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2,4–D Treatment in Tench (*Tinca tinca* L.): Pathological Processes on the Excretory Kidney

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The presence of contaminants posts a serious problem in aquatic ecosystems due to their potential and often lethal physiological and biochemical effects on the inhabitants of such environments (Ranke-Ribiya et al. 1995). One such contaminant is 2,4–dichlorophenoxyacetic acid (2,4–D), a phenoxy herbicide widely used in agriculture and forestry for weed control purposes (Arnold et al., Larson and Houglum, Rodriguez and Amin 1991).

In fish, the most common routes of entry of 2,4–D arc through the gills and, to a lesser extent, through the external tegumenta (Murty 1986). In the plasma, 2,4–D binds to protein for transport throughout the organism (Arnold and Beasley 1989). 2,4–D is rapidly eliminated as an anion, largely in urine (Hill et al. 1989), through active tubular transport. When the tubular system becomes saturated, elimination is supplemented by glomerular filtration (Arnold and Beasley 1989). The kidney is the organ most affected by long-term administration of 2,4–D in drinking water; enlargement of the kidney is accompanied by hypertrophy of proximal tubular epithelium (Erne and Bjorklund 1970). Under light microscopy, cells displaying hypertrophy show marked mitochondria1 pleomorphism and an increase in mitochondria1 liquid content. Intracytoplasmic and intranuclear inclusions are visible. These lesions give rise to metabolic dysfunction in tubular cells (Erne and Bjorklund 1970).

This study sought to examine the lesions induced by acute poisoning with 2,4–D in tenth excretory renal parenchyma. The kidney has been chosen for study by virtue of being a organ that is involved in excretion of this substance.

MATERIALS AND METHODS

The experiment was carried out in a 200 L tank with external filter without chemical filtration system, in which tench had food withheld for a day for acclimatization purposes. Adult tench of both sexes were roughly 25-30 cm in length and weighed around 200 g. The fish were supplied by the "Centro Nacional de Ciprinicultura Vegas del Guadiana" (Badajoz, Spain).

2,4–D was added at a concentration of 400 mg/L, disolved in water, i.e. half the

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lethal dose (LC_{50}) at 96 hours as has been previously reported by Gómez et al. (1998). During the experiment water was changed each 48 hours by other one with the same concentration and similar aquatic parameters. Thus, concentration of the substance was always constant for 12 days. In this period, animals had food withheld. The 25 (five each time) experimental fish were sacrificed 1, 2, 5, 8 and 12 days post-poisoning. The 10 controls (no-treated and maintained under similar conditions than those treated) were euthanasied (etomidate dissolved in other water tank) in pairs simultaneously, with aquatic parameters and handling similar to the treated animals. Subjects were opened by Salom and Cantarino's technique (1983). The kidney was immediately extracted after the euthanasia, immersed and fixed in phosphate-buffered 5% glutaraldehyde (0.1*M*, pH 7.2) for two days for structural and ultrastructural examination. Aquatic parameters (mean, S.D.) remained within normal limits for this species throughout the experiment, O_2 concentrations (9.84 mg/L, 1.02), pH (8.13, 0.11), temperature (12.63 °C, 0.31), ORP (oxidase-reduction level) (100.33 mV, 17.6), conductivity (715.3 µS, 27.24), nitrate (3.74 mg/L, 3,43) and nitrite levels (0.039 mg/L, 0.024). Samples for structural examination were routinely processed for light microscopy and embedded in paraffin. Sections (5 µm thick) were stained with hematoxylin-eosin or P.A.S. Samples for ultrastructural analysis were postfixed in osmium tetroxide and embedded in epoxy resin. Semithin samples (0.5 µm) were stained with Toluidine blue. Ultrathin sections (50-70 nm) were contrasted using lead citrate and uranyl acetate and viewed through a transmission electron microscope. Findings are shown in Figures 1-6 and tabulated in Table 1.

For statistical analysis, variables have been studied by mean ± standard desviation from 100 samples of each structure and each day of study. The possible effect of the poisoning has been determined using one way analysis of variance according to the following model:

$$X_{ij}=\mu+E_j+\varepsilon$$

E_j depends on the used treatment, X_{ij} is the value due to the treatment and the animals, µ, general mean and ε residual error. Statistical significance of the differences between means has been done by least squares difference (LSD) with 95% confidence level (Table 2).

