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EFFECT OF THE HERBICIDE AVANS 330 SL ON THE LIVER PATHOMORPHOLOGY OF CLINICALLY HEALTHY CARP (*CYPRINUS CARPIO* L.) AND CARP INFECTED BY *ICHTHYOPHTHIRIUS MULTIFILIIS*

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ABSTRACT. This study was conducted on fingerlings of carp, *Cyprinus carpio* L., that were either clinically healthy or infected with a natural invasion of the ciliate, *Ichthyophthirius multifiliis*. The fish were exposed for 96 h to Avans 330 SL in a concentration of 2 mg trimethylosulfonium glyphosate l^{-1} water in order to establish its effect on the morphological pattern of the liver. It was demonstrated that the most frequent morphological deviations, particularly in fish infected with the protozoa and bathed in water with the addition of Avans, included regressive lesions, such as parenchymatous, vacuolar degeneration, and focal necrosis. Less often in these cases, and even less often in carp infected with *I. multifiliis* or only exposed to Avans, were disorders observed in the circulation in the form of hyperaemia and minor extravasations. Changes were noted in the ultrastructures of the mitochondria and the endoplasmic reticulum. In addition, the results obtained indicate that ciliate invasion intensified the occurrence of morphological lesions in the livers of carp that were exposed to Avans 330 SL in comparison to fish that were free of this infection.

Key words: CARP (*CYPRINUS CARPIO*), *ICHTHYOPHTHIRIUS MULTIFILIIS,* LIVER, AVANS 330 SL, GLYPHOSATE, MORPHOLOGICAL LESIONS IN THE LIVER, LIVER ULTRASTRUCTURE

INTRODUCTION

As an important element of the aquatic ecosystem, fish are exposed to pollution caused by man-made compounds known as xenobiotics. Indeed, herbicides that are widely used in agriculture constitute the most frequent source of extensive water pollution (Alberdi et al. 1996, Sopińska et al. 1995, 2000, Żelazny 2002). Their harmful

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impact is intensified by the phenomenon of accumulation that occurs in living organisms and in their habitats, as well as by the condensation of xenobiotics in the food chain, which poses the greatest danger to the environment (Ghosh and Konar 1983, Gluth et al. 1985). The amount of pesticide that accumulates depends not only on its concentration in the water and exposure time but also on skin status; skin injuries, such as those produced by parasites, increase accumulation.

The adverse effect of herbicides on the aquatic environment and its organisms is demonstrated by the occurrence of pathological lesions and immunosuppression (Nešković et al. 1996, Sopińska et al. 1995, Kolman et al 2003). On the one hand, the increased herbicidal pollution of water and soil has been observed lately, which has prompted new efforts to examine their effects on widely understood ecosystems, primarily fields, forests and meadows, and less often on aquatic ecosystems (Dunier and Siwicki 1993, Sopińska et al. 2000). On the other hand, there are insufficient studies concerning the pathomorphology of the internal organs of fish exposed to small amounts of herbicides.

Research into glyphosate toxicity (the active substance in Avans 330 SL-AV) has demonstrated its moderate toxicity in mammals and fish (toxicity class IV). However, it can have a negative effect on the aquatic environment and can manifest its toxic characteristics in an indirect way by becoming a component of the fauna and flora of aquatic ecosystems (Alberdi et al. 1996, Nešković et al. 1996). Studies that have focused on the effects of glyphosate itself and have demonstrated its toxic effects on aquatic organisms and fish are relatively few (and those that examine preparations containing it are even rarer) (Nešković et al. 1996, Raszka et al. 1998, Sopińska et al. 2000, Szarek et al. 2000a, b). Some studies have found that glyphosate in the concentrations used in agriculture can have a toxic impact on hepatocytes and renal duct cells in fish (Studnicka and Siwicki 1997, Szarek et al. 1997, 2000a, b).

