study.

A very special acknowledgement is given to Dr. Sohrab Rezvani the Head of Iranian Fisheries Research Organization (IFRO), his deputies Dr. Mohammad Tokhmafshan and Dr. Mahmood Masoomian for their co-operation during the process of conducting the study.

I would also like to thank Dr. Sohrab Akbari, Dr. Bahram Kazemi and Dr. Lesa Sharifpour for their assistance with electron microscopy, purification and characterization of grass carp IgM and histopathological slides reading.
I am grateful to the Head and staff of the Mazandaran Fisheries Research Center (MFRC), Faculty of Veterinary Medicine and Research Deputy of Tehran University for their co-operation.

I would like to express my thanks to my friends who are studying at UPM: Dr. Seyyed Davood Hosseini, Dr. Broomand Chaharaein, Dr. Paimon Roostaeian, Dr. Hamid Rezaei, Dr. Hamid Khodabakhsh, Mohammad Gholizadeh, Hamid Sanatnama, Reza Khakvar, Kourosh Khaledi, Saeid Eslamian, Behnam Kamali, Usof Rostami, Mehran Keisami, Reza Motalleb, my friends and colleagues at IFRO and Faculty of Veterinary Medicine of Tehran University: Mrs. Esmaeili, Dr Hossain Rostami, Dr. Ahmad Ghoroghi, Reza Nahavandi, Dr Khazraeinia, Taheri, Bagheri, Jafari, Ali Farzanfar, Masomeh Khadem, Parvaneg Usefi and all of my friends and colleagues at UPM: Dr. Sanjoy, Dr. Lee, Dr. Wang Yin Geng, Dr. Najiah, Dr. Abeer, Dr Tan, Azah, Shamini, Jonie, Sophia, Haizal, Noraini, Anarita, Azrin, Murni, Khaled, Andra, Francis, Zainal and Azmi. The camaraderie with these students and friends at UPM is memorable.

I wish to express my deepest thanks to my wife, Sekineh Dashti and my sons Hamzeh, Mohebali and Mohammadali for their patience, consistent support and understanding during my study in Malaysia.

Lastly, I would like to thank many others whose name do not appear here, who have helped me during my study period.
I certify that an Examination Committee met on 12\textsuperscript{th} May 2005 to conduct the final examination of Reza Poorgholam on his Doctor of Philosophy thesis entitled "Toxicity and Immunosuppressive Effects of Diazinon in Grass Carp, \textit{Ctenopharyngodon idella} (Cuvier and Valenciennes)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

\textbf{Rasedee Abdullah, PhD}  
Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Chairman)

\textbf{Abdul Rani Bahaman, PhD}  
Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
/Internal Examiner

\textbf{Jasni Sabri, PhD}  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Internal Examiner)

\textbf{Noor Azhar Mohd Shazilli, PhD}  
Professor  
Faculty of Science and Technology  
University College of Science and Technology Malaysia  
(External Examiner)

\begin{center}
\includegraphics[width=\textwidth]{signature.png}
\end{center}

\textbf{GULAM RUSUL RAHMAT ALI, PhD}  
Professor/Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 21 JUL 2005

xiii
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Hassan Hj Mohd Daud, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Mohamed Shariff Mohamed Din, PhD
Professor Dato'
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Mehdi Soltani, PhD
Professor
Faculty of Veterinary Medicine
Tehran University
(Member)

Abdul Rahman Omar, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Signed:
AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia
Date: 11 AUG 2005
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

[Signature]

Date: 20 Jul 2005

REZA POORGHOLAM
TABLE OF CONTENTS

DEDICATION ii
ABSTRACT iii
ABSTRAK vii
ACKNOWLEDGEMENTS xi
APPROVAL xiii
DECLARATION xv
LIST OF TABLES xix
LIST OF FIGURES xxii
LIST OF ABBREVIATIONS xxvi

CHAPTERS

1. INTRODUCTION

2. LITERATURE REVIEW 12
  2.1. Fish immunology 12
  2.2. Fish immunotoxicology 31
  2.3. Grass carp, Ctenopharyngodon idella (Cuvier and Valenciennes) 47
  2.4. Toxicant- Diazinon 54
  2.5. Immunomodulator - Aeromonas hydrophila 61