RESULTS AND DISCUSSION

2,4–D poisoning prompts a series of pathological processes in the excretory renal parenchyma of tench; these include mesangial proliferative glomerulonephritis and tubular nephrosis, which are in turn responsible for renal dysfunction. The kidney plays a major role in the elimination of toxic substances. The presence of kidney lesions from the earliest stages of the experiment serves to confirm the WHO report (WHO 1989) indicating that 2,4–D is rapidly eliminated by this route in fish. Grossly, fish equilibrium (100%), irregular swimming behavior, marked hemorrhages at the base of the fins and the anus, sero hemorrhagic fluid, enlarged kidney parenchyma were observed. Absence of such phenomena in controls indicates a toxin-induced effect.

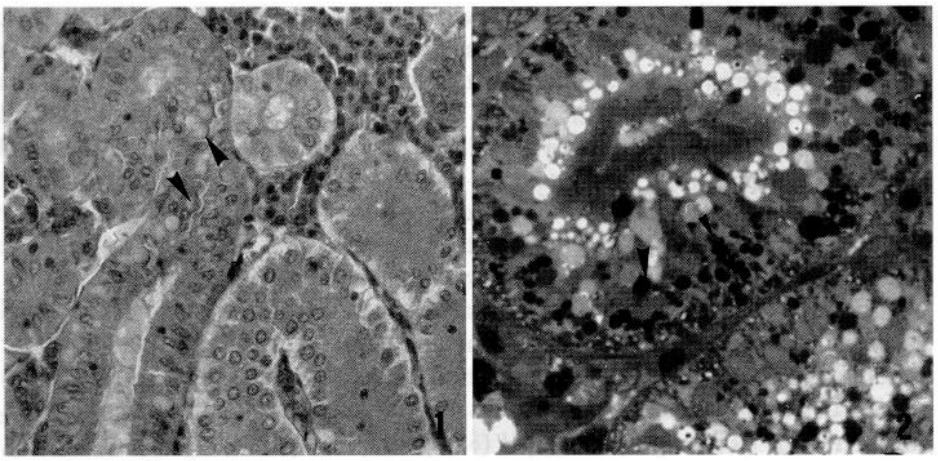

Figure 1. Excretory kidney. 2 days. Proximal tubule. Tubular degeneration; hyaline droplets visible in epithelial cytoplasm (arrows). 5% glutaraldehyde. Haematoxilin-eosin (H-E) X400.
Figure 2. Excretory kidney. 5 days. Proximal tubule. Hyaline (arrows) and vacuolar (V) degenerations with small and medium-sized droplets occupying a large area of tubular epithelial cytoplasm. 5% glutaraldehyde. Toluidine blue X1000.

Tubular system lesions are widely reported in 2,4–D poisoning of fish (Erne and Bjorklund 1970). The major lesion in the present study was marked tubular nephrosis affecting all the tubule portions involved. The progressive nature of tubular lesions characteristic of 2,4–D poisoning, has also been reported for other toxic substances (Roncero et al. 1988, 1989, Luo et al. 1992).

These lesions arc characterized by tubular swelling, leading finally to necrosis. Vacuoles with adiclectronic content were observed in the first batches (Fig. 1) but were subsequently replaced by hyaline droplets, displayed a progressive increase in size and had merged to occupy the medial and apical portions of the tubular epithelium (Fig. 2). Cytoplasmic vacuolar degeneration is reported elsewhere for the liver, in which cell nuclei are compressed and displaced (McBribe et al. 1981).

After five days, small hyaline droplets started to become visible at the basal pole of tubular cells. These droplets increased in size to the point where cell structure was disrupted. As their volume increased, droplets started to become more apparent in the apical pole (Fig. 3). This tubular degeneration was intensified when the experiment progressed together progressive increase in droplet size with the already observed under the light microscope. Droplets similar to those described in earlier days were large, with moderate to high electron density, and sometimes occupied as much as one third of total cytoplasm volume, compressing and disrupting cell structures and displacing the nuclei towards the apical pole (Fig.