Many factors causing disorders in the homeostasis of organisms (bacterial, viral, or parasitic diseases) can result in the increased susceptibility of specimens to xenobiotics and can intensify their effects. For some years in Poland there has been an increase in the frequency of the occurrence of ichthyophthiriasis (ICH). This phenomenon has been generated by limiting the scope and frequency of such maintenance works as mowing and desludging ditches in the area of fishponds (Róg 2002). Ichthyophthiriasis is caused by a parasite known as *Ichthyophthirius multifiliis*, also referred to as a cili-

ate, and it is considered a typical “transport disease”. A mature form of the ciliate parasitizes almost all freshwater fish and feeds on their tissues. When there is significant parasite density the invasion quickly spreads and reaches significant intensification (Maki et al. 2001, Kinnunen et al. 2005).

The preceding data indicate that glyphosate has various, negative effects on living organisms, including fish. The aim of the study was to define the effect of so-called toxically safe low level concentrations of glyphosate on the liver morphology in common carp, *Cyprinus carpio* L., with healthy skin and in those injured by *I. multifiliis*.

MATERIAL AND METHODS

The research was conducted on carp juveniles weighing from 81 to 98 g. The fish were obtained from two breeding centers: Wąsosze Fish Farm (central Poland) and the Experimental Fish Farm of Freshwater Fishery in Żabieniec, which is a branch of the Stanisław Sakowicz Inland Fisheries Institute in Olsztyn, Poland (IFI Olsztyn; central Poland). The fish were fed granulated feed (Aller 37/12) produced by Aller Aqua, Poland. The feed chemical composition was 37% protein, 31% carbohydrate, 12% fat, 7% ash, and 4% fiber and its energetic value was 19.5 MJ kg^{-1}. Prior to the experiment, the carp were acclimated to laboratory conditions for two weeks and after this period they were weighed.

The study was conducted as two separate experiments: A – on clinically healthy carp free of *I. multifiliis* and B – on carp infected with the protozoa (permission from Local Ethical Commission No. 31/N). The carp from group B did not exhibit any deviation from the norm either immediately after transport or throughout the acclimatization period. The histological skin lesions and the presence of parasites on the skin of the fish suggested a ciliate invasion of medium intensity that had been present for a few days (Szarek et al. 2006). In each experiment, the fish were divided into two equal groups (N = 10): A1 and B1 – control groups, A2 and B2 – exposed to Avans 330 SL (AV) at a concentration of 2 mg trimethylosulfonium glyphosate l^{-1} of water. The examined preparation, in a dose calculated as per active substance, was thoroughly mixed with a small amount of tap water and then introduced into the tanks where the fish had been placed. The AV preparation used in the experiments was produced by Zeneca Agrochemicals of Great Britain. The active substance is trimethylosulfonium glyphosate with

a three-day half-life, 360 g l^{-1} of the preparation. While determining its concentration, the following factors were taken into consideration: the presence of the examined active substance in the bodies of the fish (Banaszkiewicz 2003); the results of studies that indicated the so-called toxically safe herbicide level causes pathology in fish (Szarek et al. 2000a, b); data reported by other authors (Ghosh and Konar 1983, Demael et al. 1990, Dunier and Siwicki 1993); direct penetration of glyphosate to water reservoirs, including the shallow fishponds of fish populations (Aalbers 1996, Piska and Waghray 1997).

The fish were held in 200 l tanks under similar environmental conditions: water temperature 18-19°C; oxygen level 7.5-8.0 mg·l^{-1}; pH 7.5-8.5. Moreover, levels of total ammonia nitrogen (TAN = NH_4^+-N + NH_3-N) did not exceed 0.2 mg TAN l^{-1}. The fish were not fed for the duration of the experiment (*i.e.*, 96 hours). The study also included observations of carp behavior. After the carp had been bathed for 96 hours in the water to which AV had been added, they were removed from the tanks and placed in 10 l tanks containing water with the addition of Propiscin (a 0.2% etomidate solution manufactured by IFI Olsztyn) and anesthetized.