3 DETERMINATION OF 96 h LC$_{50}$ OF DIAZINON IN GRASS CARP
   Ctenopharyngodon idella (Cuvier and Valenciennes, 1844) 70
  3.1. Introduction 70
  3.2. Materials and Methods 71
     3.2.1. Fish and maintenance 71
     3.2.2. Toxicant 71
     3.2.3. Acute toxicity 72
        3.2.3.1. Determination of survival rate: 72
        3.2.3.2. Determination of lethal concentration of diazinon for grass
                   carp (death-limit) 72
        3.2.3.3. Determination of LC$_{50}$ at 96 hr: 73
     3.2.4. Hematological and biochemical study 74
     3.2.5. Data analysis 75
  3.3. Results 76
     3.3.1. Acute Toxicity Experiment 76
        3.3.1.1. Survival rate 76
        3.3.1.2. Determination of the lethal concentration of diazinon 76
        3.3.1.3. Determination of LC$_{50}$ of diazinon 77
     3.3.2. Hematological and biochemical study 79
     3.3.3. Clinical and behavioural signs 79
  3.4. Discussion 83
4. PURIFICATION AND PARTIAL CHARACTERIZATION OF SERUM IMMUNOGLOBULIN FROM GRASS CARP, *CTENOPHARYNGODON IDELLA* (CUVIER AND VALENCIENNES, 1844) 88

4.1. Introduction 88
4.2. Materials and Methods 90
   4.2.1. Immunization of fish 90
   4.2.2. Purification and molecular weight determination of IgM 90
      4.2.2.1. Gel filtration chromatography 90
      4.2.2.2. Affinity chromatography 90
      4.2.2.3. Ion-exchange chromatography 91
      4.2.2.4. SDS-PAGE 92
   4.2.3. Protein determination 92
   4.2.4. Western blotting assay 93
   4.2.5. Dot blotting assay 94
4.3. Results 95
   4.3.1. Purification and molecular weight determination of fish IgM 95
   4.3.2. Total serum protein 95
   4.3.3. Western blotting 96
   4.3.4. Dot blotting 96
   4.4. Discussion 103

5. ASSESSMENT OF SOME HUMORAL AND CELLULAR IMMUNORESPONSES OF NON-IMMUNIZED GRASS CARP, FOLLOWING EXPOSURE OF DIAZINON 106

5.1. Introduction 106
5.2. Materials and methods 109
   5.2.1. Fish and maintenance 109
   5.2.2. Diazinon exposure 109
   5.2.3. Collection and processing of samples 110
   5.2.4. Lysozyme assay 111
   5.2.5. Chemiluminescent assay 112
   5.2.6. Hematological and biochemical study 112
   5.2.7. Histopathological study 113
   5.2.8. Electron microscopy 113
      5.2.8.1. Scanning Electron Microscopy (SEM) 113
      5.2.8.2. Transmission Electron Microscopy (TEM) 114
   5.2.9. Statistical analysis 115
5.3. Results 115
   5.3.1. Lysozyme assay 115
   5.3.2. Chemiluminescent assay 116
   5.3.3. Hematology and biochemical studies 118
   5.3.4. Histopathology study 130
   5.3.5. Electron microscope examination 143
5.4. Discussion 149

