

CHAPTER I

INTRODUCTION

Aquaculture is the fastest growing segment of both the world and U.S. agriculture industry. Among the various species of finfish grown worldwide, cyprinids make up the largest group of species cultivated. Cyprinids have the widest continuous distribution of any freshwater fish family, with about 194 genera and 2070 species (Kestemont, 1995). This family includes the foodfish : grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), and ornamental fish, koi carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*). In 1989, the world production of common carp, *C. carpio* and three species of Chinese carp, *C. idella*, *A. nobilis*, and *H. molitrix*, reached 4 million metric tons, representing more than 65% of the freshwater finfish production and 50% of the global total finfish (marine and freshwater) aquaculture production (Table 1). In addition, according to the 1996 FAO report, carp production is predicted to increase from 6.7 million tons in 1992 to 10.2 million tons by year 2000 (Anonymous, 1996).

Aquaculture is in a phase of rapid development and growth, and the intensification of fish farming often leads to the emergence of infectious and parasitic diseases. Few vaccines are presently available and their efficacy may be questioned in several cases, at least under practical field conditions. Aquaculture and fish farming need the availability of chemotherapeutic agents to avoid severe economic losses, and chemotherapy will remain one of the main means of controlling transmissible diseases in the future (Lafont, 1992).

Disease has become a critical factor hampering the development of grass carp culture in many countries. Among the various pathogens, the Gram-negative bacteria represent the greatest potential threat to aquaculture. Carp, particularly yearling and 2-year-old fingerlings, are susceptible to a wide variety of bacterial diseases. These include hemorrhagic disease, erythrodermatitis, columnaris disease, edwardsiellosis, enteric redmouth disease and furunculosis (Jeney and Jeney, 1995 ; Grondel et al., 1987) (Table 2). Of these bacterial diseases, *Aeromonas hydrophila* and other closely related motile aeromonads are among the greatest importance for carp culture (Jeney and Jeney, 1995).

Table 1. World aquaculture production of carp (in thousands of tons) in 1989 compared with the cyprinids, total freshwater fish and global finfish aquaculture production (Kestemont, 1995)

Species	(x 1000 tons)	%
Common carp (<i>Cyprinus carpio</i>)	987	13.5
Grass carp (<i>Ctenopharyngodon idella</i>)	947	12.9
Silver carp (<i>Hypophthalmichthys molitrix</i>)	1,433	19.6
Bighead carp (<i>Aristichthys nobilis</i>)	641	8.8
Cyprinids	5,466	74.6
Freshwater fish (cyprinids + other finfish)	6,060	82.7
Total (freshwater + marine finfish)	7,324	100

Table 2. Bacteria and associated bacterial disease of carp (modified from Jeney and Jeney, 1995)

Bacterium	Disease
<i>Aeromonas hydrophila</i>	Motile <i>Aeromonas</i> septicemia
<i>Aeromonas salmonicida achromogenes</i>	Carp erythrodermatitis
<i>Edwardsiella tarda</i>	Edwardsiellosis
<i>Pseudomonas fluorescens</i>	<i>Pseudomonas</i> septicemia
<i>Flexibacter columnaris</i>	Flexibacteriosis
<i>Flavobacterium branchiophila</i>	Bacterial gill disease
<i>Streptococcus</i> sp. (<i>S. faecalis</i> , <i>S. faecium</i> , <i>S. pyogenes</i>)	Streptococcal septicemia
<i>Mycobacterium</i> sp. (<i>M. marinum</i> , <i>M. fortuitum</i> , <i>M. chelonae</i>)	Mycobacteriosis

Chemotherapeutic agents are commonly used for the control of bacterial diseases in cultured fish. Currently in the United States, there are only two available antimicrobial products approved by the U.S. Food and Drug Administration (FDA) for the treatment of bacterial diseases in fish (Plumb, 1999). Oxytetracycline (Terramycin® ; Pfizer & Co., Groton, CT) is approved for treating ulcer disease, furunculosis, hemorrhagic septicemia caused by *Aeromonas salmonicida* and *A. liquefaciens*, and pseudomonas disease in salmonids, and controlling bacterial hemorrhagic septicemia and pseudomonas disease in catfish. A 1 : 5 combination of ormethoprim and sulfadimethoxine (Romet-30® ; Hoffman-LaRoche, Inc., Nutley, NJ) is approved for controlling furunculosis caused by *Aeromonas salmonicida* in salmonids and enteric septicemia caused by *Edwardsiella ictaluri* in catfish (Dixon and Issvoran, 1992 ; Griffin, 1992 ; Plumb, 1999). However, no antibiotics are approved for the treatment of any other bacterial infections in foodfish (Plumb, 1999). In addition, the overuse of these two particular chemotherapeutics in fish has led to the development of drug resistant bacteria and multiple antibiotic resistance in the aquaculture industry (McPhearson et al., 1991). Indeed, the spread of drug resistance among *Aeromonas* spp. is of concern because recent surveys indicate the emergence of these organisms as potential primary human pathogens (Chang and Bolton, 1987 ; Ansary et al., 1992). Furthermore, oxytetracycline in fish has been reported to cause depressed cellular (inhibited anti-SRBC response) and humoral (delayed antibody response) immunity (Grondel et al., 1987 ; Rijker et al., 1980a, 1981 ; Siwicki et al., 1989).

Ceftiofur (Naxcel®, The Upjohn and Pharmacia Co., Kalamazoo, MI), a relatively new broad spectrum β -lactamase-resistant third generation cephalosporin, has been used as a therapy for treatment of bacterial disease in cattle and swine (Aaron et al., 1995c ; Brander et al., 1991). This drug inhibits synthesis of the bacterial cell wall and is most effective against young, rapidly dividing bacteria (Bill, 1993). Ceftiofur *in vitro* has already

been shown to be efficacious against *Aeromonas* spp. isolated from ornamental fish (Dixon and Issvoran, 1992).

Therefore, due to an increase in acquired resistance of bacterial pathogens to oxytetracycline and the combination of ormetoprim and sulfadimethoxine, the lack of other FDA-approved antibiotics for fish, and the need for *in vivo* data on pharmacokinetics and withdrawal periods to support the use of other chemotherapeutics in aquaculture and ornamental fish industry, this investigation examines the usefulness of the antimicrobial drug, ceftiofur sodium, and evaluates the potential of this drug for consideration as an alternative bacterial therapeutic agent for finfish. The hypothesis tested was that ceftiofur, due to its efficacy, minimal toxicity, and lack of reported resistance in major fish pathogens, is a viable chemotherapeutic agent for use in the aquaculture industry. The evaluation of ceftiofur for use in fish was studied in three different approaches, pharmacokinetics, toxicological pathology and immunotoxicity. Grass carp, *C. idella* were selected as the fish model for this research since they are a good representative of both a foodfish and an ornamental fish and are one of the major carp species grown in the United States, Thailand and other countries worldwide.

CHAPTER II

LITERATURE REVIEW

Grass carp

Grass carp or white amur (*Ctenopharyngodon idella*)(Fig. 1), members of the minnow and carp family (Cyprinidae)(Table 3)(Shireman and Smith, 1983), were imported from Malaysia to the United States by the U.S. Fish and Wildlife Service in 1963. The fish is native to the river systems of Northern China and Southern Siberia. The grass carp has been introduced worldwide into over 50 countries. Besides being a foodfish, grass carp are a phytophagous fish and have been used successfully as an effective biological control agent for some varieties of submerged aquatic soft-stemmed vascular plants and branched algae (Shireman and Maceina, 1981 ; Wanqi, 1992 ; Whyne, 1992). This fish species plays an important role in management of macrophyte overabundance and also has great potential for foodfish aquaculture.

Like other members of the cyprinid family, grass carp have pharyngeal teeth, which are adapted for chewing their food. The grass carp has a notably low conversion ratio rate of food into fish biomass. Estimates of digestion of plant material by adults range from 50-70% (Hickling, 1966 ; Vietmeyer, 1976). They can survive in a wide range of water temperatures, ranging from 1-35 °C (34-95 °F), and short periods of low dissolved oxygen of 2 to 3 ppm (mg/l).

The grass carp are a pelagophilic spawner in relatively large rivers. Breeding migrations commence when water temperature reaches 15-17 °C (Aliev, 1976). In its native range, reproduction occurs during the monsoon season, when water levels rise quickly, temperatures range between 20 °C and 30 °C and current velocities vary from 0.7 and 1.8 m/sec. (Lin, 1935 ; Dah-Shu, 1957 ; Chang, 1966). The indigenous Chinese populations spawn from late April to June. The breeding season expands and becomes less distinct in tropical areas. Spawning can occur in all months of the year in naturalized and cultured grass carp, depending upon climate and artificial conditions. A minimum water temperature



Fig. 1. Grass carp or white amur (*Ctenopharyngodon idella*)

Table 3. Taxonomy of grass carp (Shireman and Smith, 1983)

Phylum Chordata

Subphylum Vertebrata

Superclass Gnathostomata

Grade Pisces

Subgrade Teleostomi

Class Osteichthyes

Subclass Actinopterygii

Infraclass Teleostei

Division Euteleostei

Superorder Otariophysi

Series Otophysi

Suborder Cypriniformes

Family Cyprinidae

Subfamily Leuciscinae

Genus *Ctenopharyngodon* Steindachner

Generic *Ctenopharyngodon* Steindachner

Species *Ctenopharyngodon idella*

of 18 °C has been reported as necessary for successful spawning in most acclimatized populations (Inaba et al., 1957 ; Aliev, 1976). Sexual maturity occurs at ages from 1 to 11 years and standard lengths from 58 to 67 cm in females. Males mature an average of one year earlier at standard lengths from 51 to 60 cm. Absolute fecundity ranges from 10,000 to 2,000,000 eggs with an average of 500,000 for 5 kg to 7 kg brood stock.

Antibacterial Chemotherapeutics in Aquaculture

Current FDA-approved antibacterials available to veterinarians, fish health specialists and fish producers in the United States are extremely limited in number and approved usages. There are only two antimicrobials available that are approved by the U.S. Food and Drug Administration (FDA) as feed additives for the treatment of bacterial disease in fish (Stoffregen et al., 1996 ; Plumb, 1999). Oxytetracycline (Terramycin® ; Pfizer & Co., Groton, CT, USA) is a bacteriostatic antibiotic which is added to feed to control motile *Aeromonas* septicaemia, *Pseudomonas* septicaemia and enteric septicemia in catfish, as well as furunculosis, enteric redmouth, and vibriosis in salmonids, and ormethoprim and sulfadimethoxine (Romet-30® ; Hoffman-LaRoche, Inc., Nutley, NJ, USA) is approved for treating enteric septicemia of catfish and furunculosis in salmonids (Dixon and Issvoran, 1992 ; Griffin, 1992 ; Plumb, 1999). Only FDA-approved drugs may be considered for use in food-production aquatic species. Each drug is approved with limited and specific indications for use including treatable fish species and pathogens, and at a specific dosage. No other antibiotics are approved for the treatment of other bacterial infections in fish (Plumb, 1999).

The most common routes of administration of chemotherapeutics to fish are inevitably unconventional as compared to treatment of other domestic animals. Oral application of antimicrobial agents in medicated feed is the most practical route for treating

large populations of fish, while parenteral administration may be useful for large brood stock or individually important ornamental fish. The major antimicrobial groups used in aquaculture worldwide are listed in Table 4. However, the range of potential fisheries chemotherapeutic agents, which have been proposed in the scientific literatures is more than those actually in use. There are many members of several major families of antibacterial agents in the development process, including *in vitro* efficacy testing against selected fish pathogens, and *in vivo* dose-titration studies, pharmacokinetic studies, target animal safety, and field trials to test the efficacy and safety of these products under controlled field conditions. Current registration efforts in the U.S. to develop and approve new antibacterials to treat commercial aquaculture species are summarized in Table 5 (Stoffregen et al., 1996).

Table 4. Major antimicrobial drug groups used in aquaculture worldwide (Alderman and Michel, 1992)

Drug group	Drug name	Route	Dose
<u>ANTIBIOTICS</u>			
β-lactams	Ampicillin	oral	50-80 mg/kg for 10 days
	Amoxicillin		
Aminoglycosides	Neomycin	oral/bath	50-80 mg/kg for 10 days
	Kanamycin		20 mg/l
Tetracyclines	Tetracycline	oral/bath	50-80 mg/kg for 10 days
	Oxytetracycline		20 mg/l
	Doxycycline		
Macrolides	Erythromycin	oral/bath	50 mg/kg for 10 days 2 mg/l for 1 h
Fenicoles	Chloramphenicol	oral/bath	50-80 mg/kg for 10 days 20 mg/l for 1 h
<u>SYNTHETIC BACTERIAL AGENTS</u>			
Sulfonamides	Sulfamerazine	oral	200 mg/kg for 10 days
	Sulfadimethoxine		
Potentiated Sulfonamides	Trimethoprim +	oral	50 mg/kg for 10 days
Sulfonamides	Sulfadiazine		
Nitrofurans	Furazolidone	oral	50-80 mg/kg for 10 days
	Furaltadone		
	Nifurpirinol	oral	10-50 mg/kg for 10 days
Quinolones	Oxolinic acid	oral	12 mg/kg for 10 days
	Flumequine		

Table 5. Current research efforts to support development and approval of new antibacterials for fish (Stoffregen et al., 1996)

1. Fluoroquinolones and Quinolones

1. Nalidixic acid (Kitao et al., 1989 ; Elston, 1992 ; Uno et al., 1992 ; Jarboe et al., 1993 ; Stoffregen et al., 1996)
1. Oxolinic acid (Rodgers and Austin, 1983 ; Austin et al., 1983 ; Cravedi et al., 1987 ; Archimbault et al., 1988 ; Barker et al., 1990 ; Bowser, et al., 1990 ; Hustvedt et al., 1991 ; Hustvedt and Salte, 1991 ; Rogstad et al., 1993 ; Kleinow et al., 1994 ; Samuelsen and Lunestad, 1996 ;
1. Piromidic acid (Katae et al., 1979a ; Katae et al., 1979b ; Katae et al., 1979c ; Oida et al., 1982)
1. Flumequine (Scallan and Smith, 1985 ; Sohlberg et al., 1990 ; Takahashi et al., 1990 ; Barnes et al., 1991 ; Rogstad et al., 1993 ; Elema et al., 1994 ; Van der Heijden et al., 1994 ; Della Rocca et al., 1996 ; Malvisi et al., 1997 ; Samuelsen, 1997 ; Samuelsen and Ervik, 1997 ; Torkildsen et al., 2000)
1. Norfloxacin (Bowser and Babish, 1991 ; Heo and Kim, 1996)
1. Enrofloxacin (Baytril® ; Bayer Animal Health, Shawnee Mission, Kansas)
(Bowser and Babish, 1990 ; Bowser et al., 1990 ; Bowser and Babish, 1991 ; Dalsgaard and Bjerregaard, 1991 ; Stoffregen et al., 1993 ; Bowser et al., 1994 ; Hsu et al., 1994a, 1994b ; Hsu et al., 1995 ; Stoffregen et al., 1996 ; Williams et al., 1997 ; Intorre et al., 2000)
1. Ciprofloxacin (Cipro® ; Miles Pharmaceuticals, West Haven, Connecticut)
(Nouws et al., 1988 ; Lewin and Hastings, 1990 ; Chatteriee and Chatteriee, 1992 ; French, 1990 ; Hanson and Thune, 1994 ; Heo and Seo, 1997)

Table 5. (Continued)

1. Sarafloxacin (Sarafin® ; Abbott Labs, Chicago)

(Stamm, 1989 ; Plumb and Vinitnantharat, 1990 ; Wilson and MacMillan, 1989 ; Martinsen et al., 1991 ; Johnson et al., 1992 ; Thune and Johnson, 1992 ; Johnson et al., 1993 ; Martinsen et al., 1994 ; Gingerich et al., 1995)
9. Difloxacin (A-56619, Abbot Labs)

(Stamm, 1989 ; Wilson and MacMillan, 1989 ; Elston et al., 1994)
2. Sulfonamides (Horseberg et al., 1997 ; Samuelsen et al., 1997)
 1. Sulfadimidine (Grondel et al., 1986 ; van Ginneken et al., 1991 ; Grondel et al., 1992)
 1. Sulfachloropyridazine (Alavi et al., 1993 ; Oppegaard et al., 1995)
 1. Trimethoprim (Bergsjø et al., 1979 ; Bergsjø and Sognen, 1980 ; Kimura et al., 1983 ; Salte and Liestoel, 1983 ; Fonseca et al., 1991 ; Torkildsen et al., 2000)
3. Macrolides
 1. Erythromycin (Erythro® ; Sanofi Animal Health, Inc., Overland Park, Kansas)

(Shitomitsu et al., 1980 ; Katae, 1982 ; Groman and Klontz, 1983 ; Evelyn et al., 1986a, 1986b ; Kawakami and Kusuda, 1990 ; Moffitt, 1991 ; Moffitt, 1992 ; Lee and Evelyn, 1994 ; Moffitt and Haukenes, 1995 ; Peters and Moffitt, 1996 ; Moffitt and Kiryu, 1999)
4. Penicillin derivatives
 1. Amoxicillin (Amoxi® ; Pfizer Inc., North American Animal Division, West Chester, Pennsylvania)

(Nakauchi et al., 1988 ; Kitao et al., 1989 ; Elston, 1992 ; Brown and Grant, 1993 ; Inglis et al., 1993 ; Barnes et al., 1995 ; Della Rocca et al., 1998)

Table 5. (Continued)

5. Aminoglycosides

Gentamicin (Gentocin® ; Schering-Plough Corp., Lafayette, New Jersey)

(Setser, 1985 ; Rolf et al., 1986 ; Ceschia et al., 1987 ; French, 1990 ; Crow and Brock, 1993 ; Jones et al., 1997)

6. Chloramphenicol and derivatives (Cravedi et al., 1985)

1. Flofenicol (FLOR ; Schering-Plough Corp., Lafayette, New Jersey)

(Fukui, et al., 1987 ; Inglis and Richards, 1991 ; Martinsen et al, 1993 ; Horsberg et al., 1994 ; Nordmo et al., 1994 : Stoffregen et al., 1996 ; Torkildsen et al., 2000)

Approved antibacterial agents in US

1. Oxytetracycline (Terramycin for Fish®)

Tetracyclines are a group of broad spectrum antibiotics whose action is bacteriostatic, binding the 30S subunit of the microbial 70S ribosomes and inhibiting protein synthesis (mRNA translation) by blocking the attachment of aminoacyl-tRNA units. The most widely used and only FDA approved tetracycline in aquaculture is oxytetracycline, although doxycycline and chlortetracycline have been historically used.

In salmonids, oxytetracycline is approved for the control of furunculosis caused by *Aeromonas salmonicida*, bacterial hemorrhagic septicemia caused by *Aeromonas hydrophila*, ulcer disease caused by *Hemophilus piscium* and pseudomonas disease caused by *Pseudomonas* spp. In catfish, oxytetracycline is approved for the control of bacterial hemorrhagic septicemia caused by *A. hydrophila* and pseudomonal disease caused by *Pseudomonas* spp. In lobsters, *Homarus americanus*, oxytetracycline is approved for the control of gaffkemia caused by *Aerococcus viridans*. The pharmacokinetics of oxytetracycline in finfish have been studied in ayu (Uno, 1996), rainbow trout (Bjorklund and Bylund, 1990 ; Black et al., 1991 ; Rogstad et al., 1991) , Atlantic salmon (Bruno, 1989 ; Elema et al., 1996), carp (Grondel et al., 1987), catfish (Grondel et al., 1989), sea bream and sea bass (Malvisi et al., 1996), striped bass (Xu and Rogers, 1994) and tilapia (Xu and Rogers, 1995). The recommended oral dose for fish is 50-75 mg/kg given once daily for 10 consecutive days or 20 mg/kg three times a day for 10 consecutive days (Plumb, 1999 ; Stoskopf, 1993). The withdrawal time is 21 days for catfish and salmonids and 30 days for lobsters (Stoffregen et al., 1996).