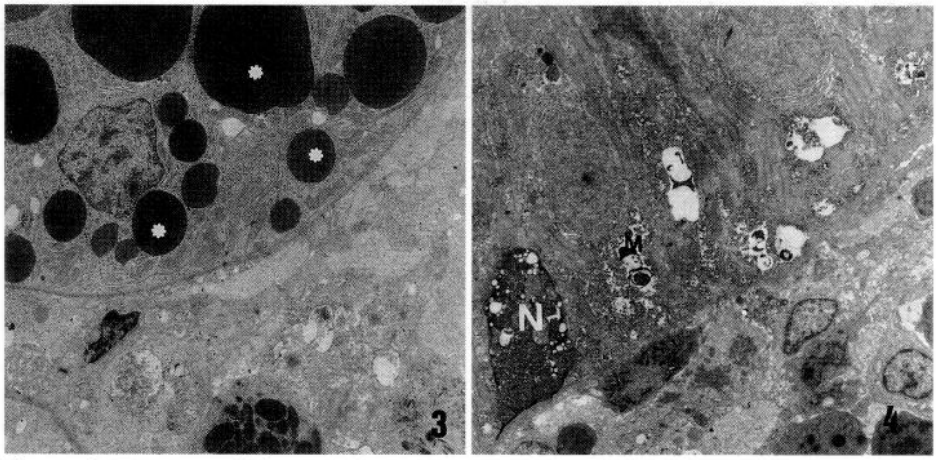

Figure 3. Excretory kidney. 8 days. Numerous small and medium-sized moderate to highly electron-dense hyaline droplets (*), filling tubular cell cytoplasm. 5% glutaraldehyde. Uranyl acetate X2300.
Figure 4. Excretory kidney. 1 day. Proximal tubule showing simple cell necrosis (N). Fine electron-dense granulate and numerous myelin figures (M), mainly located at the basal pole of the tubular epithelium. 5% glutaraldehyde. Uranyl acetate X3000.

3). Intracytoplasmic and intranuclear inclusions have been reported in chickens receiving 1.000 mg/L doses in drinking water (Erne and Bjorklund 1970); in the present study, however, there was no evidence of intranuclear inclusions.

Both small and large hyaline droplets contained protein, possibly due to the high glomerular filtration rate resulting from extensive damage to the filtration barrier; protein was subsequently reabsorbed at tubular level, giving rise to these inclusions. Epithelial cell cytoplasm contained abundant, fine electron-dense granulate; there was also evidence of degeneration of cytoplasmic organelles, giving rise finally to the production of necrosis and the formation of myelin figures (Fig. 4). Degenerative necrosis is also reported in poisoning by other toxic products (Roncero et al. 1988). In the present study, lesions primarily affected type I proximal convoluted tubules, perhaps because they are the first to contact with the toxin; subsequently, however, lesions extended to the whole tubular system. Variations of the findings are tabulated in table 1.

These facts show changes in tubular cell size. The lumen/area ratio in type I proximal convoluted tubules displayed a decrease observed the day 5 and lasted until the end of the experiment (Table 2). On the other hand, there was a clear increase of the ratio in type II proximal tubules and distal tubules in the first day, decreasing gradually until the end of the experiment. The ratio in type distal tubule in the last day suffered a slight increase (Table 2).

Table 1. Structural and ultratructural findings.

	Control	1 day	2 days	5 days	8 days	12 days
Tubular vacuoles	0/0	0/0	++/**	++/***	++/**	++/*
Hyaline droplets	0/0	0/0	++/**	++/***	+++/***	+++/***
MP Glomeruloneph.	0/0	0/0	++/**	+++/***	+++/***	+++/***
Degener./necrosis	0/0	+/**	+/**	+/**	++/**	++/**
Myelin figures	0/0	+/**	+/**	++/**	++/**	++/**
BM[a] thickening	0/0	0/0	++/**	++/**	++/***	++/***
Electrondense struct.	0/0	0/0	++/*	++/**	++/**	++/**

Note. Frequency/severity. Frequency: 0:absence, +:1-50% affected, ++:51-99%, +++:100% Severity: 0:absence, *:slight, **:moderate, ***:grave.
[a]BM: Basement membrane.

Table 2. Effect of the poisoning on the excretory structures

		Corpuscle[1]		ICP Tubule[2]		IICP Tubule[2]		Distal Tubule[2]	
Treatment*	Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	100	89.5a	5.4	6.3a	1.9	4.8ab	1.1	7.7a	3.2
1 day	100	93.5d	3.1	6.1a	2.9	5.5d	2.2	8.1ab	2.4
2 days	100	90.7ad	6.3	6.9a	1.8	5.6bd	1.9	7.9a	2.4
5 days	100	77.8bc	9.6	4.1cd	1.7	3.3c	1.5	5.8c	2.1
8 days	100	77.5bc	11.4	4.5d	1.2	3.3c	1.5	5.8c	2.1
12 days	100	75.7ce	10.3	2.8b	1.1	2.3e	0.9	6.1c	1.8

*p<0.001.
Different letters point to different means (P<0.05). E.g. a is similar to ab, ab is similar to b, but a is different from b. Values are shown as %.
Corpuscle[1]: glomerulus/corpuscle ratio. Tubule[2]: lumen/area.

Increase in cell size, evident here in the decreased lumen/area ratio, is due to droplet formation in cytoplasm. In the earlier batches, the ratio oscillated around

control values, but from the day 5 onwards values fell, remaining low until the end of the experiment. This decrease points to a increase of cell size. Erne and Bjorklund (1970), noted that this fact is attributable to cell hypertrophy through increased mitotic activity. However, we think that it is due to the growing abundance of hyaline droplets, and constant low values thereafter to the persistence and indeed increase of droplets, which finally occupied a large portion of cell cytoplasm.