6. ASSESSMENT OF SOME HUMORAL AND CELLULAR IMMUNORESPONSES OF IMMUNIZED GRASS CARP, 159

6.1. Introduction 159
6.2. Materials and methods 164
   6.2.1. Fish and maintenance 164
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Aquaculture and inland fish production (metric tonne) in I.R.IRAN</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>Aquaculture and inland fish production (metric tonne) in I.R.IRAN in 2003</td>
<td>5</td>
</tr>
<tr>
<td>3.1</td>
<td>The lethal concentration of diazinon for grass carps</td>
<td>76</td>
</tr>
<tr>
<td>3.2</td>
<td>Cumulative mortality of grass carp at 24, 48, 72 and 96 hrs exposure to various concentrations of diazinon</td>
<td>77</td>
</tr>
<tr>
<td>3.3</td>
<td>Determination of diazinon lethal concentration for grass carps</td>
<td>77</td>
</tr>
<tr>
<td>3.4</td>
<td>Erythrocyte profile of grass carp following exposure to diazinon (5.6 mg/L) at 16°C</td>
<td>80</td>
</tr>
<tr>
<td>3.5</td>
<td>Leucocyte profile of grass carp following exposure to diazinon (5.6 mg/L) at 16°C</td>
<td>81</td>
</tr>
<tr>
<td>3.6</td>
<td>The effect of diazinon (5.6 mg/L) on enzyme activities, cholesterol, triglyceride, glucose and total protein of blood plasma of grass carp at 16°C</td>
<td>82</td>
</tr>
<tr>
<td>5.1</td>
<td>Levels of lysozyme (µg/mg of tissue) in spleen, kidney and serum of grass carp exposed to diazinon at 20-22°C</td>
<td>117</td>
</tr>
<tr>
<td>5.2</td>
<td>Intensity of spontaneous and activated chemiluminescent response (impulse/second) of leucocytes of grass carp exposed to various concentrations of diazinon at 20-22°C</td>
<td>119</td>
</tr>
<tr>
<td>5.3(a)</td>
<td>Effects of various concentrations of diazinon on haematological indices of grass carp at day one post-exposure at 20-22°C.</td>
<td>121</td>
</tr>
<tr>
<td>5.3(b)</td>
<td>Effects of various concentrations of diazinon on biochemical indices of grass carp at day one post-exposure at 20-22°C.</td>
<td>122</td>
</tr>
<tr>
<td>5.4(a)</td>
<td>Effects of various concentrations of diazinon on hematological and biochemical indices of grass carp at day 7 post-exposure at 20-22°C.</td>
<td>123</td>
</tr>
<tr>
<td>5.4(b)</td>
<td>Effects of various concentrations of diazinon on biochemical indices of grass carp at day 7 post-exposure at 20-22°C.</td>
<td>124</td>
</tr>
</tbody>
</table>
5.5(a) Effects of various concentrations of diazinon hematological indices of grass carp at day 15 post-exposure 20-22°C

5.5(b) Effects of various concentrations of diazinon on biochemical indices of grass carp at day 15 post-exposure 20-22°C

5.6(a) Effects of various concentrations of diazinon on hematological indices of grass carp at day 30 post-exposure at 20-22°C.

5.6(b) Effects of various concentrations of diazinon on biochemical indices of grass carp at day 30 post-exposure at 20-22°C.

5.7(a) Effects of various concentrations of diazinon on haematological indices of grass carp at day 45 post-exposure at 20-22°C.

5.7(b) Effects of various concentrations of diazinon on biochemical indices of grass carp at day 45 post-exposure at 20-22°C.

5.8 Histopathological scores of grass carp’s liver exposed to various concentrations of diazinon at 20-22°C.

5.9 Histopathological scores of grass carp’s spleen exposed to various concentrations of diazinon at 20-22°C.

5.10 Histopathological scores of grass carp’s kidney exposed to various concentrations of diazinon at 20-22°C.

5.11 Histopathological scores of grass carp’s gills exposed to various concentrations of diazinon at 20-22°C.

6.1 Immunization, diazinon exposure and bacteria challenge of grass carp held at 18-20°C (n=320)

6.2 Levels of lysozyme (μg/mg tissue) in spleen, kidney and serum of grass carp exposed to diazinon (2 mg/L) at 18-20°C (n=90)

6.3 Intensity of spontaneous and activated chemilumoxcent response (impulse/second) of leucocytes of grass carp exposed to diazinon (2 mg/L) at 18-20°C (n=90)

6.4 Antibody titers of grass carp immunized with A. hydrophila, exposed and unexposed to diazinon (2 mg/L) at 18-20°C (n=90)

6.5 Antibody production in grass carp immunized with A. hydrophila antigens and exposed to diazinon and PBS at 18-20°C

6.6 Cumulative mortality of immunized and unimmunized grass carp challenged with A. hydrophila and exposed to diazinon
6.7 Effects of 2 mg/L of diazinon exposure on hematological and biochemical indices of grass carp (n = 90) at day one post-exposure.

6.8 Effects of 2 mg/L of diazinon exposure on hematological and biochemical indices of grass carp (n = 90) at week one post-exposure.

6.9 Effects of 2 mg/L of diazinon exposure on hematological and biochemical indices of grass carp (n = 90) at week 2 post-exposure.

6.10 Effects of 2 mg/L of diazinon exposure on hematological and biochemical indices of grass carp (n = 90) at week 3 post-exposure.