Because oxytetracycline is bacteriostatic, careful consideration regarding prolonged administration of these tetracycline chemotherapeutic agents is essential, especially when

the immune system of fish may already be compromised by environmental conditions, husbandry techniques, and infectious diseases (Grondel and Boesten, 1982). Oxytetracycline has been reported to cause depressed cellular (inhibited anti-SRBC response) and humoral (delayed antibody response) immunity in carp, *Cyprinus carpio* (Grondel et al., 1987 ; Rijkers et al., 1980a, 1981). In rainbow trout, high organ drug concentrations were found in liver, gills, kidney, gonads, gastrointestinal tract, vertebral body, skin and mucus (Black et al., 1998 ; Rogstad et al., 1991). Bioavailability in common carp ranged from 0.6 % (PO in feed) to 80 % (IM)(Grondel et al., 1987). The oral route resulted in very poor bioavailability, which may reflect the lack of a stomach and absence of pH variation in common carp.

2. Ormethoprim + Sulfadimethoxine (Romet-30®)

Romet-30® is a 1 : 5 mixture of ormethoprim and sulfadimethoxine. Potentiated sulfonamides are bactericidal, affecting bacterial nucleic acid synthesis by inhibition of folic acid metabolism. The sulfonamide blocks dihydrofolic acid synthesis and ormethoprim inhibits tetrahydrofolic acid synthesis by blocking the enzyme dihydrofolate reductase (Stoffregen et al., 1996). Romet 30® was approved by the FDA to control enteric septicemia in catfish caused by *Edwardsiella ictaluri* (Wise and Johnson, 1998) and furunculosis caused by *Aeromonas salmonicida* in salmonids (Plumb, 1999).

The pharmacokinetics and tissue distribution of sulfadimethoxine have been studied in catfish (Squibb et al., 1988 ; Michel et al., 1990), trout (Kleinow and Lech, 1988 ; Kleinow et al., 1992 ; Uno et al., 1993), and the American lobster (James and Barron, 1988 ; Barron et al., 1988). The pharmacokinetics of ormethoprim have also been characterized in rainbow trout (Droy et al., 1990), catfish (Plakas et al., 1990) and the American lobster (James and Herbert, 1988).

Romet-30® is incorporated into feed to provide a 50 mg/kg dose per day for 5 days (Plumb, 1999). Withdrawal time is 42 days for salmonids, but only 3 days for catfish (Stoskopf, 1993 ; Plumb, 1999). Unfortunately, this drug has a problem with palatability (Wilson and Poe, 1989 ; Robinson et al., 1990). In addition, Romet-30® has also been reported to induce antibiotic resistance in *E. ictaluri* (Taylor and Johnson, 1991).

Concerns regarding antibacterials use

Protection of fish against infectious and parasitic diseases has mainly relied upon chemotherapeutic agents (drugs and chemicals) and this has led to the use, overuse, and misuse of chemotherapeutics, especially antibiotics. The most serious side effects of appropriate and inappropriate drug usage include the following :

1. The possibility for development of resistant bacterial strains

Overuse of chemotherapeutic agents in fish and the use of medicated feeds for a long period of time throughout a disease epizootic have led to the development of drug resistant bacteria and multiple antibiotic resistance (McPhearson et al., 1991 ; Wise and Johnson, 1991). The drug resistant properties of bacteria can be transferred to pathogenic bacteria, resulting in reduced efficacy of antibiotic treatment for animal and human diseases caused by resistant pathogens. There are two major mechanisms of bacterial resistance to antimicrobial drugs. First, plasmids (extra-chromosomal DNA) can be passed horizontally to other bacteria of the same or different species. This method occurs with most of the commonly used antimicrobials such as tetracyclines, chloramphenicol, sulfonamides and penicillin derivatives. The second mechanism of bacterial resistance is via chromosomal alteration, thus creating mutations passed to progeny but not neighbor cells. This pattern is used for resistance to quinolones and fluoroquinolones (Stoffregen et al., 1996).

Transferable R-plasmids have been found in high frequencies in drug-resistant strains of *A. hydrophila* isolated from cultured carp (Akashi and Aoki, 1986). Plasmid DNA has also been found in wild type aeromonads. Multiple drug resistant strains of fish pathogens are encoded with resistance to a wide variety of drugs, and the drug resistant markers depend on each fish pathogen (Hastein, 1995). *Aeromonas hydrophila* has been shown to possess two to nine plasmids (Ansary et al., 1992). The drug-resistant marker of R-plasmids in *A. hydrophila* were almost exclusively against tetracycline and sulfonamide, while R-plasmids encoded with resistant markers to chloramphenicol, streptomycin and sulfonamide were only frequently detected from *A. salmonicida* (Akashi and Aoki, 1986). Therefore, the spread of drug resistance among *Aeromonas* spp. is of some concern because recent surveys indicate the emergence of these organisms as primary human pathogens (Chang and Bolton, 1987 ; Ansary et al., 1992).

The emergence of resistant bacteria may be considered a result of misuse of antibiotics such as to treat an infection of unknown origin and excessive dosage utilization (Conn and Gebhart, 1989). Repeated use of the same drug to control bacterial diseases almost invariably results in development of resistance among the bacterial fish pathogens. The development of resistance occurs because of increased disease problems and the limited number of drugs really suited for the control of fish pathogens (Hastein, 1995). When prophylactic drug use is for the generalized prevention of infections, it is almost always a failure (Sande and Mandell, 1985 ; Weinstein, 1954). Regardless of whether the resistance is mediated by excessive or multiple exposure to antibiotics or by another mechanisms, the resulting pool of antibiotic-resistant bacteria may have public health significance (McPhearson et al., 1991). Dixon et al. (1990) reported that resistance of *Aeromonas* spp. to the two approved antibiotics in the U.S., oxytetracycline and Romet, was extremely high, with 67 % of the isolates being resistant to Romet and 97 % resistant to tetracycline.

2. Possible residues in food fish

During clinical treatment of susceptible bacterial strains, drug levels in plasma and tissue should be maintained higher than the MIC expected for mutant strains to prevent their occurrence (Martinsen et al., 1992). Clinical resistance depends on the MIC for the bacterial strain together with the pharmacokinetic profile of the drug in fish (Martinsen et al., 1992).

Fish are poikilotherms whose metabolic rates are largely controlled by the temperature of the water environment. Since an increase of 1 °C in water temperature can increase metabolic rates by 10 % (Alderman and Michel, 1992), the pharmacokinetic parameters of uptake, distribution and elimination may be expected to vary markedly with water temperature. Pharmacokinetic studies clearly demonstrated that drug residues were more persistent in cold water species of fish. Also, estimates of residues in fish cannot be extrapolated between routes of administration or for other formulations of the drug.

The illegal excessive use of drugs for other purposes, such as the use of antimicrobials as growth promoters in fish feed and the prophylactic use of drugs to prevent bacterial infections, is unfortunately all too often a permanent part of production. This additional use of drugs has encouraged not only the selection of drug-resistant bacteria, but also affects the amount of residues accumulated in the fish.

Drug elimination in fish is closely related to the water temperature. Therefore, withdrawal periods have been determined depending on the water temperature. Fish treated with oxytetracycline at a dose of up to 75 mg/kg of fish body weight for 10 consecutive days have a withholding period of 60 days at water temperatures above 10 °C and of 100 days at water temperatures between 7 to 10 °C (Salte and Liestol, 1983). Fish treated with potentiated sulfonamides (sulfadiazine + trimethoprim) at a dose of up to 30 mg/kg of fish body weight for 10 consecutive days, have a withholding period of 60 days at water temperatures above 10 °C. However, the drug should not be used below 10 °C because of

prolonged residue persistence at lower temperature (Salte and Liestol, 1983). Withdrawal times for any given antibacterial exhibit considerable variation among countries. The required withdrawal periods for oxytetracycline among six selected countries which produce Atlantic salmon and/or rainbow trout are shown in Table 6. The use of antibacterials in the culture of fish is also of concern with regard to human health. Unintended ingestion of antibacterials can have a number of adverse consequences including (1) toxic effects, which may lead to mutagenic and teratogenic effects, (2) hypersensitivity, which may manifest itself as a skin reaction and/or symptoms involving the respiratory system and (3) antibacterial resistance in microflora, especially species of pathogenic enteric bacteria (Yndestad, 1992).

Table 6. Required withdrawal times following oxytetracycline chemotherapy in Atlantic salmon and/or rainbow trout (Weston, 1996).

Country	Required withdrawal time (days)	
	at 8-12 °C	at > 12 °C
Canada	42	42
Finland	60	40
Norway	80	40
Sweden	60	30
United States	21	21
United Kingdom	50	33

3. Environmental effects

Other consequences of the intensive use of antibiotics in aquaculture are the accumulation of drugs and their metabolites in aquatic environments, on-site sediment contamination of drugs administered orally, dispersion in the whole aquatic ecosystem and their effects on indigenous microbes and non-target organisms of drugs applied by bath treatment or with the feed. The major routes by which drugs end up in the environment are through the excessive feeding of medicated feeds, or from uneaten food due to reduced appetite in fish. Residues can also occur indirectly by passing through the fish during treatment, or effluent from bath treatments. Some drugs entering the environment may be taken up by wild fish, shellfish and crustaceans, thus resulting in a public health problem, while others may end up in the sediments. The sediments serve as a long-term reservoir for residues of many drugs. Of the antibacterials used, oxytetracycline is among the most persistent. The drug does not appear to be microbially degraded and is lost from the sediment only by dissolution and diffusion into the overlying water (Samuelsen, 1989). Oxytetracycline in sea water is stable for a period of at least two months (Lunestad, 1992). The oxytetracycline residue persists in sediments for months and can be detected as long as 7 months after medication (Hansen et al., 1992). In addition to the detrimental effect of the antibacterial agents on the total number of bacteria in the sediment, an increased resistance against oxytetracycline and oxolinic acid may be detected for weeks and thus create a potential risk for transferable resistance to fish pathogenic bacteria and hence possible reduced effect for future treatment (Samuelsen, 1992). In addition, elevated resistance may be observed in conjunction with reductions in total microbial density or changes in functional properties of the sedimentary microbial community as evidenced by high rates of oxygen consumption and sulfate reduction rates (Hansen et al., 1992). Antibacterial-induced reduction in microbial densities could also have indirect consequences to meio- and

macro-faunal invertebrates, for which sediment microbes may be an important food source (Weston, 1996).

Ceftiofur

Ceftiofur (Naxcel®, The Upjohn and Pharmacia Co., Kalamazoo, MI, USA) is a third generation cephalosporin that has broad spectrum β -lactamase-resistant activity and is very effective in the control of Gram-positive and Gram-negative bacterial pathogens of veterinary importance both *in vivo* and *in vitro*. Ceftiofur has been approved by the U.S. Food and Drug Administration for intramuscular injection for the treatment of certain bacterial respiratory diseases in beef cattle, dairy cattle, swine and day-old chicks (FDA, 1988, 1991, 1992). Ceftiofur sodium is the sodium salt of (6R,7R)-7-[(2-amino-4-thiazolyl)-Z- (methoxyimino)acetyl]amino-3-[(2-furanylcarbonyl)thio]methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (Yancey et al., 1987)(Figure 2).

Ceftiofur, like other cephalosporins, is bactericidal against susceptible bacteria by inhibiting synthesis of the bacterial cell wall and is most effective against young, rapidly dividing bacteria (Bill, 1993). The mechanisms of action of cephalosporins is analogous to that of penicillins which bind to specific penicillin-binding proteins that serve as drug receptors on bacteria, inhibit cell wall synthesis by blocking transpeptidation of peptidoglycan (due to a structural similarity to acyl-D-alanyl-D-alanine) and activate autolytic enzymes in the cell wall, which result in a defective barrier and an osmotically unstable spheroplast that results in bacterial cell death (Jawetz, 1992 ; Plumb, 1995).

Cephalosporins are semi-synthetic derivatives of 7-amino cephalosporanic acid, a close analog of 6-penicillanic acid (Descotes, 1990). Compared to the original β -lactamase penicillin, cephalosporins differ in the nucleus of their chemical structures (Conn and Gebhart, 1989) (Fig. 3). Cephalosporins differ from each other in their acyl side chain at

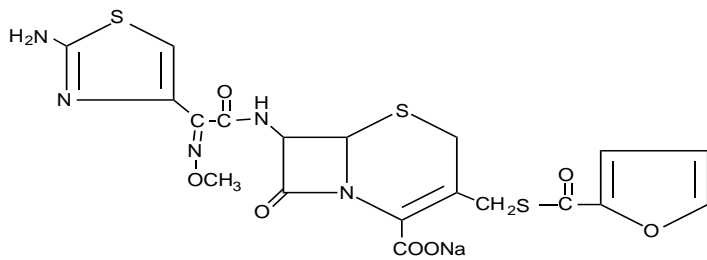
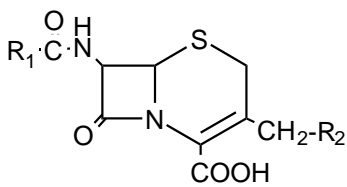
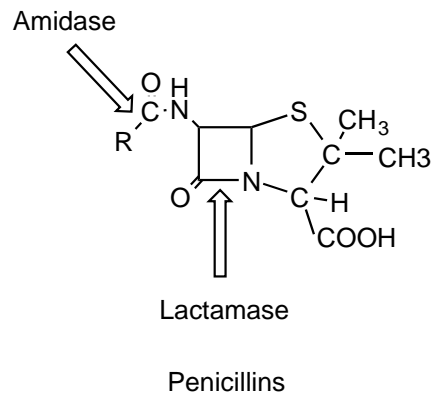


Fig. 2. Chemical structure of the ceftriaxone sodium.



Cephalosporins (Substituted 7-aminoccephalosporanic acid)

Fig. 3. The structure of cephalosporin compared to penicillin (Conn and Gebhart, 1989)

the C-7 position (Descotes, 1990). Classifications of cephalosporins are based on their antibacterial spectrum, their metabolic stability and stability against hydrolysis of β -lactamases from different organisms, or their chemistry (Dalhoff, 1998)(Table 7). Due to the aminothiazol-containing group of 3rd-generation cephalosporins and the presence of the methoxy side chain of imino group, ceftiofur typically is active against Gram-negative bacteria, retains good activity for Gram-positive bacteria, and is resistant to many β -lactamase enzymes (Neu, 1982 ; Yancey et al., 1987). Ceftiofur *in vitro* has been shown to be efficacious against *Aeromonas* spp. isolated from ornamental fish (Dixon and Issavoran, 1992)(Table 8).

The recommended dose of ceftiofur sodium for cattle and horses is 1.1 – 2.2 mg/kg IM once daily for 3 days and 2.2-4.4 mg/kg at 24-hour intervals, respectively. No slaughter withdrawal or milk withholding time is required in these animals when administered as recommended (FDA, 1988). The metabolism of ceftiofur has been studied in rats (Jaglan et al. 1989), dairy cows (Jaglan et al., 1992) and swine (Gilbertson et al., 1995). Ceftiofur is rapidly metabolized to desfuroylceftiofur and furoic acid (Fig 4). Desfuroylceftiofur is further metabolized to disulfides and also bound to macromolecules in plasma and tissues. No desfuroylceftiofur was detected free in the plasma but was always found conjugated to protein and to cysteine (Olson et al., 1998). The scheme on the metabolism of ceftiofur is summarized in Fig. 5.

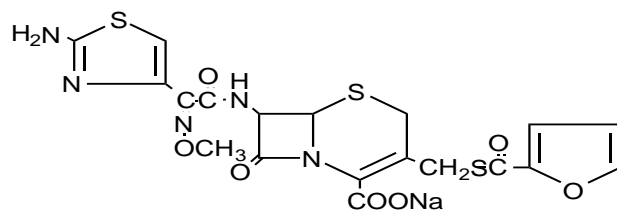
Cephalosporins are widely distributed to most tissues and fluids (Halstead et al., 1992 ; Cervantes et al., 1993). In rats, cattle and dogs, most of the drug was excreted in the urine and feces, with the highest residues being observed in the kidney tissue (Jaglan et al., 1989 ; Brown et al., 1991 ; Soback et al., 1991 ; Brown et al., 1995). An average of 62.6% of the total residue in the kidney was covalently bound to proteins and the remaining 37.4% was free. The free metabolites in the kidney were unknown polar A, unknown polar B, polar C and desfuroylceftiofur cysteine disulfide.

Table 7. The classification of cephalosporins (Dalhoff, 1998).