Corpuscle lesions, visible from the outset, became more marked as the study progressed. After two days there was evidence of mesangial proliferative glomerulonephritis (Fig. 5). Under electron microscopy there was evident disruption of the glomerular filtration barrier, with apparent loss of continuity of the vascular endothelium much more evident in 12 days (Fig. 6). A marked thickening of the basement membrane (BM) was also observed, associated with the presence of both abundant moderately electron-dense fibrillar matter and granular material (Fig. 6). Similar changes, including marked thickening, were observed in pedicels, which contained rounded, highly electron-dense substances. Podocyte cytoplasm contained a considerably higher number of electron-dense structures (Fig. 6).

The growing intensity of lesions was reflected ultrastructurally in a considerable thickening of the filtration barrier; irregularly-shaped protuberances of varying electron density were distributed unevenly over the whole barrier. There was marked thickening of the basement membrane, which contained fibrillar and granular matter of varying electron-density. These protuberances probably arise from a pesticide-induced subendothelial edema, indicative of impaired permeability and selectivity of the membrane. In the latter stages, evident and severe disruption of the vascular endothelium directly affected the filtration system. The cytoplasm of filtration-barrier podocytes was filled with a fine electron-dense granulate and abundant, highly electron-dense vacuoles. Pedicels contained a similar granulate, together with a number of electron-dense structures and a variety of adiclectronic dilations. Alterations in pedicels and podocytes were similar to those observed in the filtration barrier.

Renal corpuscle lesions varied significantly with exposure time. Corpuscle ratio increased in the first two days, thereafter recording a considerable decrease due to cell damage (Table 2).

Circulatory lesions such as edema are probably due to impaired permeability, and to the greater fragility of cell membranes. As the pesticide enters the organism, it binds to proteins for transport through the organism (Arnold and Beasley 1989), and also affects membrane-forming proteins, thus altering membrane morphology and selectivity. Impaired selectivity leads to tubular cell degeneration, altered cell equilibrium and, ultimately, necrosis.

The 2,4–D concentration used here may be seen as high for an aquatic medium.

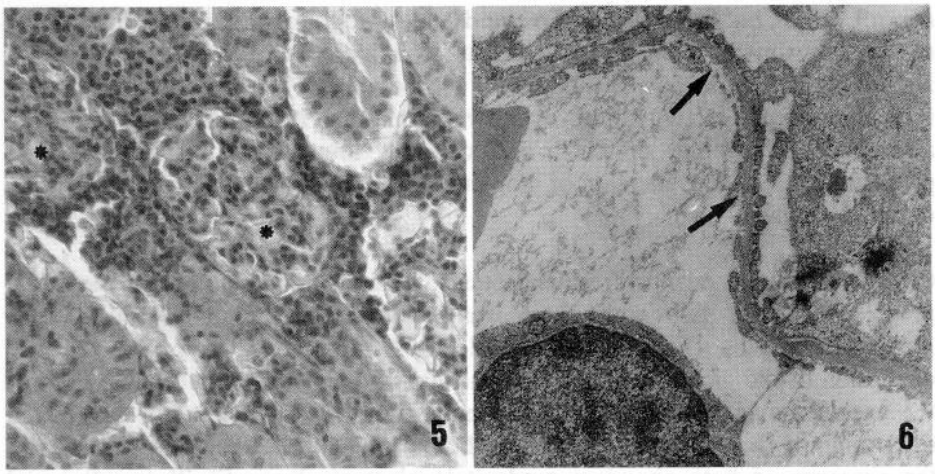

Figure 5. Excretory kidney. 5 days. Renal glomeruli. Proliferative mesangial glomerulonephritis, enlarged mesangial cells and matrix, and consequent reduction of Bowman's space (*). 5% glutaraldehyde. H–E X400.
Figure 6. Excretory kidney. 12 days. Glomerular capillary. Endothelial disruption (arrows). Marked thickening of filtration barrier basement membran podocytes (P). 5% glutaraldehyde. Uranyl acetate X 13000.

According to other authors (Arnold et al. 1991) elimination of the toxin is achieved largely through active tubular transport; when this mechanism is saturated, elimination is supplemented by glomerular filtration. In the present study, lesions in both the corpuscle and the tubular system were evident from the outset, suggesting that the administered dose saturated both elimination systems from the first day of poisoning.

All the facts note that acute 2,4–D poisoning at a concentration of 400 mg/L. provoked changes on excretory cell components, which in turn gave rise to impairment of excretory kidney tissue function.

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