Macroscopic examinations were conducted directly after the fish had been sacrificed. At the same time, liver sections were collected for ultrastructure assessment and for microscopic analysis. The liver sections for microscopic analysis were fixed in 5% neutralized formalin and after dehydration in a series of alcohols and acetone, they were sealed in paraffin blocks. Microscopic fragments were stained with haematoxylin and eosin (Bancroft and Cook 2000) and the PAS method according to McManus (1948) was applied in order to establish the level and arrangement of polysaccharides. The polysaccharide content in the liver was determined by taking into consideration Pearse's (1968) indications and the semiquantitative assessment provided by Szarek et al. (1985). Cryostat fragments of heptopancreas were also stained with Sudan III, using the Lillie Ashburn method in order to expose fat (Bancroft and Cook 2000). The liver for the ultrastructural examination was fixed in glutaraldehyde in a phosphatic buffer of pH 7.2 and sealed in Epon 812. Semi-thin sections were stained according to the method of Levis and Knight (1977); the appropriate place for preparing ultrathin sections was determined under a light microscope. Structural analysis was conducted using an Opton 900 PC electron microscope (Germany).

RESULTS AND DISCUSSION

In experiment A and in the control group of experiment B (group B1), it was established clinically that the carp behaved normally during the experiment and responded distinctly to external stimuli. However, the fish infected with ciliates and exposed to AV (B2) behaved normally only on day one of the experiment. On day two, the liveliness and excitability of the carp in this group increased slightly (the fish exhibited violent movements without any external stimuli), but on days three and four heaviness was observed in their movements. The results of clinical observations and especially the comparison of the behavior of the carp from groups A2 and B2 indicate that the intake of AV increased due to injuries to the carp gills and skin. Macroscopic examinations of fingerling from all groups revealed their correct morphological structure.

The livers of clinically healthy fish from control group A1 were characterized by proper microscopic (Fig. 1.1) and ultrastructural structures. Hepatocytes observed in electronograms contained numerous properly formed organelles, and quite abundantly scattered glycogen grains. As regards cell nuclei, only one nucleus was apparent in most cases. In 15% of the cells two or more nuclei were noted and were usually placed centrally or on the edge and had a varied structure (packed, capitulum, ring-shaped, trabecular, or indirect).

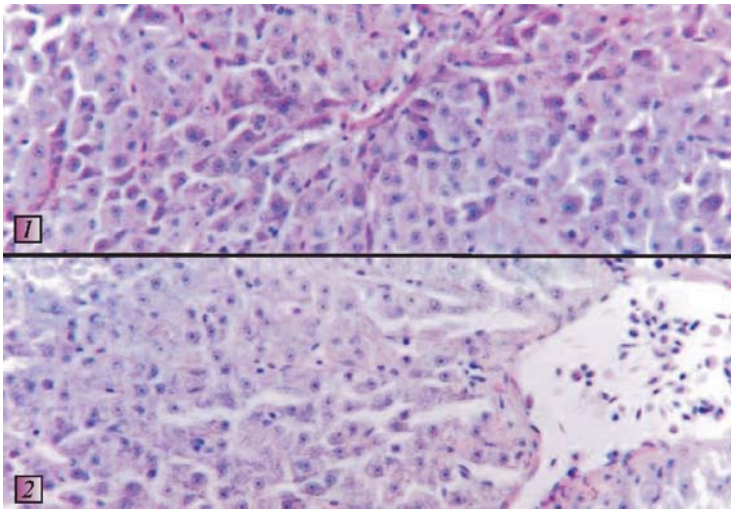

Fig. 1. Liver of carp from experiment A. 1. Liver exhibits normal morphology – carp from group A1. 2. Parenchymatous degeneration and extravasations – liver of carp from group A2. HE stain. Magnification × 500.