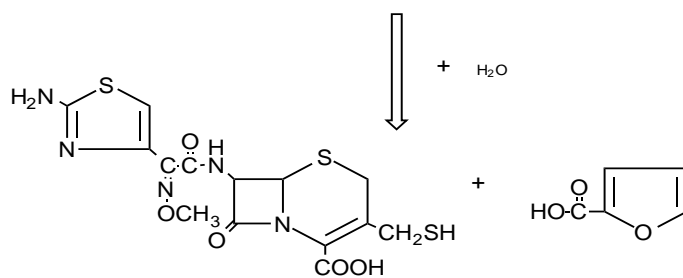
First-generation cephalosporins	Second-generation cephalosporins	Third-generation cephalosporins	Fourth –generation cephalosporins	Orally active cephalosporins
Cefadroxil	Cefaclor	Cefoperazone	Cefetamet	Cephaloglycin
Cefazolin	Cefamandole	Cefotaxime	Cefotetan	Cefaclor
Cephalexin	Cefonicid	Ceftazidime	Cefodizime	Cephalexin
Cephalothin	Ceforanide	Ceftizoxime	Cefpiramide	Cephradine
Cephapirin	Cefoxitin	Ceftriaxone		Cefadroxil
Cephradine	Cefmetazole	Cefixime		Cefroxadine
Cephaloridine	Cefotetan	Latamoxef (Moxalactam)		Cefixime
Cephacertril	Cefuroxime	Cefsulodine		Cefprozil
Cefazedone	Cefotiam	Ceffriaxone		Ceftibuten
		Cefmenoxime		Loracarbef
		Cefpirome		
		Cefepime		
		Ceftiofur		

Table 8. Activity of various antimicrobials, including ceftiofur, against *Aeromonas* spp. isolated from ornamental fish

Antibiotic	% resistant isolates		Comments
	(Dixon et al., 1990)	(Dixon and Issvoran, 1992)	
Ampicillin	100	100	
Tetracycline	96	71	FDA approved for food fish
Nalidixic acid	30	26	
Erythromycin	64	19	
Romet-30®	67	12	FDA approved for food fish
Sulfamethoxazole/ trimethoprim	60	12	
Oxolinic acid	27	12	
Trimethoprim	44	9.5	
Ceftiofur	0	-	Proposed antimicrobial



Ceftiofur Sodium



Desfuroylceftiofur

Furoic acid

Fig. 4. Metabolism scheme of ceftiofur in bovine plasma (Jaglan et al., 1990)

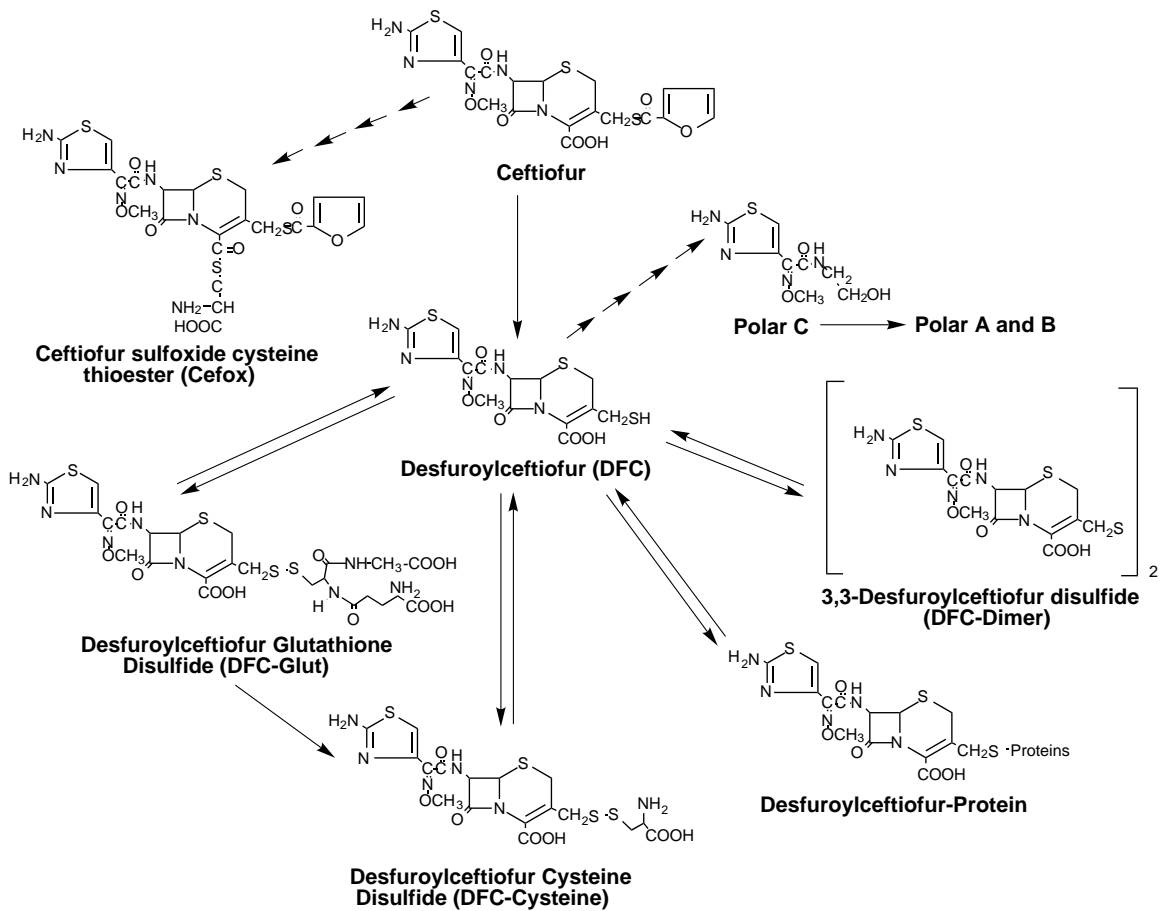


Fig. 5. Metabolism of ceftiofur proposed in rats, cattle and swine (Modified from Beconi-Barker et al., 1995).

Protein-binding of the drug is widely variable and species specific. Cephalosporins and their metabolites are excreted by the kidneys, via tubular secretion and/or glomerular filtration (Plumb, 1995). About 65 % of residue was bound to macromolecules and 35 % was free in the kidneys of both rats and pigs. Desfuroylceftiofur cysteine disulfide was a major metabolite in the kidneys of both rats and pigs. Protein-bound (i.e. albumins) metabolites accounted for 62.6 %, desfuroylceftiofur cysteine disulfide for 12.3 %, unknown polar A for 7.6 %, unknown polar B for 12.3 % and polar C for 11.3 %. However, quantitative differences in the amount of metabolites between animals, as well as species, are probably due to the reversibility of the disulfide metabolites and the amount of endogenous reactants such as cysteine and glutathione and their oxidative products (Beconi-Barker et al., 1995).

Resistance to cephalosporins can occur by various mechanisms such as poor penetration of the drug into bacteria, lack of the penicillin-binding proteins for a specific drug, degradation of drug by beta-lactamase (cephalosporinase), appearance of special beta-lactamases or failure of activation of autolytic enzymes in the cell wall (Jawetz, 1992).

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CHAPTER III

PLASMA PHARMACOKINETICS OF CEFTIOFUR IN GRASS CARP, *Ctenopharyngodon idella*, AFTER VARIOUS ROUTES OF ADMINISTRATION

(Submitting to Journal of Veterinary Pharmacology and Therapeutics)

**Plasma pharmacokinetics of ceftiofur in grass carp,
Ctenopharyngodon idella, after various routes of administration**

ABSTRACT

Ceftiofur sodium, a third generation cephalosporin, was administered as a single dose to grass carp (*Ctenopharyngodon idella*) by four different routes : intracardiac (IC), intraperitoneal (IP), intramuscular (IM) and oral (PO) administration at a dosage of 8 mg/kg body weight. Serial blood samples were obtained and plasma samples were analyzed by high performance liquid chromatography for ceftiofur (as measured by its metabolite, desfuroylceftiofur (DFC) and DFC-related metabolite concentration). Disposition pharmacokinetic data were best described by a two compartment open model for IC and non-compartment model with no lag time for IP and IM administrations. Oral absorption of ceftiofur was not observed in this species. Following IC, IP and IM ceftiofur sodium administration, the final elimination half-lives, maximum plasma concentration, time to reach maximum concentration, volume of distribution and plasma clearance were 0.38, 0.45 and 13.86 hours ; 157.09, 31.54 and 8.86 µg/ml ; 0, 0.25 and 0.5 hours ; 0.09, 0.17, 0.53 l/kg ; and 0.21, 0.26, 0.26 ml/min.kg, respectively. Desfuroylceftiofur metabolite was highly bounded with plasma protein at pH 7.0 and 8.0.

INTRODUCTION

Outbreaks of disease have become a critical factor which has hampered the development of aquaculture in many countries. Among the various disease agents, Gram-negative bacteria cause a significant amount of infection and loss of cultured fish (Austin and Austin, 1985). However, only two antibacterials are legally available in the United

States for foodfish, oxytetracycline (Terramycin®, Pfizer, Inc., Groton, CT) and sulfadimethoxine with ormetoprim in a 5: 1 combination (Romet-30®, Hoffmann-La Roche, Inc., Nutley, NJ)(Stoffregen et al., 1996 ; Plumb, 1999).

Ceftiofur sodium (Naxcel®, Upjohn and Pharmacia Co. Kalamazoo, MI), a semisynthetic third generation cephalosporin, has a broad range of *in vitro* activity against a variety of bacterial pathogens, especially Gram-negative bacteria, while still retaining the Gram-positive activity of the first and second generation cephalosporins. This drug is bactericidal against susceptible bacteria and affects cell wall synthesis by inhibiting mucopeptide synthesis in the cell wall resulting in a defective barrier and an osmotically unstable spheroplast (Plumb, 1999).

In aquaculture, diseases caused by bacteria of the genus *Aeromonas* sp., especially *Aeromonas hydrophila*, are among the most common bacterial infections in freshwater fish. There is a need to augment the traditionally used drugs with other antimicrobial agents because a number of these bacterial fish pathogens are resistant to the two currently approved drugs (Smith et al., 1994). Ceftiofur sodium has been shown to be highly effective *in vitro* against 42 isolates of *Aeromonas* spp. from ornamental fish, while resistance to the FDA-approved antimicrobial drug, oxytetracycline, was as high as 71 % (Dixon and Issvoran, 1992). Thus, information about the pharmacokinetic profile of ceftiofur sodium in a commercially important species of fish is necessary for responsible therapeutic use of the drug. Pharmacokinetics of ceftiofur sodium have been studied in a variety of other animal species (Brown et al., 1991a ; Craigmill et al., 1991 ; Soback et al., 1991 ; Jaglan et al., 1994), but not in fish. Thus, this study was designed to characterize the plasma disposition of ceftiofur sodium and its metabolites in grass carp (*Ctenopharyngodon idella*) after a single dose via intracardiac, intraperitoneal, intramuscular and oral routes. Grass carp were used as a representative of both ornamental and food fish species.

MATERIALS AND METHODS

Experimental animals and aquaria conditions

Juvenile triploid grass carp obtained from a commercial fish farm (Hopper-Stephen Hatcheries, Inc. Lonoke, AK), were placed in 1900-liter tanks at a density of 30 fish per tank and allowed to acclimate at 25 ± 3 °C for at least two weeks before starting the experiment. Water temperature, pH, total ammonia and nitrite concentrations were monitored (HACH Company, Loveland, CO) and maintained daily at 25-27 °C, 7.0 – 7.5, less than 0.2 ppm and 0.02 ppm, respectively, during the experiment. Fish were fed a commercial brood stock (35-40 % protein) pelleted diet (Zeigler Bros., Inc., Gardners, PA) daily at 2 % of their body weight. The study included 78 grass carp for each of the IP, IM and PO routes and 60 fish for the IC route of administration. During the chemotherapeutic trials, the fish were temporarily held in 5 m³ fiberglass tanks supplied with aerated filtered water.

Chemotherapeutic and Dosing Regimen

Ceftiofur sodium (Naxcel®, 1g vial, Upjohn and Pharmacia Co. Kalamazoo, MI) was reconstituted according to the label directions with 20 ml sterile water to achieve a solution with a final concentration of 50 mg ceftiofur free acid equivalents/ml. To facilitate ceftiofur sodium administration, grass carp were anesthetized in water containing approximately 100 mg/l of tricaine methanesulfonate (MS-222, Sigma Chemical Company, St. Louis, MO). Each fish was weighed and then received a single injection of ceftiofur sodium at a dose of 8 mg ceftiofur sodium free acid equivalents/kg. A single dose of 8 mg/kg in this study was based on the disposition kinetics of ceftiofur sodium in lactating cows (Soback et al., 1991) and the minimum inhibitory concentration of *Aeromonas* spp.

isolated from ornamental fish (Dixon and Issvoran, 1992). Ceftiofur sodium was delivered to grass carp by injecting the drug into the heart for intracardiac administration (IC), along the ventral midline between the pelvic and anal fin for intraperitoneal administration (IP), or into the epaxial muscle (approximately two rows of scale below the dorsal fin) for intramuscular administration (IM) using a 23 gauge 1" needle. For oral administration (PO), a stainless steel gavage needle was used to place the ceftiofur sodium directly into the digestive tract.

Blood Sample collection

At each sampling time, six fish were arbitrarily removed from the experimentally exposed fish and anesthetized with MS-222. Blood samples (approximately 0.7 ml) were serially collected at 0.08, 0.25, 0.5, 1, 2, 4, 8, 18, 24, 48, 72, 96 hours after IP (n = 6), IM (n = 78) and PO (n = 78) administration from the caudal blood vessels with a 3 cc heparinized syringe attached to a 23 gauge 1" needle. For the IC study, blood samples were collected at 0.08, 0.25, 0.5, 1, 2, 4, 8, 18 and 24 hr post-administration (n = 6). Six fish from each route were bled as controls. Plasma was obtained from blood samples in Microtainer Tubes (Plasma Separator Tube with lithium heparin, Becton Dickinson & Company, Franklin Lakes, NJ) by centrifugation at 1500 x g and frozen at - 80 °C until analysis.

Chemical analysis of ceftiofur sodium and metabolites

Plasma samples were analyzed for ceftiofur (as measured by combined desfuroylceftiofur (DFC) and DFC-related metabolite concentration) using a modification of the high performance liquid chromatographic (HPLC) procedure developed by Hamlow et al. (1994a, 1994b). Briefly, DFC, the metabolite from hydrolysis of ceftiofur sodium,

associated with plasma protein or other sulfur-containing compounds in the plasma samples was cleaved with dithioerythritol ($C_4H_{10}O_2S_2$; Sigma -Aldrich, Inc., Saint Louis, MO), yielding DFC. The DFC was then derivatized with iodoacetamide (C_2H_4INO ; Sigma -Aldrich, Inc.) to form the more stable derivative desfuoylceftiofur acetamide (DCA). Finally, all DCA in plasma samples was analyzed via gradient HPLC on a C-18 column (BDS-Hypersil C-18, 5 μ m, 250 x 4.6 mm, Keystone Scientific, Inc., Bellefonte Park, NJ) with UV detection (Beckman model 166; Altex Scientific Operations, Berkeley, CA) at wavelength 254 nm. The detection limit was 0.1 μ g/ml.

Protein binding

The degree of plasma protein binding of ceftiofur sodium was determined by equilibrium dialysis (Alavi et al., 1993) using pooled plasma from untreated grass carp ($n = 16$). Plasma protein binding was determined by enclosing one millimeter aliquots of plasma in weighted dialysis units (Spectra/Por DispoDialyzer, Spectrum®, Laguna Hills, CA). The dialysis unit was composed of regenerated cellulose membrane with a 10,000 dalton molecular weight cut off. The dialysis units were securely closed with polycarbonate end-pieces and placed in glass beakers containing 50 and 100 μ g/ml ceftiofur sodium in Dulbecco's Phosphate Buffered Saline (DPBS)(Sigma BioSciences, St. Louis, MO, USA)(pH 6.0, 7.0, 8.0 or 9.0). The beakers were covered and shaken at room temperature (21°C) for 48 hours after which time ceftiofur concentrations both sides of dialysis membrane were measured by HPLC using the procedure described by Hamlow et al. (1994a, 1994b).

Data analysis

The mean \pm SEM of plasma concentrations of ceftiofur after IC, IP, IM and PO dosing at 8 mg/kg vs. time data was calculated by SAS® computer analysis package version 7.0 (SAS Institute, Inc., NC). The best model to characterize the plasma kinetic profile of the drug in each route was determined by using the computer program WinNonlin™ version 1.5 (Pharsight, Palo Alto, CA). This computer program has been developed for linear regression or nonlinear modeling including compartmental and noncompartmental analysis. Goodness of fit of the pharmacokinetic equation was evaluated using Akaike's information criterion (Yamaoka et al., 1978). Statistical analyses for the degree of protein binding were also calculated using SAS®. A probability level of $p < 0.05$ was considered significant. The difference of means was compared by using Tukey-Kramer Methods (Sokal and Rohlf 1995).

RESULTS

Following IC administration, mean plasma concentration of ceftiofur was best described by first-order kinetics and a two-compartment open model with no lag time (Fig. 1) while plasma kinetic profiles following IP or IM administration were best described by non-compartment pharmacokinetic models with no lag time (Fig. 2-3). No ceftiofur was detected in the plasma of grass carp at any sampling time following oral administration (data not shown).

The concentrations of DFC and DFC containing metabolites measured as ceftiofur equivalents in the plasma of grass carp was 89.56 ± 4.87 $\mu\text{g/ml}$ for IC, 24.54 ± 3.72 $\mu\text{g/ml}$ for IP and 6.19 ± 1.84 $\mu\text{g/ml}$ for IM at 5 minutes post-administration. The ceftiofur

concentrations in the plasma declined to or below the limit of detection at 8 h after IC and IP administration, and at 96 h after IM administration. The calculated maximum concentration of ceftiofur (C_{max}) of 157.09, 31.54 and 8.86 μg ceftiofur equivalents/ml for IC, IP and IM administration occurred at the peak time (T_{max}) of 0, 15 and 30 min, respectively. The elimination half-lives ($t_{1/2}$), area under the curve (AUC) and total body clearance (Cl_b) for DFC after IC, IP and IM administration were 0.38, 0.45 and 13.86 hours, 38.51, 31.15 and 30.96 $\mu\text{g}\cdot\text{h}/\text{ml}$, and 0.21, 0.26 and 0.26 $\text{ml}/\text{min}\cdot\text{kg}$, respectively. Pharmacokinetic parameter estimates for IC, IP and IM administration are summarized in Table 1.

The degree of protein binding was pH related. Maximum binding occurred at pH 7 and 8 with decreased binding at pH 6 and pH 9. The degree of protein binding of ceftiofur sodium at various pH is shown in Table 2.

DISCUSSION

This is the first pharmacokinetic investigation of ceftiofur sodium in fish. In this study, ceftiofur sodium did not appear to be absorbed from the gastrointestinal tract of the fish after PO administration as no drug or its metabolites were detected in plasma at any sampling time. This is similar to what has been reported for other domestic animals (Prescott and Baggot, 1993). This would limit use of this drug by the oral route. The plasma kinetic profiles of ceftiofur in grass carp were best described by two-compartment model for IC administration and by a non-compartment model for IP and IM administration. Previous studies demonstrated that the models described as the best fit for ceftiofur sodium disposition are species dependent. The kinetics of ceftiofur sodium were described by one or two-compartment models in avian species (Tell et al., 1998), by a non-

compartment model in sheep (Craigmill et al., 1997), and by a two-compartment model in goats (Courtin et al., 1997).

The large volumes of distribution observed in grass carp following IM (0.63 l/kg) compared with IC (0.09 l/kg) and IP (0.17 l/kg) administration (Table 1) suggest that the concentration of the drug in the tissues is greater than in the plasma (Horsberg, 1994). A lower volume of distribution and higher elimination rate constant of ceftiofur sodium following IC and IP administration compared with the IM route results in faster clearance (Cl_b) after IC and IP dosing.

The elimination half-life of ceftiofur sodium in grass carp following IC administration in this study (0.38 h) was significantly shorter than the elimination half-life in sheep (4.87 h) (Craigmill et al., 1997) or goats (4.23 h) (Courtin et al., 1997). However, terminal half-life of ceftiofur sodium in grass carp administered this drug by the IM route (13.5 h) was longer than the half-life in sheep (7.65 h) (Craigmill et al., 1997), lactating goats (2.6 h) (Courtin et al., 1997), lactating cows (8.25 h) (Soback et al., 1991), calves (9.65 h), (Brown et al., 1991a), or swine (13.3 h) (Brown et al., 1991a). In this study, IM administration of ceftiofur sodium in grass carp also caused a mild inflammatory reaction at the site of injection (data not shown). This may limit the bioavailability of the drug and contribute to the drug's slower distribution in muscle mass. It is suggested that the muscle may act as a reservoir of this drug. In addition, biotransformation of ceftiofur sodium to DFC is more rapid when given IC and IP due to the first-pass effect (Smith and Reynard, 1995).

Ceftiofur sodium is rapidly metabolized to DFC and furoic acid in rats and cattle (Jaglan et al., 1989 ; Olson et al., 1998). No parent ceftiofur could be determined by HPLC after 1 h of intramuscular injection in cattle and swine (Brown et al., 1991a). Free DFC (which contains an intact β -lactam ring) is the microbiologically active metabolite of ceftiofur sodium (Beconi-Barker et al., 1995). DFC is further metabolized to disulfides and is bound to macromolecules in plasma and tissues. The binding of DFC to disulfides or

proteins is reversible (Jaglan et al., 1989), therefore, the biological molecule conjugates can serve as reservoirs for DFC in plasma and tissue (Olson et al., 1998). These disulfides and bound conjugates of DFC are suspected to play a role in the activity and efficacy of administered ceftiofur sodium (Jaglan et al., 1994).