6.11 Effects of 2 mg/L of diazinon exposure on hematological and biochemical indices of grass carp (n = 90) at week 4 post-exposure.
LIST OF FIGURES

Figures

1.1 Map of I.R. IRAN showing the areas of warm water aquaculture

2.1 Structural formula of diazinon (Eisler, 2000)

3.1 Experimental design of acute toxicity examination of diazinon in grass carp

3.2 The sigmoid curve of dose response for the 96h LC50 determination of diazinon

3.3 Probit of the mortality versus log-dose of exposure grass carp for the diazinon 96h LC50 determination

4.1 Gel filtration of 1ml grass carp serum on Sephadex G-150. The peak indicated by the arrow is rich in IgM

4.2 Protein elution profile of grass carp serum from affinity column. Application of whole serum to the column (A) was followed by the detection of a large protein peak (A-B). The column was washed with PBS buffer (B) and the bound protein was eluted from the agarose beads (C) by the glycine elution buffer, resulting in the second protein peak containing the affinity purified grass carp Ig (C-D). Each line segment between two points represents individual fractions

4.3 Chromatography of grass carp IgM on Ion-exchange column. Elution was carried out using a linear gradient 0-0.6mM NaCl. The IgM eluted as two separated peaks (peak 1 and 2)

4.4 SDS-PAGE with 12% polyacrylamide gel stained with Coomassie Blue under non-reducing conditions for estimation of molecular weight of grass carp IgM. Lanes 1 and 7: Urease (marker), Lanes 2 and 8: BSA (marker), Lanes 3 and 4: normal fish serum, Lanes 5 and 6: grass carp affinity-purified IgM
Degeneration of interstitial tissue of kidney (DI) and severe necrosis of basement membrane of tubule cells (arrow) and necrosis of glomerulus (arrow head) were also seen at 7 days post-exposure to 4 mg/L diazinon (H&E, x488).  

Normal tissue of liver (H&E, x488)  

Generalized liver degeneration as indicated by pyknotic nuclei (arrow) in the paranchyma. Also seen hepatopancreas showing loss of structural integrity (arrow head) in fish exposed to 2 mg/L diazinon at day 30 post-exposure (H&E, x122).  

Focal necrosis (arrow) in the liver manifested by the presence of pyknotic cells and pale-staining area, seen at day 45 post-exposure to 2 mg/L of diazinon (H&E, x488).  

Vacuolar degeneration of hepatocytes (arrow), at day 7 post exposure to 2 mg/L of diazinon (H&E, x488).  

Generalised vacuolar degeneration and pyknosis of hepatocytes nuclei (arrow) at day 7 post-exposure to 4 mg/L diazinon (H&E, x488).  

Normal structure of gills: primary lamellae (PL), secondary lamellae (arrow head) and mucosal cell (arrow) (H&E, x740)  

Gills lamellae of grass carp at day 7 post-exposure to 2 mg/L diazinon showing proliferation of secondary lamellae (arrow). Separation and sloughing-off epithelium (arrow head) from the underlying basement were also seen (H&E, x122).  

Gills lamellae of grass carp at day 15 post-exposure to 2 mg/L diazinon showing hyperplasia and fusion of secondary lamellae (arrow). Separation and sloughing-off epithelium (arrow head) from the underlying basement were also seen (H&E, a= x244 and b= x488).  

Normal structure of cells lining grass carp’s nostril. Note the sensory cell (SC) epithelial cells (EC), mucous cells (MC), basement membrane (BM) and connective tissue (CT), (H&E, x488).
Grass carp’s nostril at day one post-exposure to 1 mg/L diazinon showing denudation of epithelial surface (arrow) (H&E, x488).

EM micrograph of vacuolated epithelial cells (arrow) of grass carp’s nostril exposed to 4 ppm diazinon at 20-22°C and normal epithelial cells (EC), (x3, 439 um)

(A) TEM micrograph of vacuolated epithelial cell of grass carp’s nostril with abnormal nuclei (AN), (x7, 410) and (B) Normal epithelial cell with normal nuclei (NN), (x7, 410).

SEM micrographs of nasal epithelial cells of normal grass carp showing amorphous proteinaceous materials, vesicles and cell surface canals, Mag: A= x800, B= x2500, C= x5000, D= x8000.

SEM micrograph of nasal epithelial cells of grass carp exposed to 1 ppm diazinon showing an increase in droplet on the cell surface. Mag: A= x500, B= x1000, C= x2500, D= x5000.

SEM micrograph of nasal epithelial cells of grass carp exposed to 2 ppm diazinon showing a reduction in excretion of amorphous proteinaceous materials, vesicles numbers and blockage of cell surface canals. Mag: A= x800, B= x1000, C= x2500, D= x1500.

SEM micrograph of nasal epithelial cells of grass carp exposed to 4 ppm diazinon showing a severe reduction in excretion of amorphous proteinaceous materials and vesicles. The cell surface canals were blocked. Mag: A= x250, B= x2600, C= x2500, D= x2500.

Diagram of fish immunization procedure, with positive and negative control groups

Diagram of serial dilution for microagglutination test