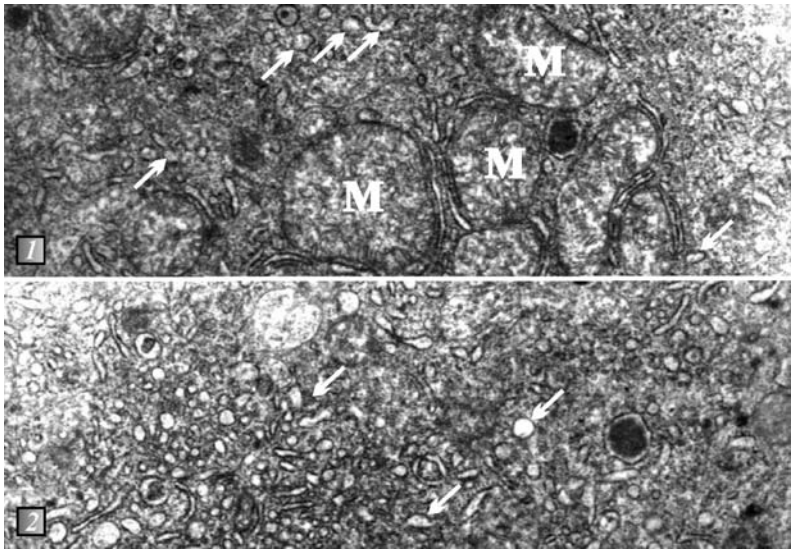

Fig. 2. Liver of carp from group A2. 1. Fragment of hepatocyte with swollen mitochondria with indistinct cristae structure (M) and partial vesicular transformation of rough endoplasmic reticulum – RER (arrows). 2. Clear visible vesicular transformation of RER (arrows). Magnification × 14600.

The microscopic examination of liver in carp from group A2 revealed that there was parenchymatous degeneration at various intensification levels in the majority of the fish (Fig. 1.2). In one case, parenchymatous degeneration was accompanied by vacuolar degeneration. However, hyperaemia was noted more frequently and was usually accompanied by blood extravasations covering small areas (Fig. 1.2). In most of the fish, the cells of the endothelium of blood vessels and sinuses demonstrated a normal structure, and only in two carp did they show swelling. Occasionally, there were changes in behavior of the stellate cells, consisting of their proliferation or hypertrophy. In ultrastructural analysis, hepatocytes in seven carp showed mitochondria swelling, with the partial or total blurring of crests (Fig. 2.1). These were the cases where the growth and broadening of the canals of the rough endoplasmic reticulum (RER) were most often observed with diverse vesicular transformation (Fig. 2).

In four carp from group B1, the hepatocytes displayed characteristics of parenchymatous degeneration (Fig. 3.1), which predominantly covered small areas of the examined fragment. Slightly more often (in five fish), vacuolar degeneration was observed (Fig. 3.1). Hyperaemia occurred in some cases, and sporadically, extravasations were observed. In two carp, connective tissue hypertrophy was observed

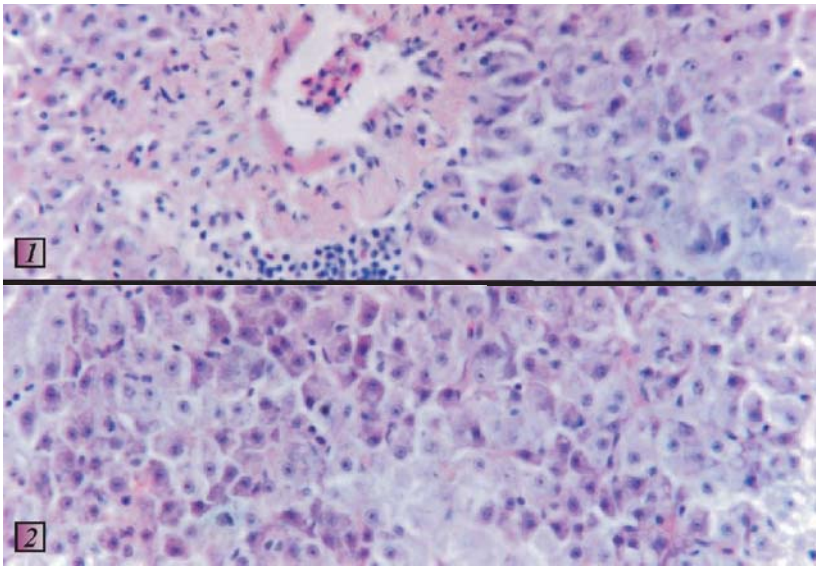

Fig. 3. Liver of carp from experiment B. 1. Liver of fish from group B1 – partial parenchymatous and vacuolar degeneration, hypertrophy of the blood vessel wall with connective tissue and the presence of lymphoid cells in its vicinity. 2. The liver of fish from group B2 – vacuolar degeneration, foci of necrosis. HE stain. Magnification × 500.

in the walls of blood vessels, and in three cases, lymphoid cells were found in this vicinity (Fig. 3.1). Additionally, in three carp, the proliferation and hypertrophy of stellate cells was observed. Ultrastructural examination most often revealed mitochondria swelling (sometimes with the presence of dense bodies in their matrix), together with the blurring of their cristae structure, and hyperplasia and the widening of the canals of the rough endoplasmic reticulum, with slight vesicular transformation and fragmentation (Fig. 4). Additionally, the cytoplasm of hepatocytes sometimes revealed vacuoles, single myelin-like structures, as well as phagosomes and lysosomes. In some cases, the endothelium of the blood vessels was swollen.