In vitro, desfuroylceftiofur formation was significantly greater at alkaline pH compared with acidic pH in phosphate buffer solution (Table 2). Ceftiofur metabolism accelerated the most at pH 7.4 ($1.27 \pm 0.05/\text{day}$) (Sunkara et al., 1999). The degree of protein binding *in vitro* from this study was high compared with other third generation cephalosporins : 35 % for cefotaxime, 30 % for ceftizoxime, 30 % for cefsulodine, 17 % for ceftazidime, 43-75 % for cefmenoxime and more than 50 % for latamoxef (moxalactam) (Dalhoff, 1998). The disulfhydryl moiety (-SH) of DFC formed disulfide bonds with proteins containing disulfhydryl moiety in the plasma. In plasma, DFC was conjugated to serum albumin, as has been previously found in rats (Jaglan et al., 1989) and swine (Beconi-Barker et al., 1995). The binding of DFC to macromolecules is reversible (Jaglan et al., 1989). The rate of metabolism increased with an increase in the incubation temperature (Sunkara et al., 1999). The degree of plasma protein binding was also reported to be age-related in cattle (Brown et al., 1991a).

Water temperature plays a major role in the efficiency of drug metabolism in poikiothermic animals. Water temperature also contributes to regulating absorption, distribution and excretion of drugs given to fish. Temperature-related absorption and excretion of chemotherapeutic agents have been studied in many fish species (Bjorklund and Bylund, 1990 ; Bowser et al., 1992 ; Sohlberg et al., 1994). Lower temperatures which decrease absorption rate probably contribute to the decreased bioavailability generally seen in cold water species of fish (Bowser et al., 1992). Conversely, the relatively warm water temperature in this study ($25 \pm 3 \text{ }^\circ\text{C}$) may have contributed to the faster absorption and elimination of ceftiofur sodium in grass carp.

Cephalosporins and their metabolites are primarily excreted by the kidneys, via tubular secretion and/or glomerular filtration, while some metabolites are partially biotransformed by the liver to desacetyl compounds (Beconi-Barker et al., 1995 ; Plumb, 1999). In mammals, ceftiofur sodium is excreted rapidly within 24 hours (Brown et al., 1991a). In our study, similar results were shown for IC and IP administration, as most of the ceftiofur could no longer be detected in plasma at 8 h after administration. However, the drug could be measured in the plasma longer (ca. 4 days) after IM administration.

The major ceftiofur sodium metabolite, DFC, is noted to be less active than the parent drug against some bacteria. However, this is probably not clinically important in fish since both ceftiofur sodium and DFC are highly active against most Gram-negative bacteria, the major pathogens of fish. Minimum inhibitory concentration (MICs) of the least sensitive bacteria, *Staphylococcus* spp., isolated from bovine to ceftiofur sodium and DFC ranged from 0.0019 to 0.03 µg/ml (Salmon et al., 1996), 0.06 – 0.25 µg/ml against the Gram-negative bacteria *Streptococcus* spp. isolated from swine (Brown et al., 1991b), and 0.25 - 1.0 µg/ml against the Gram-negative *Aeromonas* spp. isolated from ornamental fish (Dixon and Issvoran, 1992). At a dosage of 8 mg/kg, the mean plasma concentration of ceftiofur sodium exceeded or remained equal to 1 µg/ml for at least 2 h after IP or IC administration, and 2 days after IM administration. These results of achievable serum concentrations in grass carp suggest that ceftiofur sodium has the potential to be efficacious *in vivo* against a number of Gram-negative fish pathogens. The prolonged half-life of the ceftiofur in the plasma at the therapeutic level after IM administration has a benefit for this drug to be used to treat bacterial infection in fish since this decreases the need for multiple injections and also the stress from handling.

The peak concentration (C_{max}) of ceftiofur in the present study was substantially higher (157.09 from IC, 31.54 µg/ml from IP and 8.86 µg/ml from IM administration)

than the MICs of the *Aeromonas* spp. (0.25 – 0.1 µg/ml) isolated from ornamental fish. In addition, *Aeromonas* spp., common pathogens of freshwater fish, showed no resistance to ceftiofur sodium at MIC concentrations. This species of bacteria exposed to oxytetracycline demonstrated resistance (Dixon and Issvoran, 1992). Ceftiofur sodium therefore, offers alternative therapy for resistant bacteria to currently approved antibiotics. However, even though ceftiofur sodium may be a good chemotherapeutic agent against common bacterial infections in brood stock or ornamental fish where the injection route of drug administration might be potentially considered, ceftiofur sodium is probably not appropriate for production food fish because of its lack of oral absorption.

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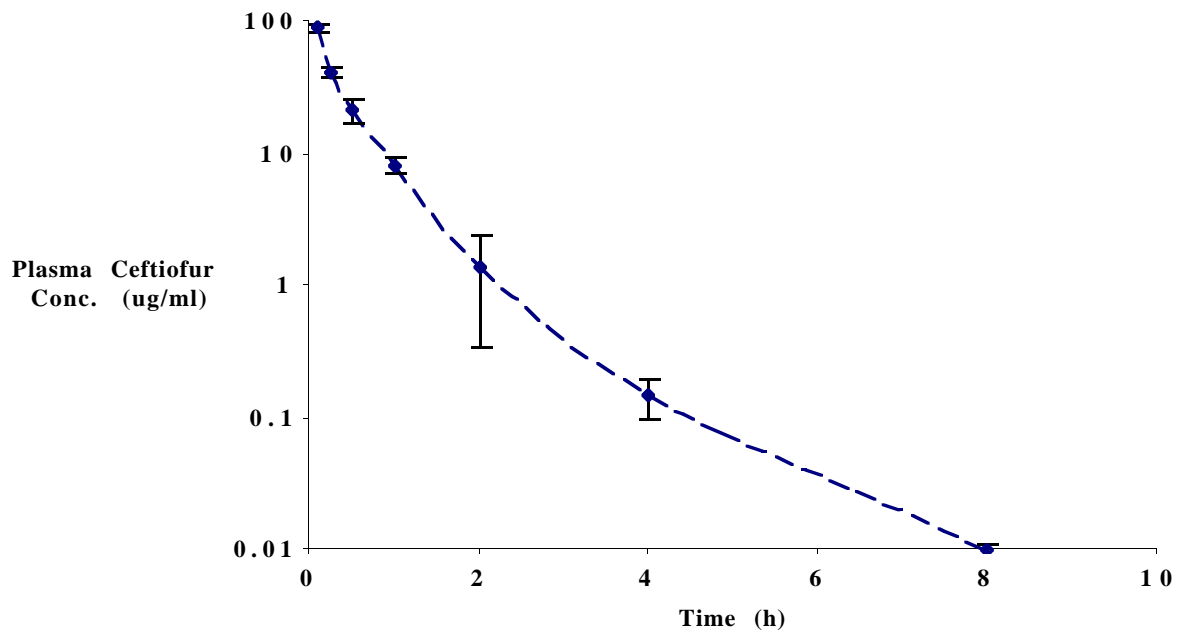


Fig. 1. The mean plasma concentration profile of ceftiofur in grass carp (n = 6) following a single intracardiac (IC) administration of 8 mg/kg. (Error bars = SEM)

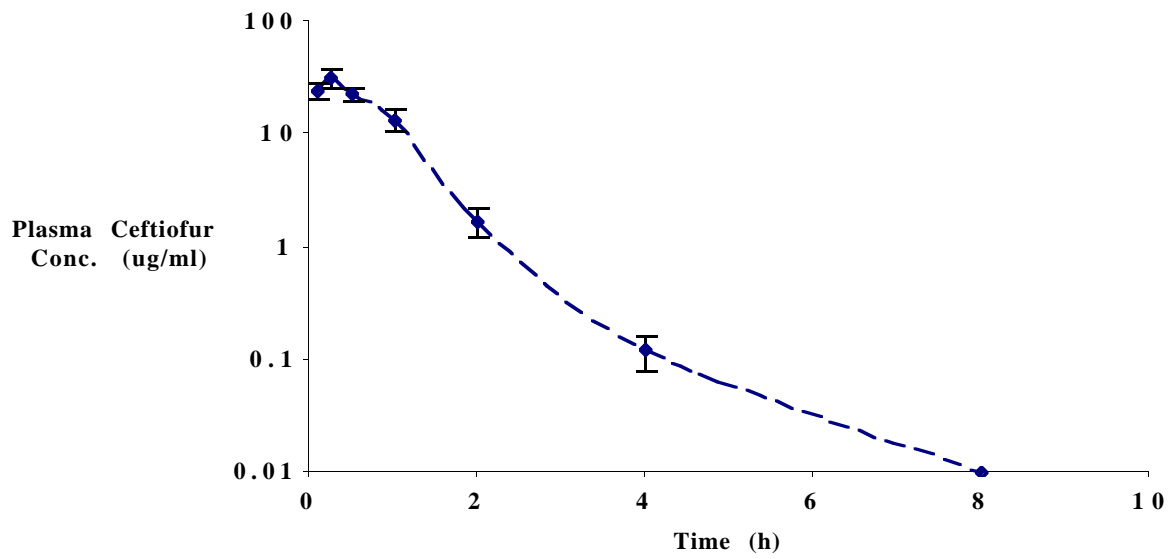


Fig. 2. The mean plasma concentration profile of ceftiofur in grass carp (n = 6) following a single intraperitoneal (IP) administration of 8 mg/kg. (Error bars = SEM)

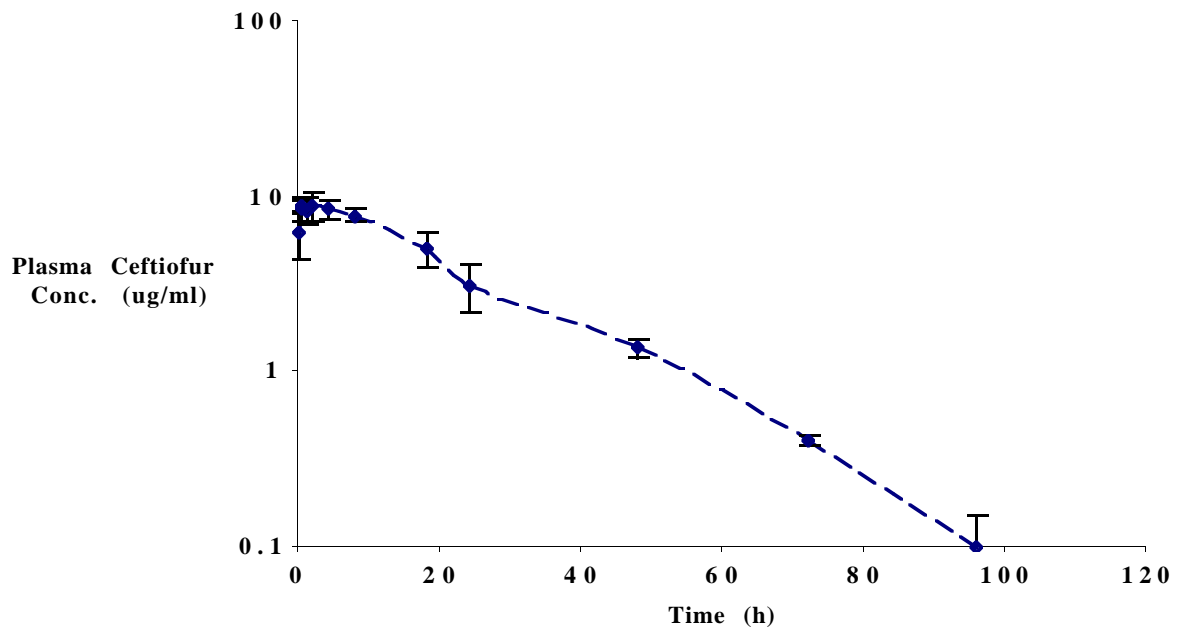


Fig. 3. The mean plasma concentration profile of ceftiofur in grass carp (n = 6) following a single intramuscular (IM) administration of 8 mg/kg. (Error bars = SEM)

Table 1. Pharmacokinetic parameters for ceftiofur sodium administered by intracardiac (IC), intraperitoneal (IP) and intramuscular (IM) routes to grass carp.

Pharmacokinetic parameters	Route of administration		
	IC	IP	IM
Model	2-Comp	Non-Comp	Non-Comp
Weight of fish (g)	578.98 ± 77.97	430.06 ± 75.29	423.79 ± 71.21
AUC (µg.h/ml)	38.51	31.15	30.96
k_{el} (h ⁻¹)	1.82	1.52	0.05
$t_{1/2}$ (h)	0.38	0.45	13.86
C_{max} (µg/ml)	157.09	31.54	8.86
T_{max} (h)	0	0.25	0.50
Cl_b (ml/min.kg)	0.21	0.26	0.26
MRT	0.43	0.71	2.03
Vd_{ss} (l/kg)	0.09	0.17	0.53

Water temperature : 25 ± 3 °C

Pharmacokinetic abbreviations

AUC	Area under the plasma drug interaction-time curve after administration of a single dose.
k_{el}	Elimination rate constant for disappearance of a drug from the central compartment (this parameters was designated by β for the 2-compartment model and λ for noncompartment model)
$t_{1/2}$	Elimination half-life (this parameters was designated by $t_{1/2,\beta}$ for the 2-compartment model)($t_{1/2,\beta} = \ln 2 / \beta$, $t_{1/2,\lambda} = \ln 2 / \lambda$, $t_{1/2} = (\ln 2)(Vd_{ss}) / Cl_b$)
C_{max}	Peak concentration
T_{max}	Time to reach peak serum concentration

- Cl_b Body clearance of a drug, which represents the sum of all clearance process in the body.
 $(Cl_b = Vd_{ss} / MRT = Dose/AUC)$ (Baggot, 1977)
- MRT Mean residence time which is average time the drug molecules that are introduced reside in fish
- Vd_{ss} Apparent volume into which a drug distributes in a fish at steady state
 $(Vd_{ss} = (Dose)(AUMC)/(AUC)^2 = (MRT)(Cl_b))$ (Baggot, 1977)

Table 2. Degree of protein binding of ceftiofur in various pH (n = 16)

pH	Percentage of protein-bound ceftiofur	
	50 µg/ml	100 µg/ml
6.0	89.68 ^a	88.54 ^a
7.0	100.00 ^b	100.00 ^b
8.0	100.00 ^b	100.00 ^b
9.0	81.16 ^a	78.64 ^a

Within columns, treatment means followed by the different superscript letter are significantly different ($p < 0.05$)

CHAPTER IV

HISTOLOGICAL ALTERATIONS IN THE POSTERIOR KIDNEY OF GRASS CARP, *Ctenopharyngodon idella* INDUCED BY SINGLE DOSES ADMINISTRATION OF CEFTIOFUR SODIUM

(Submitting to Journal of Aquatic Animal Health)

**Histological alterations in the posterior kidney of grass carp,
Ctenopharyngodon idella, induced by
single dose administration of ceftiofur sodium**

ABSTRACT

Ceftiofur sodium is an antimicrobial agent that may have potential for treating drug-resistant bacterial infections in aquaculture. A single intramuscular dose of ceftiofur sodium at three different concentrations, 8 (1X), 40 (5X) and 80 (10X) mg/kg, was administered intramuscularly to separate groups of grass carp for evaluation of the potential toxicity to major tissues involved in metabolism and excretion of this drug. These included the anterior kidney, posterior kidney, liver, and spleen. After 48 hours, lesions were seen in the posterior kidney at the highest dose of ceftiofur (10X). Morphological alterations observed microscopically included increased number of renal tubules, tubular necrosis and infiltration of inflammatory cells. No adverse effects on the glomeruli were observed at any concentration of the drug.

INTRODUCTION

Ceftiofur, a third generation cephalosporin, is the sodium salt of (6R,7R)-7-[(2-amino-4-thiazolyl)-Z-(methoxyimino)acetyl]amino-3-[(2-furanylcarbonyl)thio]methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (Koshy and Cazars, 1997). It is a broad spectrum β -lactamase-resistant antibiotic and has been used to treat a variety of bacterial infections in various animal species.

In our previous work, we studied the pharmacokinetics of ceftiofur sodium in tilapia (*Oreochromis nilotica*)(Prater et al., Unpublished data), and grass carp

(*Ctenopharyngodon idella*)(Somjetlertcharoen and Smith, in press). The pathological effects of ceftiofur have not been studied in fish even though several compounds in the cephalosporin group have been reported as causing nephrotoxicity in humans and domestic animals (Atkinson et al., 1966 ; Silverblatt et al., 1970 ; Linton et al., 1972 ; Foord, 1975 ; Tune, 1975). Thus, the present investigation was aimed to evaluate the possible toxic effect of ceftiofur on the tissues involved in the metabolism and excretion of ceftiofur in grass carp, a freshwater fish used for both food and weed control worldwide.

MATERIALS AND METHODS

Chemical agents.

Ceftiofur sodium, (Naxcel® 1g vial, Upjohn and Pharmacia Co. Kalamazoo, MI) was reconstituted according to the label directions with 20 ml sterile water to achieve a solution with a final concentration of 50 mg ceftiofur free acid equivalents/ml.

Animals and antibiotic administration.

Experimental fish were obtained from a commercial fish farm (Hopper-Stephen Hatcheries, Inc. Lonoke, AK). The study consisted of 48 grass carp (weighing 250 ± 20 gm) held in 5 m³ fiberglass tanks supplied with aerated filtered water and acclimatized at 23 ± 2 °C for at least two weeks before starting the experiment. Fish were arbitrarily separated into four groups and dosed with ceftiofur intramuscularly at a single dose of 8 mg/kg (1X), 40 mg/kg (5X) or 80 mg/kg (10X). A single dose of 8 mg/kg used as the 1X dose in this study was based on the disposition kinetic of ceftiofur sodium in lactating cows (Soback

et al., 1991) and the minimum inhibitory concentration of *Aeromonas* spp. isolated from ornamental fish (Dixon and Issvoran, 1992). The fourth group acted as a control and was administered an equal volume of sterile phosphate buffered saline intramuscularly.

Histopathological studies.

Six fish from each dose regime (1X, 5X, 10X) and control group were euthanized at 48 hours post-administration by an overdose of tricane methanesulfonate (MS-222 ; Sigma Chemical Co., St. Louis, MO). Tissue samples (anterior kidney, posterior kidney, liver and spleen) were collected and fixed in 10 % neutral buffered formalin for at least 48 hours. Histological examination of these tissues was carried out by standard histological techniques. Sections of 5 µm thickness were cut and stained with haematoxylin and eosin. Histological observations were made using a light microscope.

Quantitative analysis on pathological observation.

Quantitative morphologic abnormalities were read blindly and ranked on a 0-3 scale in order of apparent severity (0 = normal, 1 = little, 2 = mild or moderate and 3 = severe) (Bointnott and Solez 1984). The scores from each criterion were analyzed statistically by SAS® version 7.0 (SAS Institute, Inc., NC) to find the difference of the mean value between control and treatment and among treatment groups. A probability level of $p < 0.05$ was considered to be significantly different.