The livers of most carp from group B2 displayed characteristics of parenchymatous and/or vacuolar degeneration (Fig. 3.2). Additionally, foci of necrosis of various sizes were found in a similar number of fish (Fig. 3.2). In some cases, enlarged blood vessels were observed which were filled with morphotic blood elements and extravasations of blood occurring over a small area. In most fish, the endothelium cells of blood vessels and sinuses displayed the proper structure, and only in two fish did they reveal swelling. In four fish, stellate cells showed proliferation, while in three – hypertrophy.

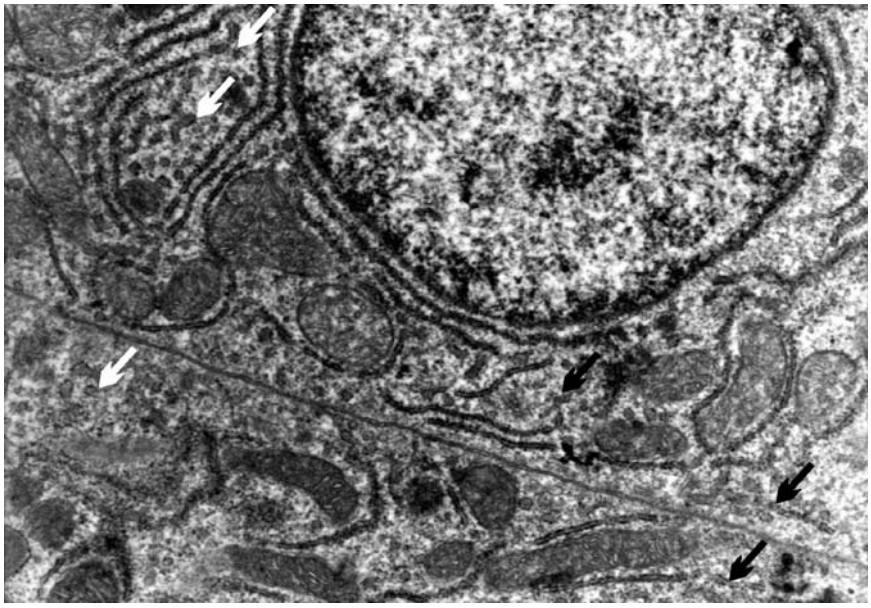

Fig. 4. Fragment of hepatocytes of carp from group B1 – slight vesicular transformation of RER (black arrows) with fragmentation (white arrows). Magnification × 14600.

The hypertrophy of blood vessel walls with connective tissue and the infiltration of lymphoid cells were found in three carp. Ultrastructural examination most often revealed the occurrence of the previously-described lesions in the mitochondria as well as hyperplasia and the widening of the canals of the rough endoplasmic reticulum with diverse vesicular transformation (Figs. 5 and 6). Foci of cytoplasm degradation were observed relatively often and were accompanied by lysosomes, phagosomes, autophage vacuoles, and myelin-like structures (Figs. 5 and 6). Additionally, the cytoplasm revealed glycogen clusters of various sizes (Fig. 6) and sporadically, lipids.

Liver sections from eight fish from group A1 were stained with the PAS method, and it was established that the content of polysaccharides was average and their arrangement regular (Fig. 7.1). In the two remaining specimens, the hepatic cells contained a slightly higher level of polysaccharides that were located mostly far from the blood vessels. In most fish from the other groups (A2, B1, B2), the livers were characterized by a varied polysaccharide content (Figs. 6, 7.2 and 8). Next to places that were rich in this substance, there were areas poor in it, and the polysaccharide grains were of various sizes.

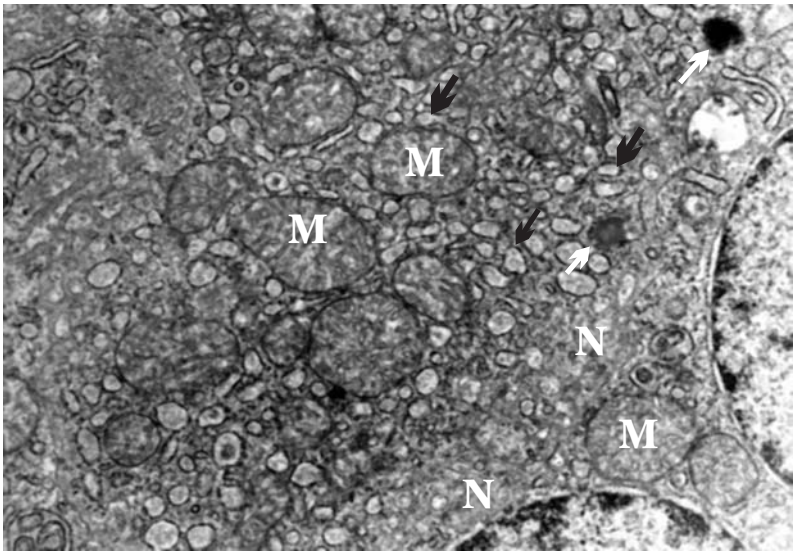

Fig. 5. Fragment of hepatocytes from carp from group B2 – vesicular transformation of RER (black arrows) with insignificant swelling of mitochondria (M) and foci of cytoplasm degradation (N) and lysosomes (white arrows). Magnification × 14600.

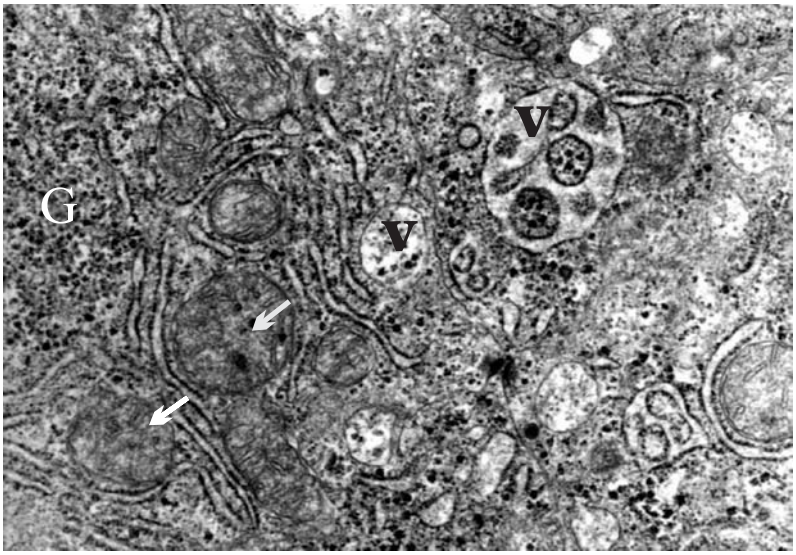

Fig. 6. Fragment of hepatocytes from carp from group B2 – autophage vacuoles (V), dense bodies in mitochondria (white arrows) and glycogen clusters (G). Magnification × 25000.

Herbicides, like other toxic substances, can influence the processes that occur at various biological levels ranging from cells to the tissues and organs, while their impact