RESULTS

No overt clinical signs of toxicity (i.e. sluggish behavior, lethargy, depression, altered respiration or pigmentation) were observed during the study. The anterior kidney, spleen and liver did not show any histological alterations within 48 hours post-administration with ceftiofur sodium at any of the dosages. Histological alterations were however, seen in the posterior kidney. The pathology found in the proximal tubules was primarily structural alterations. Tubular lesions accounted for the majority of the observed renal injuries after high dose (10X) ceftiofur administration. Ruptured tubules and accumulation of tubular fluid into the lumen were commonly observed. The tubular necrosis did not typically involve all tubules, but appeared selective. At the early stage of injury after exposure to the drug, proximal epithelium was observed sloughing from the basement membrane. The injured tubular cells were shrunk and detached from the epithelium appearing as a tubule without a lumen. However, cytoplasm of these affected tubules remained eosinophilic. Basophilic-stained tubules were also found in all treatment groups, but these did not appear to be significantly associated with ceftiofur nephrotoxicity in this study. In addition to these progressive changes, there was also a significant infiltration of inflammatory cells into the interstitial tissues of the posterior kidney. Lesions seen in the posterior kidney are summarized in Table 1.

Quantitative analysis of these results showed that the density of tubules, amount of tubular necrosis, and inflammatory cells were statistically significant among the 10X group and other groups ($p < 0.05$). The number of tubules decreased as the dose of ceftiofur increased (Fig. 1). On the other hand, the number of inflammatory cells and necrotic tubules increased with increased dosage (Fig. 2). No statistical difference ($p < 0.05$) in the number of glomeruli and the number of glomerular cells were found post-administration at any dose.

DISCUSSION

The proximal tubule is one of the most vulnerable sites of toxicant-induced renal injury because it is the first segment that comes into contact with toxins after they are filtered by the glomerulus (Solez 1984). In addition, ceftiofur sodium is converted to desfuroylceftiofur metabolite by esterase enzyme, which is likely active in the kidney (Olson et al. 1998). Therefore, the kidney is often a target tissue for many drug toxicities. The severity of the renal damage depends on the nature and dose of toxicants, as well as many individual host factors.

In fish, renal injury has been described for aminoglycoside antimicrobials, especially gentamicin (Svec et al. 1982 ; Chamie and Reimschuessel 1994 ; Reimschuessel and Williams 1995 ; Reimschuessel et al. 1996 ; Augusto et al. 1996). Extensive necrosis of proximal tubular cells has been reported in tilapia, *Oreochromis nilitica* (Augusto et al. 1996), goldfish, *Carassius auratus* (Reimschuessel and Williams 1995) and toadfish, *Opsanus tau* (Reimschuessel et al. 1996).

Cephaloridine is the most widely studied nephrotoxic β -lactam and has been used as a prototype of cephalosporin nephrotoxicant in human and animals. Similar to the adverse effect of gentamicin on the kidney, cephaloridine has been reported as inducing renal tubular necrosis both *in vivo* and *in vitro* in various species. (James et al. 1981 ; Solez 1984 ; Rush et al. 1992 ; Lash et al. 1994 ; Tune 1994 ; Halligan et al. 1995 ; Greaves 1998 ; Haschek and Rousseaux 1998 ; Cojocel and Lock 1999 ; Plumb 1999). Acute deterioration of renal function after exposure to cephaloridine is manifested by direct tubular injury (Bennett and Porter 1993). Other β -lactam antibiotics, such as imipenem and paipenem, have been shown to cause proximal acute tubular necrosis in the monkey and rabbit (Cojocel and Lock 1999). Cephalothin has also been found to produce extensive

acute tubular necrosis in rats while no significant renal damage was observed with cephalexin or cephalixin (Linton et al. 1972).

Several mechanisms have been proposed for cephaloridine nephrotoxicity, although the exact mechanism of cephalosporin nephrotoxicity is not fully established. The proximal tubular injury induced by cephaloridine is due to high intracellular concentrations during transport activity (Berndt 1998). The tubular damage is possibly explained by the drug's ability to induce lipid-peroxidation-type injury to membranes (Rush and Hook 1986 ; Haschek and Rousseaux 1998). The cytotoxic effect of the drug was due to the increases in hydrogen peroxide and lipid peroxide levels (Kiyomiya et al. 2000). The capacity of specific β -lactam to generate superoxide, hydrogen peroxide, and other more reactive oxygen species is dependent on the pyridinium ring containing structure of the cephalosporin molecule (Cojocel and Lock 1999). Cephalosporins have little or no influence on the structure and/or function of the glomerular cells, depending on chemical structure, dose, pharmacokinetic and metabolic properties, period of administration, species and sex (Cojocel and Lock 1999). In this study, the glomeruli showed no significant changes at the light microscopic level.

Silverblatt et al. (1970) reported that the alteration in proximal tubular brush border was seen 1 hr after administration of a cephalosporin to rabbits. The affected tubules contained degenerate tubular cells or cell debris, and loss of microvilli also was observed with the electron microscope. As the injury progressed, the tubules became dilated and the permeability of the tubular cell membrane was altered. This was characterized by basophilic-stained cytoplasm, hyperchromatic irregular nuclei and occasional mitotic figures (Greaves 1998). Edema and inflammatory cells were found in the interstitial tissue. In this study, the number of inflammatory cells was significantly different between the 10X group and other treatment groups. The accumulation of inflammatory cells between individual renal tubules was a common response to the presence of necrotic debris of this nephrotoxicity. A mild interstitial infiltrate of inflammatory cells was commonly seen in all

groups, but the increased accumulation of inflammatory cells in the interstitial area appeared to be dose dependent.

In summary, this study did not show tissue pathology at either the 8 mg/kg or the 40 mg/kg cefiofur sodium dose given to the grass carp. Thus, a dose of 40 mg/kg (5X) is apparently safe for this fish species. However, a dose of 80 mg/kg (10X) ceftiofur sodium caused renal tubular damage including proximal tubular necrosis and decreased density of tubules. Low doses of nephrotoxins that do not induce overt light microscopic tubular injury, may still injure the kidney. Physiological mechanisms of the renal tissue may be affected without any obvious alterations at the microscopic or even the ultrastructural level. In addition, agents that induce proximal tubular necrosis at higher doses may induce only accelerated apoptosis at lower doses (Haschek and Rousseaux 1998).

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Table 1. Comparison of pathological alterations (Mean \pm SE) in the posterior kidney of grass carp (*C. idella*) at 48 hours after a single intramuscular administration with 3 different doses of ceftiofur sodium.

Lesion	Experimental group			
	Control (0 mg/kg) (n = 6)	1X (8 mg/kg) (n = 6)	5X (40 mg/kg) (n = 6)	10X (80 mg/kg) (n = 6)
1. Glomerulus				
Number of glomeruli ¹	12.14 \pm 4.24	12.75 \pm 3.63	12.86 \pm 3.35	13.04 \pm 3.45
Number of glomerular cells ²	40.25 \pm 4.37	38.97 \pm 4.58	39.25 \pm 4.16	39.69 \pm 4.66
Basophilic glomerulus	0.50 \pm 0.55	0.50 \pm 0.55	0.50 \pm 0.55	0.00 \pm 0.00
2. Renal tubules				
Density of tubules ³	40.00 \pm 6.14	39.64 \pm 4.35	39.28 \pm 4.00	35.48 \pm 5.30*
Basophilic tubules	1.00 \pm 0.63	1.17 \pm 0.41	1.00 \pm 0.00	1.00 \pm 0.00
Tubular necrosis	0.67 \pm 0.35	1.00 \pm 0.53	1.33 \pm 0.52	2.50 \pm 0.55*
3. Inflammatory cells	1.17 \pm 0.41	1.33 \pm 0.52	1.67 \pm 0.52	2.83 \pm 0.41*

Note : ¹ The number of glomeruli were counted at 100X magnification/field with 6 replication fields.

² The number of glomerular cells were counted at 400X magnification with 6 glomeruli

³ Density of tubules is counted at 400X magnification/field with 6 replication fields.

* Significantly different from other treatment in the same criterion ($p < 0.05$, mean \pm SE, n = 6)

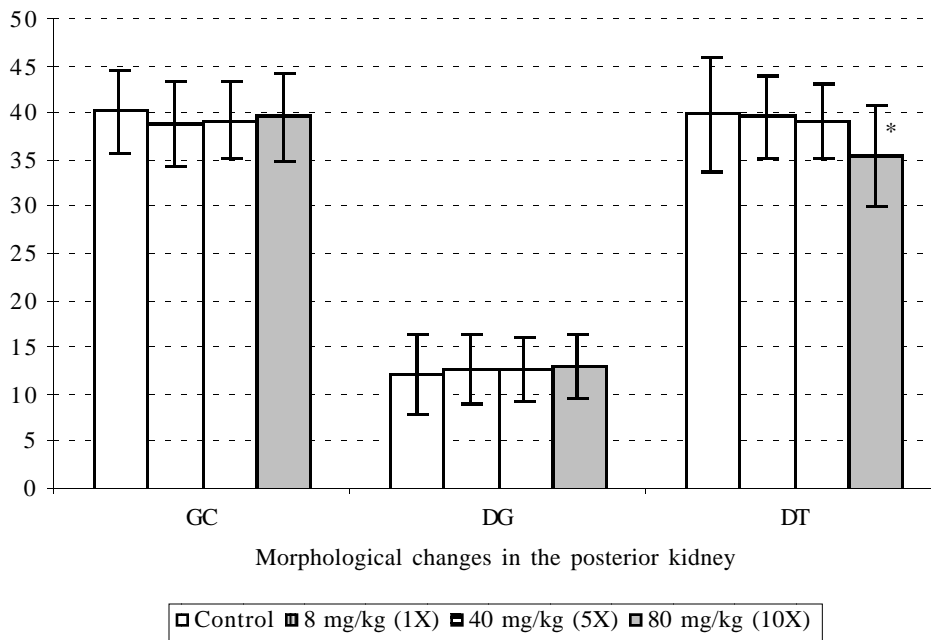


Fig. 1. Morphological changes (Mean \pm SE) in the posterior kidney at 48 hours after ceftiofur sodium administration in grass carp (*Ctenopharyngodon idella*) ; GC = the number of glomerular cells/glomerulus (at 400X magnification), DG = density of glomeruli/field (at 100X magnification), and DT = density of tubules/field (at 400 X magnification) ; (* Significantly different at $p \leq 0.05$).

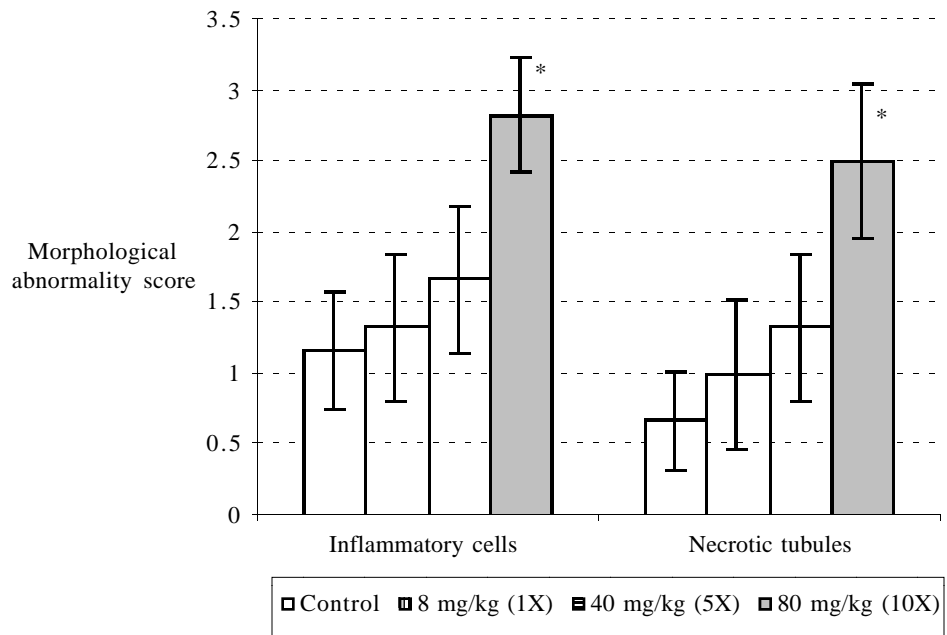


Fig. 2. Amount of inflammatory cells and necrotic tubules in the posterior kidney at 48 hours after ceftiofur sodium administration in grass carp (*Ctenopharyngodon idella*)(* Significantly different from other treatment in the same criterion at $p \leq 0.05$)

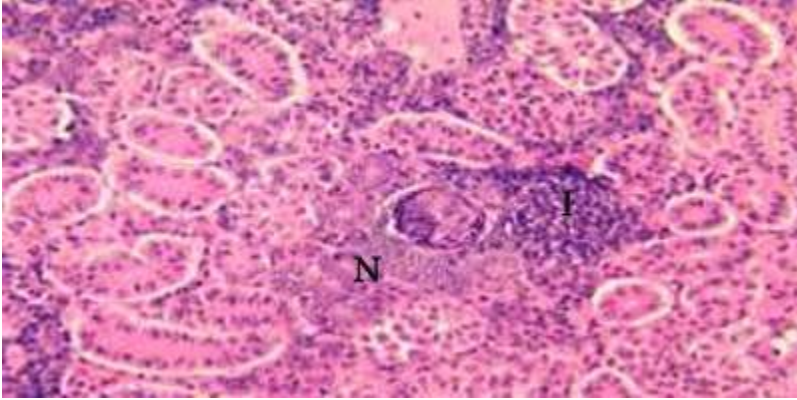


Fig. 3. Posterior kidney from grass carp (*C. idella*) administered ceftiofur sodium intramuscularly at a single dose of 80 mg/kg (10X). (H&E, 200X) Note : necrotic tubule (N) surrounded with inflammatory cells (I).

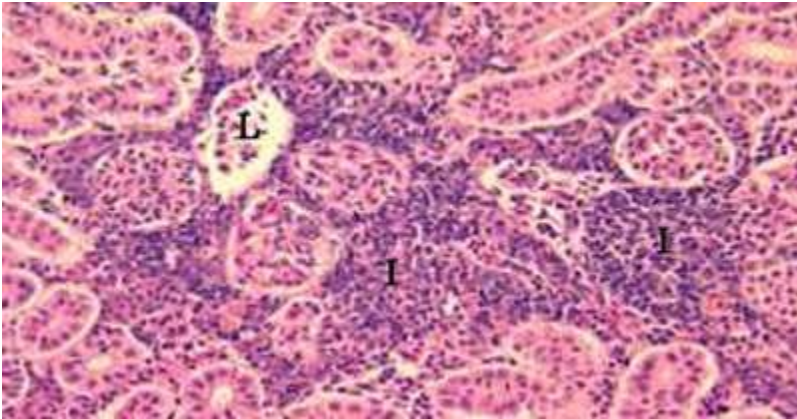


Fig. 4. Loss of tubules (L) and increased appearance of many inflammatory cells (I) in the group of grass carp (*C. idella*) dosed with 80 mg/kg (10X) ceftiofur sodium (H&E, 200X).

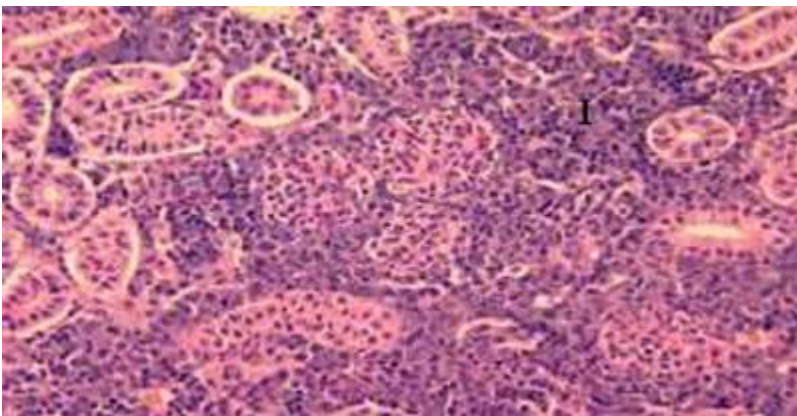


Fig. 5. Infiltration of inflammatory cells (I) in the posterior kidney of grass carp (*C. idella*) treated with 80 mg/kg (10X) of ceftiofur and the decrease in number of tubules (H&E, 200X).

CHAPTER V

EFFECT OF CEFTIOFUR SODIUM ON NON-SPECIFIC IMMUNE RESPONSE AFTER INTRAMUSCULAR ADMINISTRATION TO GRASS CARP, *Ctenopharyngodon idella*

(Submitting to Journal of Fish Diseases).

**Effect of ceftiofur sodium on non-specific immune response
after intramuscular administration to grass carp,
*Ctenopharyngodon idella***

ABSTRACT

The effect of ceftiofur sodium (Naxcel®), a third generation cephalosporin, on the non-specific immune response of grass carp (*Ctenopharyngodon idella*) was investigated. Dosages of either 8 or 40 mg/kg body weight were administered intramuscularly. After 24 and 48 h, leukocyte number, phagocytic ability and H₂O₂ production were examined in the cells of the pronephros. The results showed that neither dosage had an effect on the number of leukocytes in the pronephros. Phagocytosis was also not significantly altered at either dosage in macrophages from the pronephros. Hydrogen peroxide production was not altered in the pronephros of fish dosed at 8 mg/kg, while at a dosage of 40 mg/kg, H₂O₂ production was significantly increased.

INTRODUCTION

Chemotherapeutics, mostly in the form of antibiotics, have been widely used in aquaculture for the past decade. Recently, attention has been focused on residues in food fish, the risk of developing resistant pathogens, and environmental bioaccumulation. In addition, other deleterious effects, such as immunosuppression, nephrotoxicity, and growth retardation have been associated with drug use in aquatic organisms (Lunden et al., 1998).

Antimicrobial agents directly affect microorganisms by different modes of action. The bactericidal or bacteriostatic actions of a drug indirectly enable the immune system of

the fish to mount an appropriate response against invading pathogens. The infectious agent can then be eliminated as a result of the combined action of the immune system and antimicrobial activity (Grondel et al., 1987). However, exposure to immunotoxicants can also result in increased susceptibility to diseases (Anderson and Zeeman, 1995). Thus, immunomodulation resulting from chemotherapeutic use may have a significant effect on the outcome of disease control.

A number of studies in fish have examined the interaction between antibiotics and the immune system. Non-specific defense mechanisms are important in fish, because fish have fewer complex specific immune capabilities than higher vertebrates (Anderson and Zeeman, 1995). Specific studies concerning the effects of antimicrobials on specific and non-specific immune responses in fish have been reported in the most widely used aquaculture drug, oxytetracycline (Rijkers et al., 1980, 1981 ; Grondel et al., 1987 ; Siwicki et al., 1989 ; Lunden et al., 1998) and oxolinic acid (Siwicki et al., 1989 ; Lunden et al., 1998). Flumequine also has reported immunomodulating effects in fish (van der Heijden et al., 1995). Studies in mammals indicate that cephalosporins may also produce immunotoxicity (Descotes, 1986, 1998). However, no corresponding data are available in fish. This present study examines the effects of ceftiofur sodium on the non-specific immune response in grass carp (*Ctenopharyngodon idella*).

MATERIALS AND METHODS

Animals

Grass carp (*C. idella*) weighing 200-400 g were purchased from a commercial source (Hopper-Stephen Hatcheries, Inc. Lonoke, AK, USA). Fish were maintained in 150 l fiberglass tanks with a continuous flow of aerated filtered water at 24 ± 2 °C during the month prior to and during the experiment. Water quality parameters (temperature,

ammonia, nitrite and pH) were monitored (HACH Company, Loveland, CO, USA) daily and adjusted as necessary. Fish were maintained on a 12 hour light/dark cycle, and were fed daily with a commercial pelleted brood stock feed (35-40 % protein) (Zeigler Bros., Inc., Gardners, PA, USA) at 1 % body weight. Fish were allowed at least one week acclimation in the experimental tanks prior to initiation of the experiment.