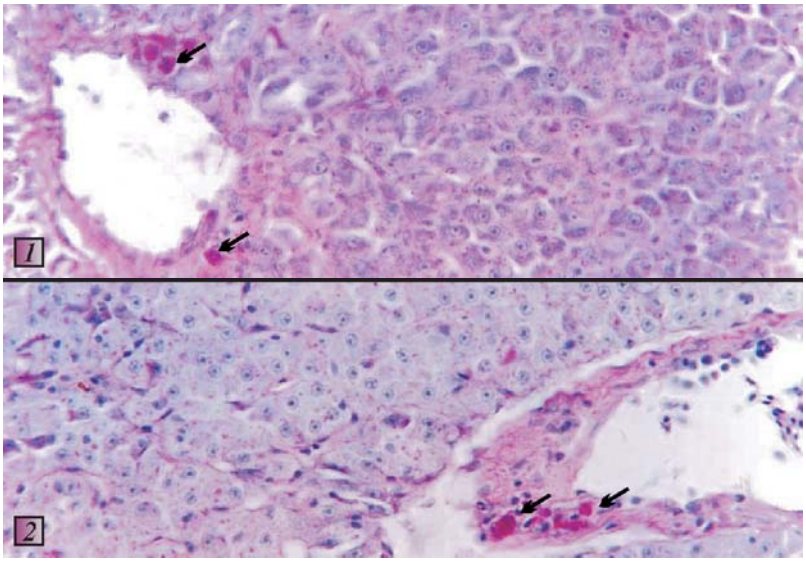

Fig. 7. Livers of carp from experiment A. 1. Liver of carp from group A1 – average content of polysaccharides with regular arrangement in hepatocytes and high content in blood vessel wall (arrows). 2. Liver of carp from group A2 – varied content of polysaccharides and high content in blood vessel wall (arrows), hyperaemia. Staining with the PAS method according to McManus (1948). Magnification × 500.

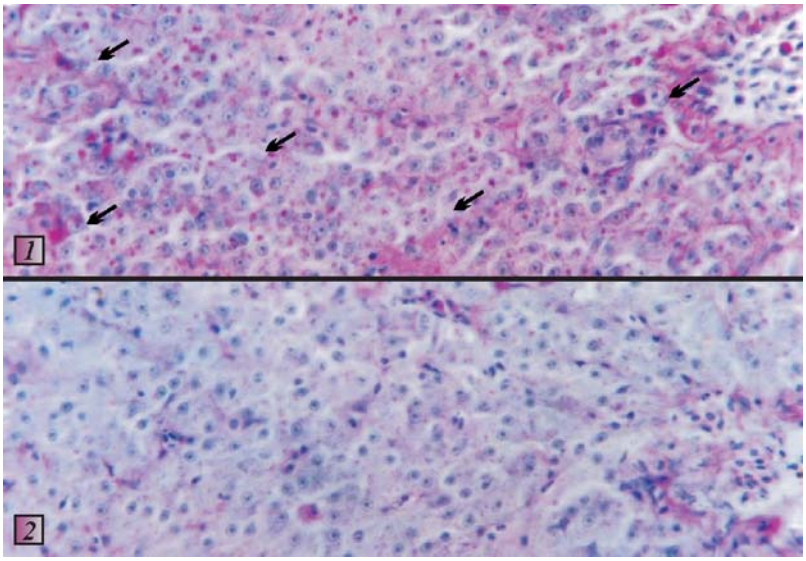

Fig. 8. Livers of carp from experiment B. 1. Liver of carp from group B1 – varied content of polysaccharides (arrows), hyperaemia. 2. Liver of carp from group B2 – low content of polysaccharides, hyperaemia. Staining with the PAS method according to McManus (1948). Magnification × 500.

level can range from affecting just individuals, whole populations, and even the environment (Gluth et al. 1985, Nešković et al. 1996, Piska and Waghray 1997). Therefore, there are several methods that can be used to assess the major effects of various (as regards their chemical composition) toxic compounds from the varied group of xenobiotics that are herbicides. That is why the criteria for selecting proper ecotoxicological biomarkers (*i.e.*, biological indicators) are so important (Adams et al. 1996, Triebskorn et al. 1997). The carp that were used in the authors' previous research constitute good material for studying the effects of glyphosate on liver pathomorphology.

Histological lesions are the final result of adverse biochemical and physiological changes in the bodies of AV-exposed fish. As such, they can provide insight into the mechanism of the toxic operations of these factors and indicate which organs are most susceptible to their harmful impact. Numerous studies on xenobiotic toxicity, both in lethal doses and at extremely low concentrations, conducted on various species of fish have demonstrated changes in the circulation in the liver (hyperaemia and extravasations) and hepatic damage that consists, not exclusively, of the vacuolization of hepatocytes, necrosis, and lesions in the endoplasmic reticulum (Nešković et al. 1996, Sopińska et al. 2000, Szarek et al. 1997, 2000a). The vacuolization of hepatocytes is a pathology that is noted quite commonly in breeding carp and is sometimes observed even in control groups and at low herbicide concentrations. These lesions were observed in the authors' previous research in both clinically healthy carp and in fish infected with ciliates.