Antibiotic and dosage

Ceftiofur sodium (Naxcel®) was obtained from a commercial source (Upjohn and Pharmacia Co., Kalamazoo, MI, USA). A single dose of 8 mg/kg (1X dose, n = 12) or 40 mg/kg (5X dose, n = 12) of ceftiofur sodium was administered intramuscularly in the dorsal musculature just anterior to the dorsal fin. A control group (n = 6) was injected with sterile saline by the same route. The single dose of 8 mg/kg used as the 1X in this study was based on the disposition kinetics of ceftiofur sodium in lactating cows (Soback et al., 1991) and the minimum inhibitory concentration of *Aeromonas* spp. isolated from ornamental fish (Dixon and Issvoran, 1992).

Isolation of phagocytes

On the first and second day following administration of ceftiofur sodium, six grass carp from each experimental group were anesthetized with MS-222 (tricaine methanesulfonate; 150 mg/l, Sigma Chemical Company, St. Louis, MO, USA) and then humanely euthanized by cervical separation. The pronephros of each grass carp from the experimental and control groups was aseptically collected and placed into separate sterile 60 x 15 mm polystyrene tissue culture dishes containing 5 ml Dulbecco's Phosphate Buffered Saline (DPBS)(Sigma BioSciences, St. Louis, MO, USA). Single cell suspensions were obtained by dissociation of the tissues by mechanical disruption through a 100 µm wire

mesh screen. Pronephric cells were washed twice by centrifugation at 200 x g for 5 minutes and resuspended with DPBS. The number of leukocytes in the suspension was appropriately diluted as per manufacturer's recommendation and counted with a CASY1 Cell Counter (Cell Tools, Inc., South San Francisco, CA, USA).

Oxidative product (H₂O₂) formation assay

The number of the cells in pronephric suspension was standardized with DPBS to a density of 5 x 10⁶ cells/ml. A 300 µl aliquot of each pronephric cell suspension was incubated with 5 µl dichlorofluorescein-diacetate (DCF-DA ; Molecular Probes, Eugene, OR, USA ; 5 mM) for 15 minutes and then incubated with 10 µl of phorbolmyristate acetate (PMA ; Sigma, St. Louis, MO, USA ; 10 ng/ml) at 22 °C for 30 minutes. Immediately following the second incubation, cells were analyzed using a Coulter Epics XL flow cytometer interfaced with a MDADS data analysis computer (Coulter Electronics, Hialeah, FL, USA). For each sample, 5,000 viable cells were counted by electronic gate and analyzed the fluorescence intensity activated by oxidized DCF-DA in the presence of hydrogen peroxide which was trapped within the reacting cells. This reaction has been employed to measure the hydrogen peroxide released by polymorphonuclear leukocytes (Tam and Hinsdill, 1990).

Phagocytosis assay

The number of the cells in pronephric suspension was standardized with DPBS to a density of 5 x 10⁶ cells/ml. Aliquots of 300 µl from cell suspensions were incubated with 10 µl of sonicated fluorescent latex particles (Fluoresbrite microspheres®, Polyscience, Inc. ; Warrington, PA, USA ; 1.75 µm) and 4 ml culture medium (RPMI 1640 ; Mediatech, Cellgro, VA, USA) for 18 h at 22 °C. Following incubation, cells were washed

twice and resuspended in 0.3 ml fresh media, and immediately analyzed by flow cytometry as previously described. For each sample, 5,000 viable cells were counted by electronic gate and analyzed the fluorescence intensity resulted from phagocytizing the fluorescent beads by phagocytic cells in the tissue.

Statistical analysis

The data were analyzed for statistical differences using one way analysis of variance (ANOVA) and Tukey-Kramer Methods (Sokal and Rohlf, 1995). A probability level of $p < 0.05$ was considered significant. The results were expressed as mean \pm SEM.

RESULTS

The administration of ceftiofur sodium at a dose of 8 or 40 mg/kg did not significantly affect the total number of leukocytes in the pronephros ($p = 0.2571$)(Table 1). The ability to phagocytize latex beads by cells in the pronephros ($p = 0.4097$) was also not significantly affected (Table 2). However, H_2O_2 production in the pronephros of the fish was significantly increased when grass carp were treated with 40 mg/kg ceftiofur sodium as compared to control fish and fish treated with 8 mg/kg ($p = 0.0233$)(Table 3).

DISCUSSION

The major lymphoid tissues in teleost fishes are the pronephros, spleen, thymus, and mucosa-associated lymphoid tissues, including skin and gills (Press and Evensen, 1999). In carp, the pronephros is a granulocyte-rich tissue that takes up pathogens from the circulation (Steinhagen and Jendrysek, 1994). Unlike mammals, only neutrophils have been obviously identified in carp while basophils and eosinophils did not differ

distinctively in end stage of development line, ultrastructural and cytochemical characteristics (Temmink and Bayne, 1987 ; Hine, 1992).

Many mammalian assays have been modified to evaluate the effect of drugs on the fish immune system. Hematological examination is a simple way of comparing the change in the number of various types of leukocytes in the leukocyte-rich tissue, spleen and pronephros, where these cells proliferate and differentiate. Many immune assays have been developed for use in fish. In addition, measurements of both humoral and cell mediated immune responses have been measured. Methods used include the plaque assay (Pourreau et al, 1986 ; Rijkers et al., 1981), chemiluminescence assay (Cossarini et al., 1988), phagocytosis assay (Siwicki et al., 1990), complement activity (Yano et al., 1991), lysozyme level (Siwicki et al., 1990), challenging with pathogens (Cossarini et al., 1988 ; Karunasagar et al., 1991 ; Yano et al., 1991), mitogen assays (Pourreau et al., 1987 ; Bly and Clem, 1991) and tissue allograft response (Pourreau et al., 1986 ; Khangarot and Tripathi, 1991). The overall influence of drugs on the defense system must be evaluated in large scale challenge tests with relevant pathogens (van der Heijden et al., 1995). Non-specific immune response played a more important role than specific defense for the survival of the fish challenged. The surviving fish from an experimented bacterial infection had a specific and non-specific protective immunity but the non-specific response (level of circulating granulocytes as well as hydrogen peroxide formation) existed for a longer period of time than the specific response, the serum antibody (Lunden et al., 1998). Thus, the non-specific immune responses, phagocytosis and hydrogen peroxide production were the main focus of this study.

Antibiotics are commonly used for the control of diseases in cultured fish. In order to prevent the development of drug resistant bacteria, the concentration of antibiotics used is generally high (Rijkers et al., 1980). The interaction of drugs with the immune system may result in immunosuppression, inhibited cell proliferation, and alterations of host defense mechanisms against pathogens (Anderson and Zeeman, 1995). Oxytetracycline, as

an example of a drug frequently used in fish culture, can suppress both humoral immunity and cellular immunity in fish (Rijkers et al., 1980, 1981 ; Grondel and Boesten 1982 ; Grondel et al., 1987 ; Siwicki et al., 1989 ; Lunden et al., 1998).

Cephalosporins are known to alter the immune response in mammals, with non-specific host defense mechanisms the main target of cephalosporin toxicity (Descotes, 1998). Regarding the effect of the third generation cephalosporins on hydrogen peroxide production, cefoperazone, cefotaxime and ceftazidime, caused alterations in hydrogen peroxide production. These alterations were an 11 % decrease in guinea-pig, a 50 % increase in human and a 8-35 % decrease in humans (Descotes, 1986). For the effect on phagocytosis, cefoperazone and ceftazidime caused a 30-40 % decrease in mice and 30 % increase in humans, respectively (Descotes, 1986). No study to date has examined the effects of cephalosporins on the non-specific immune response in fish. In this study, administration of ceftiofur sodium to grass carp at the dose of 8 mg/kg body weight had no effect on the non-specific immune response. Thus, cellular immunity as measured by the three assays used in this study was not affected at this dosage. However at a dosage of 40 mg/kg, ceftiofur sodium produced an 18-19 % increase in H₂O₂ production which suggests an enhanced non-specific immune response in this fish species.

Ceftiofur sodium, a cephalosporin with little bacterial resistance, was studied as an alternative antimicrobial for finfish. The pharmacokinetics and toxicities of this drug have already been studied (Somjetlertcharoen and Smith, submitted). Pharmacokinetically, due to the complete lack of oral absorption, ceftiofur sodium is of limited use in aquatic animals since administration would have to be by a parenteral route. Thus, this agent may have no practical use in food fish production. However, brood stock or ornamental fish that are relatively more expensive or more important individually might benefit from ceftiofur sodium use.

The present results have shown that ceftiofur sodium at a dose of 8 mg/kg did not alter any of the non-specific immune responses of grass carp assayed in this study. In

contrast, a high dose of 40 mg/kg (5X recommended dose) slightly increased one of the three assayed non-specific immune responses of grass carp. However, since only hydrogen peroxide production appeared to be affected, it should not be concluded that treatment with ceftiofur sodium at the high dose is capable of enhancing the immune response in this fish species.

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Table 1. Effect of ceftiofur sodium on the number of leukocytes in pronephros of grass carp, *Ctenopharyngodon idella*

Experimental groups (n = 6)	The number of leucocytes ($\times 10^6$)(Mean \pm SEM)
<u>24 hours</u>	
Control	57.14 \pm 7.95 ^a
8 mg/kg	54.23 \pm 4.16 ^a
40 mg/kg	50.32 \pm 7.95 ^a
<u>48 hours</u>	
Control	57.14 \pm 7.95 ^a
8 mg/kg	43.39 \pm 4.44 ^a
40 mg/kg	46.19 \pm 6.38 ^a

Within columns, treatment means followed by the same superscript letter are not significantly different ($p \geq 0.2571$)

Table 2. Effect of ceftiofur sodium on the phagocytosis response (Mean \pm SEM) of pronephros leukocytes in grass carp, *Ctenopharyngodon idella*.

Experimental group (n = 6)	% Response from Flow Cytometer (Mean \pm SEM)
<u>24 hours</u>	
Control	28.86 \pm 2.64 ^a
8 mg/kg	25.53 \pm 1.15 ^a
40 mg/kg	26.47 \pm 2.64 ^a
<u>48 hours</u>	
Control	28.86 \pm 2.64 ^a
8 mg/kg	22.28 \pm 1.78 ^a
40 mg/kg	26.16 \pm 1.38 ^a

Within columns, treatment means followed by the same superscript letter are not significantly different ($p \geq 0.4097$)

Table 3. Effect of ceftiofur sodium on the production of H₂O₂ (Mean \pm SEM) from pronephros in grass carp, *Ctenopharyngodon idella*.

Experimental group (n = 6)	% Responses from Flow Cytometer (Mean \pm SEM)
<u>24 hours</u>	
Control	67.37 \pm 4.80 ^a
8 mg/kg	68.86 \pm 4.80 ^a
40 mg/kg	86.76 \pm 5.88 ^b
<u>48 hours</u>	
Control	67.37 \pm 4.80 ^a
8 mg/kg	68.97 \pm 5.09 ^a
40 mg/kg	86.18 \pm 5.88 ^b

Within columns, treatment means followed by the different superscript letters are significantly different ($p < 0.05$)

SUMMARY AND CONCLUSIONS

Resistance of bacterial fish pathogen to commonly used antibiotics is an emerging problem in the aquaculture industry. Antimicrobial resistance has been documented in food fish and ornamental fish. Oxytetracycline (Terramycin®) and ormethoprim/sulfadimethoxine (Romet-30®), the only two antibiotics approved for use on food fish in the United States has been reported by numerous studies. Many of the commonly used antimicrobial drugs may no longer prove efficacious for the treatment of Gram-negative fish pathogen. It may be necessary to begin screening antimicrobial compounds for efficacy against those resistant Gram-negative bacteria.

Ceftiofur sodium offers alternative therapy for resistant bacteria to currently approved antibiotics. The hypothesis for use ceftiofur sodium in fish was proved that ceftiofur sodium may be a good chemotherapeutic agent against common bacterial infections in brood stock or ornamental fish where the injection route of drug administration might be potentially considered and the recommended dose is 8 mg/kg. The dose of 40 mg/kg can be use with careful consideration. This dosage may not injure the posterior kidney. However, it may affect non-specific immune response of the fish. The prolonged half-life of the ceftiofur in the plasma at the therapeutic level after IM administration at the dose of 8 mg/kg has a benefit for this drug to be used to treat bacterial infection in fish since this decreases the number of multiple injection and also the stress from handling when multiple dose is needed. Ceftiofur sodium is probably not appropriate for production food fish because of its lack of oral absorption.

Histologically, ceftiofur sodium did not cause tissue pathology at either the 8 mg/kg or the 40 mg/kg ceftiofur sodium dose given to the grass carp. However, a dose of 80 mg/kg ceftiofur sodium caused renal tubular damage including proximal tubular necrosis and decreased density of tubules. Thus, a dose between 8 to 40 mg/kg is apparently safe for this fish species.

Immunologically, the primary results have shown that ceftiofur sodium at a dose of 8 mg/kg did not alter any of the non-specific immune responses of grass carp. In contrast, a high dose of 40 mg/kg (5X recommended dose) slightly increased one of the three assayed non-specific immune responses. Thus, at the dosage of 8 mg/kg is likely to be safe while the dose of 40 mg/kg (5X) can be used with careful consideration since at this dose may stimulate the non-specific immune response in fish. According to this study, only hydrogen peroxide production assay appeared to be affected, more immunological assay should be done to confirm the immunostimulation result of this drug.

Appendix A

Determination of Ceftiofur sodium concentrations by HPLC

HPLC assay for determination of ceftiofur and metabolites in plasma. (Hamlow et al., 1994a, 1994b)

This procedure is for the routine quantitative assay of ceftiofur (syn-oxime) and desfuroylceftiofur related metabolites found in the plasma of cattle and pigs treated with ceftiofur sodium (NAXCEL®) or other ceftiofur formulations.

In this procedure, ceftiofur HCl anti-oxime (U98,335A) is added as an internal standard before plasma is incubated with dithioerythriol which cleaves ceftiofur and related compounds from plasma proteins or other sulfur-containing compounds, yielding the desfuroylceftiofur (DFC) moiety. The DFC is extracted onto C-18 solid-phase extraction column where it is converted to a stable derivative (Desfuroylceftiofur acetamide (DCA)) by treatment with iodoacetamide. Further purification is performed on a SCX column. After the final elution, the samples are analyzed via gradient HPLC on a C-18 column with UV detection. A standard curve is prepared by fortifying plasma with ceftiofur HCl syn-oxime. Control check plasma samples also fortified with ceftiofur syn-oxime are used for quality assurance.

Procedure

A. Preparation of ceftiofur standards : ceftiofur HCl syn-oxime (U64, 279A)

1. Prepare a 100 µg/ml stock solution of ceftiofur syn-oxime by dissolving 11.20 mg of ceftiofur HCl in 100 ml of 0.1 M ammonium acetate or using equivalent methods to achieve this concentration.
2. Transfer 1.5 ml aliquots into freezer-safe vials and store at - 15 C. May be

stored for at least two months.

3. Prepare a 10 µg/ml solution of ceftiofur syn-oxime daily by diluting 1 ml of the stock solution to 10 ml with 0.1 M ammonium acetate.
4. Prepare the standards for calibration curve and control check samples in separate vials, each contain 1 ml of control plasma. The following table describes volumes of standard solution needed for different concentrations. The range of the standard curve may be shortened as needed, but should not be used below 0.15 µg/ml or above 10 µg/ml without being validated in those ranges.

	Volume of 10 µg/ml standard solution (ml)	Volume of plasma (ml)	Final plasma equivalent concentration (µg/ml)
Standard	0.015	1.0	0.15
Standard	0.04	1.0	0.40
Standard	0.10	1.0	1.0
Standard	0.20	1.0	2.0
Standard	0.50	1.0	5.0
Standard	1.0	1.0	10.0
Control	0.03	1.0	0.30
Control	0.10	1.0	1.0
Control	0.40	1.0	4.0

If control plasma is not available, standards may be prepared without it by substituting 0.1 M ammonium acetate. This was not part of the method development or

validation, but should provide acceptable results, and should be certified through experimentation. A bias has been observed, with solution standards giving higher efficiency in the extraction and derivatization process, which can give slightly low estimates for plasma concentrations.

B. Internal standard stock solution preparation : ceftiofur HCl anti-oxime (U-98335A)

1. Prepare a stock solution of ~ 250 µg/ml (ceftiofur syn-oxime equivalent) internal standard in 0.1 M ammonium acetate. The concentration can be estimated by dissolving purified anti-oxime ceftiofur HCl (U-98335A) in a small volume of 0.1 M ammonium acetate and comparing the HPLC peak area response of the anti-oxime with a known concentration of syn-oxime ceftiofur standard prepared above.

The HPLC gradient conditions described below are sufficient to do this or others may be used as described. Use small injection volumes (5-10 µl) of these concentrated solutions and try to inject similar amounts (µg) of drug for each isomer so that peak sizes are similar. Calculate the response factor for the ceftiofur syn-oxime (area per µg) and use this to estimate the concentration of the ceftiofur anti-oxime solution. Dilute the anti-oxime solution to ~250 µg/ml using 0.1 M ammonium acetate.

2. Transfer 1.0 ml aliquots of 250 µg/ml stock solution into freezer-safe vials and store at -15 °C may be stored for at least two months.
3. Prepare a 25 µg/ml solution daily by diluting 1 : 10 with 0.1 M ammonium

acetate. For example, 1.0 ml added to 9.0 ml of 0.1 M. ammonium acetate. This is now ready to use by adding to plasma samples being assayed.

C. Plasma sample preparation and analysis :

1. Diethioerythritol incubation :

1. Pipet a 1.0 ml test portion of each plasma sample to be assayed into a vial or tube.
1. To each sample and standard tube add 100 μ l of 25 μ g/ml internal standard solution.
1. Add 5.0 ml of DTE solution to each tube and adjust the pH to 8.7 if necessary.
1. Incubate in a gently shaking water bath at 50 C for 15 minutes.
1. Centrifugation at 48000 x g for 20 minutes is optional, but should be done uniformly for all samples of a run.

2. C-18 column extraction : one column per sample or standard

1. Prepare the C-18 extraction columns by washing with one column volume of methanol (~ 6 ml). Followed with two-column volume of 0.1 M ammonium acetate. Do not allow the columns to run dry.
2. Charge the incubated plasma samples onto the columns and allow to pass through the columns slowly (use a minimum of vacuum as necessary).
3. Wash the columns with two column volumes of 0.1 M ammonium acetate and proceed to the on-column derivatization.

3. On-column derivatization :

1. Add 5 ml of iodoacetamide solution to each C-18 column. Allow the solution to pass through the cartridges in the dark for 30 minutes. Use the least amount of vacuum or positive pressure to keep the solution dripping through the columns.
1. Wash each C-18 column with two volumes of 0.1 M ammonium acetate.
1. Wash each column with one volume of 2 % acetic acid.

4. SCX column extraction : one column per sample or standard

1. Prepare the SCX extraction columns by washing with 2 ml of methanol followed by 2 ml of 20 : 80 acetonitril : 2 % acetic acid.
1. Elute the C-18 column directly onto the SCX cartridge with 6 ml of 20 : 80 acetonitril : 2 % acetic acid. The plunger from the 6 cc. monoject syringe is useful for pushing the 20 : 80 elute through the C-18 column and into the reservoir of the SCX column. Allow the solution to pass through the SCX column by gravity feed. Some mild vacuum or positive pressure may be needed.
1. Wash each SCX column with 1 ml of methanol.
1. Wash each column with 2 ml of 2 % acetic acid.