The references available provide only the morphological pattern of the organs of clinically healthy fish that have been exposed to various herbicides. It must be emphasized that the authors' previous research was the first to demonstrate how an invasion of the protozoa results in increased susceptibility to glyphosate.

The results presented indicate that AV at a concentration of 2 mg of glyphosate l^{-1} water caused regressive lesions in the livers of carp fingerlings, occasional circulation disorders, and sporadic progressive lesions. Under an electron microscope, the deviations were mostly noted in the mitochondria and rough endoplasmic reticulum. Moreover, ultrastructural analysis has proved that Avans 330 SL produced microfoci of necrosis in the liver and small droplets of lipids emerged only in carp infected with *I. multifiliis*. The lesions were accompanied by the phenomenon of necrotic structure

removal. Both these lesions and clinical observations revealed that natural infection with ciliates permitted easier and increased herbicide access to the fish through injured skin and gill epithelium.

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STRESZCZENIE

WPŁYW HERBICYDU AVANS 330 SL NA PATOMORFOLOGIĘ WĄTROBY KARPI (*CYPRINUS CARPIO* L.), KLINICZNIE ZDROWYCH I KARPI ZARAŻONYCH *ICHTHYOPHTHIRIUS MULTIFILIIS*

Badania przeprowadzono na narybku karpia, *Cyprinus carpio* L., o masie ciała 81-98 g w dwóch oddzielnych doświadczeniach: A – karpie klinicznie zdrowe, wolne od orzęska, *Ichthyophthirius multifiliis* i B – zarażone tym pierwotniakiem (naturalna inwazja). W każdym doświadczeniu ryby podzielono na dwie grupy (n = 10): A1 i B1 – kontrolne, A2 i B2 – poddane działaniu preparatu Avans 330 SL (AV) o stężeniu 2 mg trimethylosulfonium glyphosate na 1 l wody. AV, w stężeniu przeliczonym na substancję czynną, po dokładnym wymieszaniu z małą ilością wody pitnej, wprowadzono do 200 l basenów, gdzie umieszczono ryby na 96 h.

Stwierdzono, że karpie zakażone orzęskiem i poddane działaniu AV (grupa B2) zachowywały się prawidłowo tylko w pierwszym dniu eksperymentu. W drugim dniu ruchliwość i pobudliwość karpi z tej grupy nieco wzrosła, po czym w kolejnych dniach (3 i 4) obserwowano ociężałość w ich ruchach. Pozostałe ryby podczas doświadczeń zachowywały się normalnie.

Mikroskopowo wykazano, że do najczęściej występujących morfologicznych odstępstw od normy, zwłaszcza u ryb zarażonych orzęskiem i kąpanych w wodzie z dodatkiem AV, należały zmiany wsteczne, jak zwyrodnienie miąższowe (rys. 1.2), wodniczkowe (rys. 3) oraz martwica ogniskowa (rys. 3.2). Rzadziej w tych przypadkach, a szczególnie rzadko u karpi chorych na Ichthyophthiriasis (ICH) lub tylko poddanych działaniu AV, obserwowano zaburzenia w krążeniu (przekrwienie i drobne wynaczynienia) – rys. 1.2, 7.2, 8. Ultrastrukturalnie znajdowano zmiany w mitochondriach (obrzmienie – rys. 2.1, 5, zatarcie struktury grzebieni – rys. 2.1, a niekiedy, głównie w grupie B2, obecność ciałek gęstych – rys. 6) oraz w siateczce sródplazmatycznej (rozrost i poszerzenie kanałów szorstkiej siateczki sródplazmatycznej z jej niewielką transformacją pęcherzykową – rys. 2, 4, 5, a czasem defragmentacją – rys. 4). Stwierdzono też mikroogniska martwicze w cytoplazmie hepatocytów karpi z grupy B2 (rys. 5). Ponadto, uzyskane wyniki, a zwłaszcza rodzaj obserwowanych odstępstw od normy i ich zasięg, wskazują, że ICH nasilała występowanie zmian morfologicznych w wątrobie karpi znajdujących się pod wpływem AV w stosunku do ryb wolnych od wymienionej choroby.