5. Elution of derivative :

1. Elute the derivative (DCA) from the SCX column with 2,0 ml of 15 : 85 methanol : 1 M ammonium acetate, collecting the entire elute in an appropriate tube. Elute slowly, then use higher vacuum to recover all the solution.
1. Vortex the collected elute and transfer to autosampler vials for HPLC analysis.

D. HPLC system conditions and sample analysis :

1. The UV absorbance detector should be set for 254 nm. 266 nm gives maximal absorbance for the syn-oxime DCA, but is not optimal for the internal standard. 254 nm is an intermediate wavelength. Injection volumes for all samples and standards should be 100 µl, and solution of 50 : 50 acetonitril : water should be used for flushing the autosampler needle.

The gradient conditions are described in the table below. The gradient initial conditions are at 10 % acetonitril. When the injection is made a gradient to 40 % acetonitril is run over 13 minutes, followed by a 1 minute reverse gradient back to initial conditions (10 % acetonitril). A 10 minutes equilibration finishes the run and precedes the next injection. The elution times for ceftiofur and the internal standard are about 9 to 10.5 minutes, respectively. Adjustments in the gradient may be required if different chromatography results are observed due to column lot variations or if needed to accommodate different equipment.

Elapsed Time (min)	Flow (ml/min)	% Mobile Phase A (Water + 0.1 % TFA)	% Mobile Phase B (Acetonitril + 0.1 % TFA)
0	1.0	90	10
13	1.0	60	40
14	1.0	40	60
15	1.0	90	10
25	1.0	90	10

1. The first run of a set is a blank injection and gradient run to assure column conditions are stable. The fortified control extracts should run following the standards.
1. The column was washed with at least 15 column volumes of 50 : 50 acetonitril : water at the end of the run in order to remove the TFA and clean the column. The guard column was changed as necessary to maintain column efficiency (400-600 injections). New columns or guard columns was washed with 100 % acetonitril prior use. The 50 : 50 acetonitril : water was stored the column between use.

Result

1. A standard curve is generated from the peak area ratio vs the ceftiofur concentration ($\mu\text{g/ml}$) in the fortified standards. The peak area ratio is calculated as follows :

$$\text{Peak area ratio} = \frac{\text{Peak area of desfuroylceftiofur acetamide}}{\text{Peak area of internal standard}}$$

A set of standards is assayed with the samples that were derivatized on the same day as that of the standard curve. Data can be managed in any spreadsheet or data system that will conduct linear regression of standards (peak area ratio vs standard concentration).

Standard curve would be rejected if the correlation coefficient was less than 0.97 and/or if the standards were not back-calculated to within +/- 10 % (+/- 15 % at 0.150 $\mu\text{g/ml}$). Control samples were predicted within +/- 15 % of theoretical concentrations. Refer to the formula below for calculating concentrations.

Upon acceptance of the standard curve, the concentrations in unknown samples are calculated from the standard curve using the conventional formula, which describes the line. In this case it would be :

$$\text{Sample concentration} = \frac{\text{Peak area ratio} - \text{Intercept}}{\text{Slope}}$$

Extrapolation beyond the range of the standard curve was not recommended. The limit of quantitation for assay is 0.015 µg/ml.

Reagents

1. Ammonium acetate (HPLC Grade), Fisher Scientific Co., Fair Lawn, NJ
1. Sodium hydroxide, J. T. Baker Chemical Co., Phillipsburg, NJ
1. Dithioerythritol (DTE), 99 %, Sigma -Aldrich, Inc., Saint Louis, MO
1. Iodoacetamide 97 %, Sigma -Aldrich, Inc., Saint Louis, MO
1. Methanol (HPLC Grade), AlliedSignal Inc., Burdick & Jackson, Muskegon, MI
1. Acetonitril (HPLC Grade), AlliedSignal Inc., Burdick & Jackson, Muskegon, MI
1. Ceftiofur HCl, syn-oxime (U-64, 279A), Upjohn Control Reference Standard, Upjohn Control Division
1. Ceftiofur HCl, anti-oxime (U-98, 335A), Upjohn Fine Chemicals
1. Trifluoroacetic acid (TFA), 99 %, Sigma -Aldrich, Inc., Saint Louis, MO
1. Acetic acid, glacial, (AR grade), Sigma -Aldrich, Inc., Saint Louis, MO

Equipment & Supplies for HPLC

1. C-18 Cartridge : Mega Bond Elut, 6 cc, 1000 mg, Part no. 1225-6001, Varian Sample Preparation Products, Harbor City, CA
1. SCX Cartridge : Bond Elut LRC, 100 mg, Part no. 1211-3013, Varian Sample Preparation products, Harbor City, CA
1. Disposable syringes, 6 cc., Monoject #716917, Sherwood Medical, St. Louis, MO
1. Guard Column : Part No. 844-46-P, Keystone Scientific, Inc., Bellefonte, PA
1. Column : BDS-Hypersil C-18, 5 μm , 250 x 4.6 mm., Part No. 255-46-CPG, Keystone Scientific, Inc., Bellefonte Park, NJ

Appendix B

Data from the pharmacokinetic study

Table 1. The relationship of plasma concentration of ceftiofur and metabolites with retention time, peak area and peak height obtained from high performance chromatographic determination.

Ceftiofur concentration (µg/ml)	Retention time (mins)	Peak area	Peak height
10.0	17.353	1.84923	0.01449
5.0	17.269	0.72635	0.00565
2.0	17.354	0.29372	0.0023
1.0	17.365	0.16548	0.00151
0.40	17.378	0.04746	0.00076
0.15	17.396	0.03157	0.00055

$$(\text{Peak Area}) = 0.18225 * (\text{Ceftiofur Concentration}) - 0.0445$$

$$R^2 = 0.988161, p < 0.0001$$

Table 2. Plasma concentration of ceftiofur and metabolites of grass carp (*C. idella*) following intracardiac (IC) administration at 8 mg/kg.

Time (h)	Plasma concentration of ceftiofur and metabolites ($\mu\text{g/ml} \pm \text{SE}$)
0 (Control)	0
0.08	89.56 ± 4.87
0.25	40.82 ± 3.54
0.50	21.27 ± 4.56
1.00	8.23 ± 1.17
2.00	1.41 ± 1.06
4.00	0.15 ± 0.05
8.00	< LOD
18.00	< LOD
24.00	0

< LOD = less than limit of detection

Detection limit : 0.1 $\mu\text{g/ml}$

Temperature : 25 ± 3 °C No. of animal : 60 fish

Weight \pm SD (g) : 578.98 ± 77.97

Table 3. Plasma concentration of ceftiofur and metabolites of grass carp (*C. idella*) following intraperitoneal (IP) administration at 8 mg/kg.

Time (h)	Plasma concentration of ceftiofur and metabolites ($\mu\text{g/ml} \pm \text{SE}$)
0 (Control)	0
0.08	24.54 ± 3.72
0.25	31.54 ± 5.65
0.50	23.12 ± 3.11
1.00	13.57 ± 2.93
2.00	1.69 ± 0.53
4.00	0.12 ± 0.05
8.00	< LOD
18.00	< LOD
24.00	< LOD
48.00	0
72.00	0
96.00	0

< LOD = less than limit of detection

Detection limit : 0.1 $\mu\text{g/ml}$

Temperature : 25 ± 3 °C No. of animal : 78 fish

Weight \pm SD (g) : 430.06 ± 75.29

Table 4. Plasma concentration of ceftiofur and metabolites of grass carp (*C. idella*) following intramuscular (IM) administration at 8 mg/kg.

Time (h)	Plasma concentration of ceftiofur and metabolites ($\mu\text{g/ml} \pm \text{SE}$)
0 (Control)	0
0.08	6.19 ± 1.84
0.25	8.52 ± 1.23
0.50	8.86 ± 0.62
1.00	8.44 ± 1.50
2.00	8.85 ± 1.61
4.00	8.49 ± 0.99
8.00	7.85 ± 0.64
18.00	5.07 ± 1.16
24.00	3.16 ± 0.91
48.00	1.38 ± 0.16
72.00	0.40 ± 0.03
96.00	0.10 ± 0.02

Detection limit : 0.1 $\mu\text{g/ml}$

Temperature : 25 ± 3 °C No. of animal : 78 fish

Weight \pm SD (g) : 423.79 ± 71.21

Table 5. Plasma chemistry profile of grass carp (*Ctenopharyngoden idella*)

	Fish #1	Fish #2	Fish #3	Fish #4	Mean \pm SD
Total protein	2.8	1.7	2.6	2.9	2.5 \pm 0.5
Albumin	1.1	0.8	1.0	1.1	1.0 \pm 0.1
Creatinine	0.1	0.1	0.1	0.1	0.1 \pm 0.0
TB	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
AST	46	29	91	63	57.2 \pm 26.4
Alkp	53	22	66	57	49.5 \pm 19.1
Na	117	116	114	123	117.5 \pm 3.9
K	2.3	2.7	3.2	2.4	2.6 \pm 0.4
Cl	112	115	110	119	114.0 \pm 3.9
Ca	7.7	6.7	8.0	8.4	7.7 \pm 0.7
P	2.0	1.6	2.8	3.1	2.4 \pm 0.7
Mg	2.3	2.2	2.3	2.4	2.3 \pm 0.1
Clu	41	62	46	46	48.8 \pm 9.1
Cholesterol	134	82	135	143	123.5 \pm 27.9

Table 6. Comparison of blood chemistry profile in 5 different species of fish, rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), hybrid tilapia (*Oreochromis* sp.), hybrid striped bass (*Morone* sp.) and grass carp (*Ctenopharyngodon idella*)

	<u>Trout</u> ¹	<u>Catfish</u> ¹	<u>Tilapia</u> ¹	<u>Striped bass</u> ¹	<u>Tilapia</u> ²	<u>Grass carp</u> ³
Total protein	2.7	3.0	3.1	3.8	3.4	2.5
Albumin	1.2	1.2	1.3	1.5	1.2	1.0
Creatinine	0.4	0.5	-	0.2	0.2	0.1
TB	0.1	0.1	0	0.3	0.1	< 0.1
AST	102	40	15	40	87.5	57.2
Alkp	31	32	26	58	15.5	49.5
Na	152	131	146	155	149	117.5
K	2.3	3.6	3.0	3.2	2.9	2.6
Cl	137	118	141	149	137	114
Ca	9.2	10.3	13.0	11.6	13.0	7.7
P	10.5	7.3	4.3	9.2	6.8	2.4
Mg	2.3	2.3	2.5	2.0	2.4	2.3
Clu	103	50	50	86	69	49
Cholesterol	144	148	208	200	173.5	123.5

¹ Hrubec and Smith (1999)

² Prater et al. (unpublished data)

³ Data in this study

Appendix C

Pharmacokinetic model in grass carp

Pharmacokinetics in fish

(Horsberg, 1994 ; Sohlberget al., 1996)

There are two frequently employed single dose techniques to determine the basic pharmacokinetic properties of a chemical in fish. First, single individual-single sample, is to administer the chemical substance to a group of individuals, take single blood sample from several individual fish at each time point, and then use the mean values at the different time point for calculation of the pharmacokinetic parameters. This method involves sacrificing many fish at each sampling time point. Secondly, single individual-multiple samples, is to withdraw repeated blood samples from the same individuals cannulated fish through a catheter inserted into the dorsal aorta, for the determination of individual pharmacokinetic profiles of the drug. This method allows many blood samples to be taken from the sample fish and pharmacokinetic parameters to be measured in each individual, as done in mammals.

The plasma ceftiofur concentration-time profile from intracardiac administration (chapter III) was best described by two-compartment model, which can be generalized in Fig. 1.

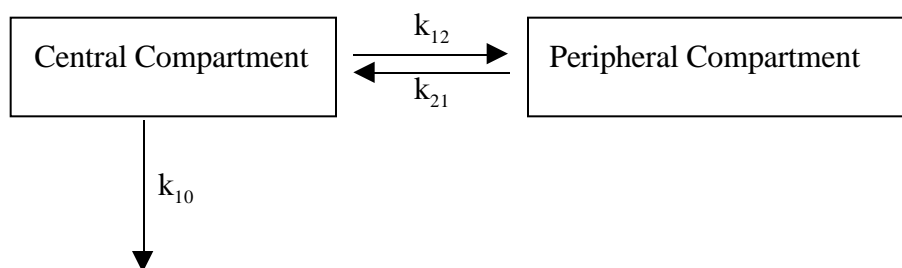


Fig. 1. Generalized open two-compartment pharmacokinetic model with elimination (k_{10}) from the central compartment, k_{12} and k_{21} represent intercompartmental micro-rate constant (Gibaldi and Perrier, 1982).

The fundamental principle involved is that the observed plasma concentration-time profile is actually the result of two separate pharmacokinetic processes, which can be described by the equation (1) and Fig. 2.

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

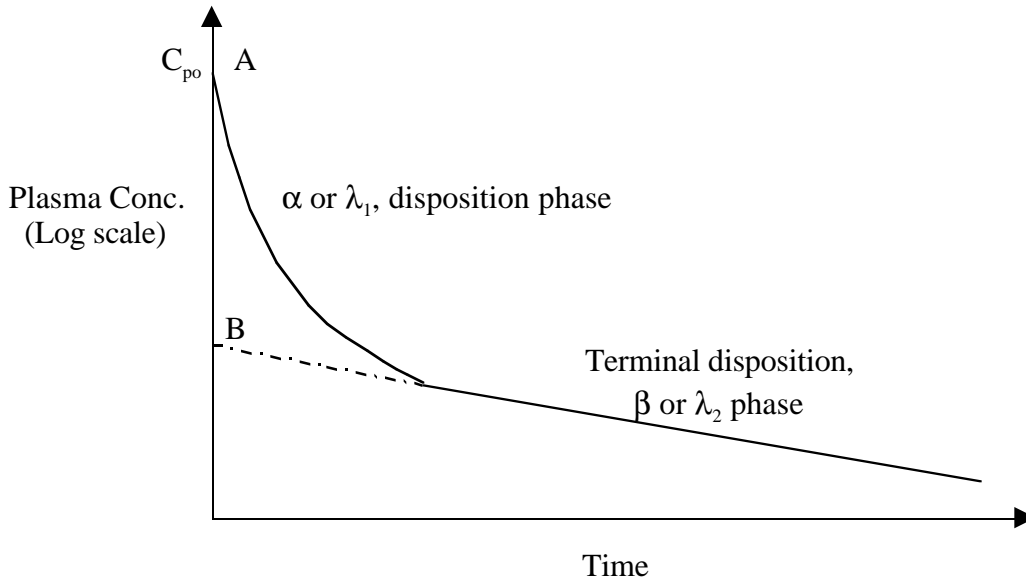


Fig. 2. Semilogarithmic plasma concentration versus time profile of a drug described by two compartment open model (Benet and Massoud, 1984).

To obtain estimates of the model parameters, equation (1) can be fit to the plasma concentration-time profile to obtain values for A, α , B and β . These values may then be used to calculate the model and associated parameters (Gibaldi and Perrier, 1982).

$$V_1 = \frac{C_{po}}{A + B} \quad (2)$$

$$k_{21} = \frac{A\beta + B\alpha}{A + B} \quad (3)$$

$$k_{10} = \frac{\alpha\beta}{k_{21}} \quad (4)$$

$$k_{12} = \alpha + \beta + k_{21} + k_{10} \quad (5)$$

$$V_2 = \alpha + \beta - k_{21} - k_{10} \quad (6)$$

$$Vd_{ss} = V_1 + V_2 \quad (7)$$

$$V_\beta = \frac{V_1(k_{12} + k_{21} - \beta)}{k_{21} - \beta} = \frac{V_1 k_{10}}{\beta} \quad (8)$$

$$Cl_b = k_{10} V_1 = \beta V_\beta \quad (9)$$

$$T_{1/2,\alpha} = \frac{\ln 2}{\alpha} \quad (10)$$

$$T_{1/2,\beta} = \frac{\ln 2}{\beta} \quad (11)$$

The plasma ceftiofur concentration-time profile from intraperitoneal and intramuscular administration (chapter III) were best described by noncompartment model. Noncompartmental pharmacokinetic parameters based on the statistic moment (compared with the method of least squares used by compartmental analysis) are usually expressed in terms of the area under the first moment curve (AUMC) and area under the plasma concentration-time curve (AUC)(Fig. 3).

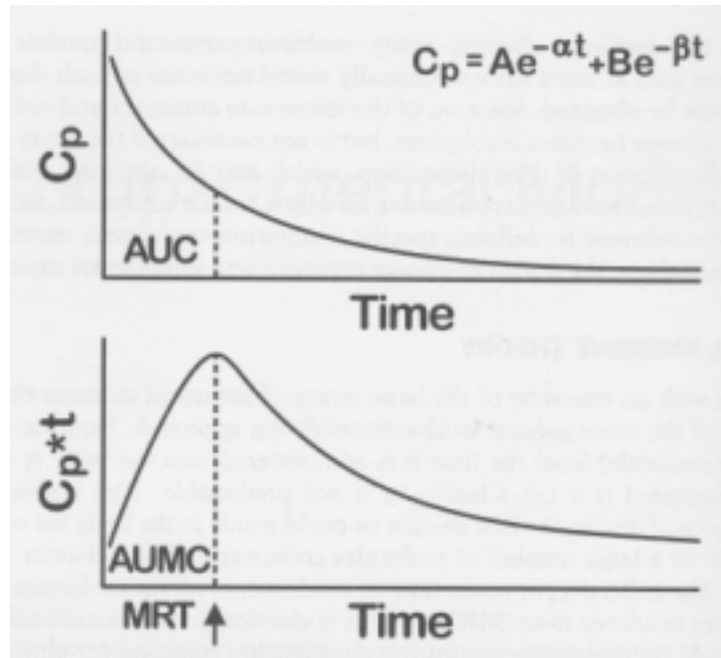


Fig. 3. Plasma concentration versus time (C-T) and its first-moment (CT-T) plots demonstrating area under the curve (AUC), area under the moment curve (AUMC) and mean residence time (MRT) (Riviere, 1999).

Noncompartmental equations for calculating common pharmacokinetic parameters from an analysis of plasma concentration-time profile are summarized below

$$Cl_b = \frac{\text{Dose}}{\text{AUC}} \quad (12)$$

$$Vd_{ss} = \frac{(\text{Dose})(\text{AUMC})}{(\text{AUC})^2} \quad (13)$$

$$V_1 = \frac{\text{Dose}}{C_{po}} \quad (14)$$

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}} = \frac{Vd_{ss}}{Cl_b} \quad (15)$$

$$t_{1/2} = 0.693 \text{ MRT} = 0.693 \frac{Cl_b}{Vd_{ss}} \quad (16)$$

$$t_{1/2, \lambda} = \frac{0.693}{\lambda} \quad (17)$$

The major advantage of the noncompartmental model structure is that any number of recirculations or exchanges can occur, with any number of noncentral pools, none of which have to be identified with any physiological structures. This is in contrast to a multiple compartmental structure, for which this connectivity must be rendered more specifically whether or not the physiological or anatomic identity of the several compartments is known. The noncompartmental model requires that the substance under study is directly eliminated from only the (central) pool or region in which a tracer, drug, or other indicator is introduced and measured in a kinetic study.

Pharmacokinetic abbreviations

C_{p0}	Plasma drug concentration at time zero.
A, B	Zero-time plasma drug concentration intercepts of biphasic disposition curve.
α, β	Values of α and β are related to the slopes of the different phases of a bi-exponential drug disposition curve (α = distribution rate constant ; β = elimination rate constant).
Cl_b	Body clearance of a drug, which represents the sum of all clearance process in the body.
AUC	Area under the plasma drug interaction-time curve after administration of a single dose.
AUMC	Area under the moment curve.

MRT	Mean residence time which is average time the drug molecules that are introduced reside in fish.
k_{el} (k_{10})	First-order elimination rate constant for disappearance of a drug from the central compartment.
k_{12} , k_{21}	Rate constant describing diffusion from the central compartment (1) to the peripheral compartment (2) and vice versa.
V_1	Apparent volume of the central compartment (1).
V_2	Apparent volume of the peripheral compartment (2).
V_β	Apparent volume of the central compartment during elimination phase.
$t_{1/2, \alpha, \beta, \lambda}$	Represent half-lives of a drug in different phases of a two- ($t_{1/2, \alpha}$ = distribution half-life ; $t_{1/2, \beta}$ = elimination half-life) and non-compartment ($t_{1/2, \lambda}$ = elimination half-life) experimental drug disposition.
Vd_{ss}	Apparent volume into which a drug distributes in a fish at steady state.

Akaike's Information Criterion (AIC)(Akaike, 1976 ; Yamaoka et al., 1978)

Akaike's Information Criterion (AIC) is a statistical technique to estimate the number of exponential term, n, for the time course of drug. Based on linear pharmacokinetics, the time course of plasma concentration, C_p , is given as a sum of exponential functions :

$$C_p = \sum a_i e^{b_i t} \quad (18)$$

where a_i and b_i are hybrid coefficients and exponential terms in the linear equation ($b_i > 0$), t is time, and n is the number of exponential terms. AIC was proposed by Akaike (1976) to determine the number of exponential terms in equation (18). AIC was defined on the assumption that the random errors obey Gaussian distribution :

$$AIC = N \ln R_e + 2p \quad (19)$$

where N is the number of exponential data points, p is the number of parameters in an estimated model, and R_e is the residual sum of squares defined by

$$R_e = \sum_{i=1}^n W_i (\hat{C}_i - C_i)^2 \quad (20)$$

where C_i is the observed concentration, \hat{C}_i is the estimated concentration obtained from the model equation using the method of W_i is the weighting for the i th observation.

To select the best models from the given set of possible models, there are two commonly used selection criteria to minimize Akaike Information Criterion, AIC (equation 19) or to minimize the residual sum of squares, R_e (equation 20). The equation with minimum AIC is regarded as the best representation of the time course plots. Therefore, this statistic method is called "minimum AIC estimation" (MAICE). When the residual sums of squares are almost equal for two different models. MAICE picks out the model with the smaller number of parameters.

Appendix D

Preparation of pronephros or spleen for cytometry

**Preparation and standardize of cell suspensions for non-specific
immune response study in grass carp,
Ctenopharyngodon idella.(Ahmed et al., 1985)**

1. Collection of spleen and pronephros

Carefully remove spleen or pronephros of the fish and place separately into 60 x 15 mm polystyrene tissue culture dishes containing 5 ml DPBS

2. Preparation of cell suspensions

- 2.1 Release cells from spleen or pronephros into culture plates by using sieve screens (60-mesh stainless steel sieve screen)
- 2.2 Collect cell suspension from culture dishes and transfer into 17 x 100 mm round bottomed polystyrene culture tubes.
- 2.3 Centrifuge the cell suspensions for 5 minutes at 1200 rpm

3. Lysing of erythrocytes.

- 3.1 Add 4 ml lysing solution into each tube.
- 3.2 Allow the cells to remain in the lysing solution for 3-5 minutes at room temperature (22 °C).
- 3.3 Add 4 ml DPBS into each tube.
- 3.4 Centrifuge the cell suspensions for 5 minutes at 1200 rpm at room temperature (22 °C).
- 3.5 Wash cell suspensions by centrifugation using 4 ml DPBS.

4. Quantification and standardization of cell suspensions

4.1 Quantification of cell suspensions

4.1.1 Resuspend the cells in 2 ml DPBS.

4.1.2 Pipette 5 μ l cell suspension into the tube containing 10 ml DPBS.

4.1.3 Count cells with the CASY Cell Counter

4.1.4 Calculate number of cells in the original 2 ml cell suspension as following :

if the CASY count is Y cells

5 μ l suspension contains Y cells

10 ml * 1000 μ l suspension contains 2000 * Y cells

The concentration of cell suspension (in 4.1.1) = 2000 * Y cells/ml

The total cell in the tissue (in 4.1.1) = Y*2000*2

4.2 Standardization of cell suspension

4.2.1 The number of cells in the suspension that need to be standardized is
5 x 10⁶ cells/ml.

4.2.2 To make the concentration of cells in sample tubes to 5 x 10⁶ cells/ml, divide the number of total cells in the sample tube by 5 x 10⁶. This will give the final volume of cell suspensions containing 5 x 10⁶ cells/ml.

4.2.3 Subtract the final volume with the resuspended volume (2 ml). This gives the number of DPBS in ml needed to be added in the sample

tubes.

Chemicals

1. Dulbecco's Phosphate Buffered Saline (DPBS)(Sigma BioSciences, St. Louis, MO, USA)

Potassium Phosphate Monobasic	0.2 gm/l
Potassium Chloride	0.2 gm/l
Sodium Chloride	8.00 gm/l
Sodium Phosphate Dibasic (Anhydrous)	1.15 gm/l

Sterilize by filtration using an membrane with porosity of 0.22 μ . Adjust pH of solution to 0.1-0.3 pH unit below the desired pH (since it may rise during filtration). The use of 1 N HCl or 1 N NaOH is recommended.

2. Lysing solution

NH ₄ Cl	800 mg/l
NaHCO ₃	84 mg/l
EDTA	29.2 mg/l

Bring the volume to 1 L with PBS

Equipment & Supplies

1. CASY1 Cell Counter (Cell Tools, Inc., South San Francisco, CA, USA)
2. IEC-Centra GP8R Centrifuge

Appendix E

Chemiluminescence assay

**Study of non-specific immune responses in grass carp,
*Ctenopharyngodon idella.***

Chemiluminescence assay (Bass et al., 1983)

1. Transfer 300 µl of samples from 5×10^6 cell/ml cell suspension tube into 12 x 75 mm culture tube.
2. Add 5 µl of DCF-DA stock solution (= 5mM in EtOH) to each tube (set aside one tube of unstained cells to set background fluorescence on the cytometer with). Incubate tubes with DCF-DA for 15 minutes at room temperature.
3. Immediately after the DCF-DA 15 min incubation, add 10 µl PMA working solution (100 ng/ml RPMI) to tubes received DCF-DA (all tubes except the unstained cells). Incubate for 30 minutes at room temperature.
4. Immediately after PMA 30 min. incubation, place tubes on ice and analyze for green fluorescence by flow cytometry.

Reagents

1. Dichlorofluorescein-diacetate (2',7'-dichlorofluorescein diacetate (DCF-DA) ; Molecular Probes, Eugene, OR, USA ; 5 mM)
2. Splenic and pronephric cultured medium (RPMI 1640 ; Mediatech, Cellgro, VA, USA)
3. Phorbolmyristate acetate (Phorbol 12-myristate 13-acetate (PMA) ; Sigma, St. Louis, MO, USA ; 10 ng/ml)

Equipment & Supplies

1. Flow cytometer (Coulter Epics XL) & Data analysis computer program (Coulter Electronics, Hialeah, FL, USA)

Appendix F

Phagocytosis assay

**Study of non-specific immune responses in grass carp,
*Ctenopharyngodon idella.***

Phagocytosis assay (Dunn and Tylor, 1981)

1. Collect spleen or pronephros, lyse RBCs, and suspend cells at 5×10^6 cells/ml according to the sample preparation.
2. Take 300 μ l samples from 5×10^6 cells/ml tube into 12 x 75 mm culture tubes
3. Prepare fluorescent beads (Fluoresbrite microspheres®) by diluting 1:2.8 in RPMI. Ten μ l of beads will be added to each tube of cells. Wash beads in RPMI by centrifugation. Examples : If you have 24 tubes of cells, you will need to add 240 μ l of beads to these tubes. Take 100 μ l of beads + 180 μ l RPMI = 280 μ l total volume at the proper dilution.
4. Sonicate beads in RPMI using miniprobe at 35 % for 30 sec. (15 sec. on, 15 sec. off, 15 sec. on). Make sure vial with beads is on ice so solution of beads will not get too hot.
5. Add 10 μ l of beads to each sample tube (set aside one tube of unstained cells to set background fluorescence on the cytometer with). Add 4 ml RPMI to each tube and incubate tubes for 18 hr. at room temperature (22-23 °C).
6. Wash cells in 2 ml RPMI to remove unphagocytized beads and resuspend cells in 0.3 ml RPMI
7. Analyze by flow cytometry.

Reagents

1. Fluoresbrite microspheres® (Fluoresbrite Yellow Green (YG) Carboxylate Microspheres (1.75 μm), Polyscience, Inc. ; Warrington, PA, USA
2. Splenic and pronephric cultured medium (RPMI 1640 ; Mediatech, Cellgro, VA, USA)

Equipment & Supplies

1. Flow cytometer (Coulter Epics XL) & Data analysis computer program (Coulter Electronics, Hialeah, FL, USA)
2. IEC-Centra GP8R Centrifuge

Appendix G

Data from the immunotoxicity study

Table 1. Effect of ceftiofur sodium on the number of leukocytes in grass carp,
Ctenopharyngodon idella

Experimental groups (n = 6)	The number of leucocytes (x 10 ⁶)(Mean ± SEM)	
	Pronephros	Spleen
<u>24 hours</u>		
Control	57.14 ± 7.95 ^a	130.49 ± 10.58 ^a
8 mg/kg	54.23 ± 4.16 ^a	128.85 ± 12.36 ^a
40 mg/kg	50.32 ± 7.95 ^a	127.39 ± 14.19 ^a
<u>48 hours</u>		
Control	57.14 ± 7.95 ^a	130.49 ± 10.58 ^a
8 mg/kg	43.39 ± 4.44 ^a	134.52 ± 11.16 ^a
40 mg/kg	46.19 ± 6.38 ^a	131.86 ± 9.47 ^a

Within columns, treatment means followed by the same superscript letter are not significantly different

($p = 0.2571$ in pronephros and $p = 0.3535$ in spleen)

Table 2. Effect of ceftiofur sodium on the phagocytosis response (Mean \pm SEM) of pronephros and spleen leukocytes in grass carp, *Ctenopharyngodon idella*.

Experimental group (n = 6)	% Response from Flow Cytometer (Mean \pm SEM)	
	Pronephros	Spleen
<u>24 hours</u>		
Control	28.86 \pm 2.64 ^a	16.13 \pm 1.77 ^a
8 mg/kg	25.53 \pm 1.15 ^a	15.72 \pm 2.97 ^a
40 mg/kg	26.47 \pm 2.64 ^a	17.64 \pm 4.67 ^a
<u>48 hours</u>		
Control	28.86 \pm 2.64 ^a	16.13 \pm 1.77 ^a
8 mg/kg	22.28 \pm 1.78 ^a	21.81 \pm 3.25 ^a
40 mg/kg	26.16 \pm 1.38 ^a	20.83 \pm 6.22 ^a

Within columns, treatment means followed by the same superscript letter are not significantly different

($p = 0.4097$ in pronephros and $p = 0.8627$ in spleen)

Table 3. Effect of ceftiofur sodium on the production of H₂O₂ (Mean ± SEM) from pronephros and spleen in grass carp, *Ctenopharyngodon idella*.

Experimental group (n = 6)	% Responses from Flow Cytometer (Mean ± SEM)	
	Pronephros	Spleen
<u>24 hours</u>		
Control	67.37 ± 4.80 ^a	NR
8 mg/kg	68.86 ± 4.80 ^a	NR
40 mg/kg	86.76 ± 5.88 ^b	NR
<u>48 hours</u>		
Control	67.37 ± 4.80 ^a	NR
8 mg/kg	68.97 ± 5.09 ^a	NR
40 mg/kg	86.18 ± 5.88 ^b	NR

Within columns, treatment means followed by the same superscript letter are significantly different

($p = 0.0233$), NR = No Response

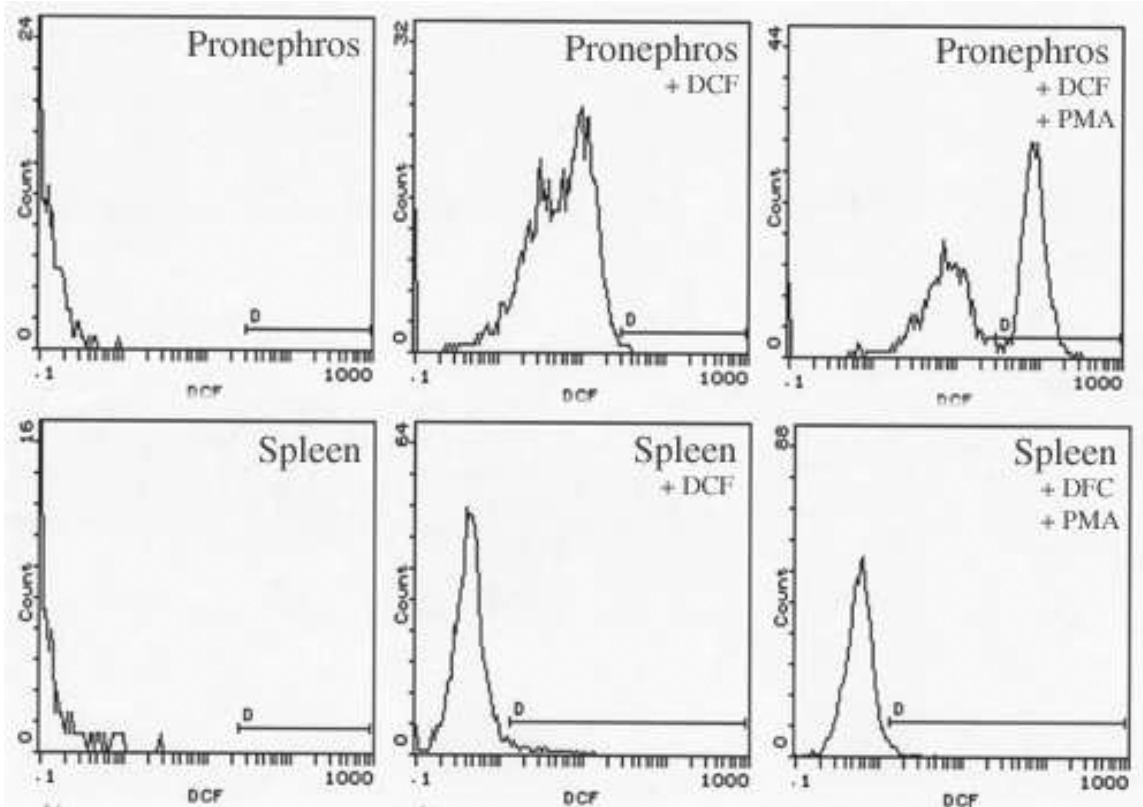


Fig. 1. Fluorescence distribution of resting and stimulated polymorphonuclear leukocytes responded to H_2O_2 production assay in pronephric and splenic tissue. The Histograms represent numbers of cells (on the ordinate) as a function of fluorescence intensity (on the abscissa).

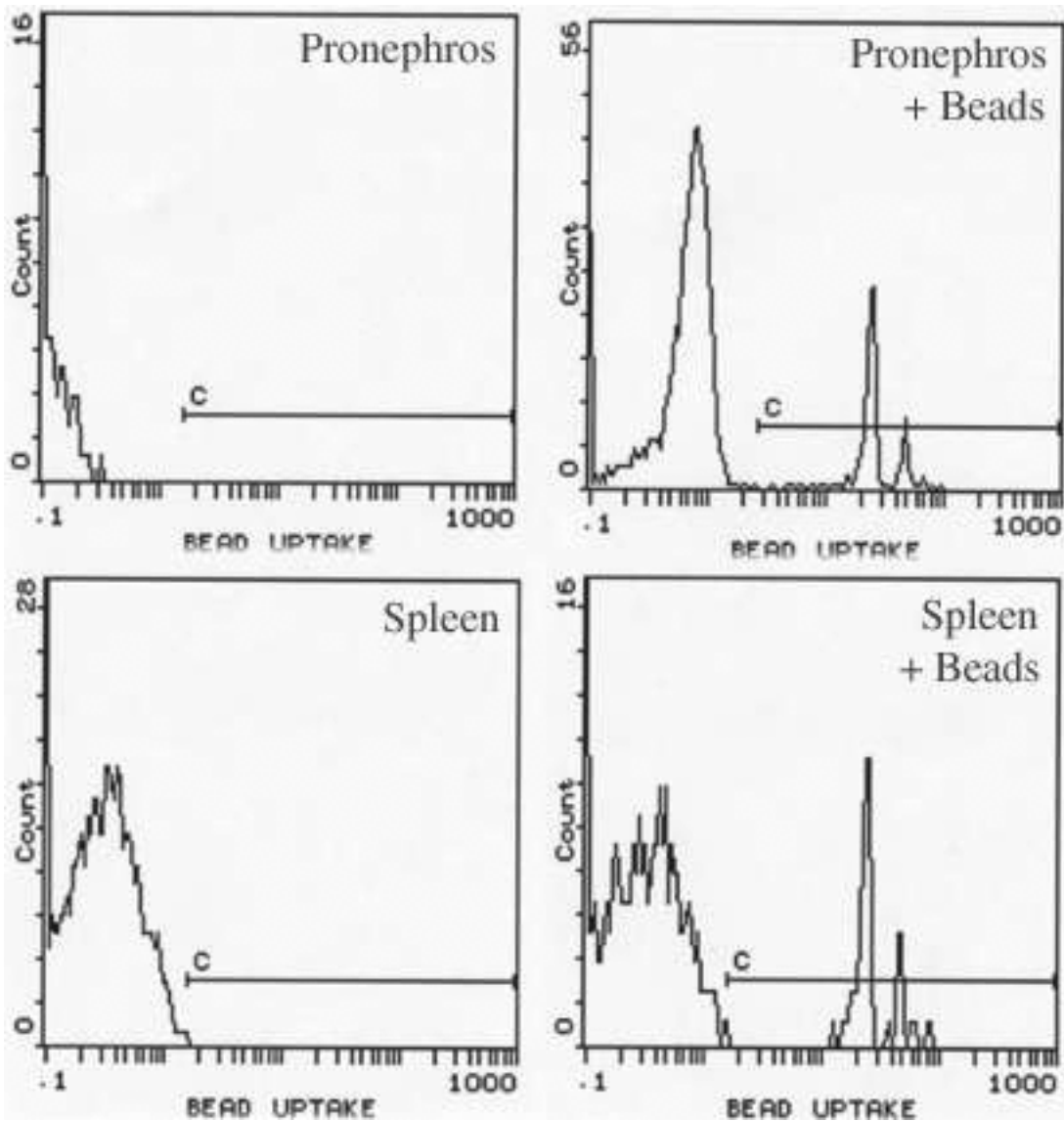


Fig. 2. Fluorescence distribution of resting and bead-phagocytosing leukocytes from phagocytosis assay in pronephric and splenic tissue. The Histograms represent the numbers of cells responding to the assay by uptaking the beads (on the ordinate) as a function of fluorescence intensity (on the abscissa).

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