THE PROTECTIVE ROLE OF MALVIDIN ON THE HEMATOLOGICAL, BIOCHEMICAL AND HISTOPATHOLOGICAL PARAMETERS OF MARSH FROG (*Pelophylax ridibundus*) EXPOSED TO SUBLETHAL DOSES OF ROUNDUP®

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Abstract

In the present paper it was investigated the protective effect of malvidin on the hematological, biochemical and histopathological parameters of marsh frog (*Pelophylax ridibundus*) exposed to sublethal concentration of Roundup® herbicide. The animals were divided in 3 experimental groups: lot of control individuals, second lot containing animals which were subjected to Roundup® in a dose of 0.138mL/L administered in water for 3 weeks and third lot containing animals which were subjected to malvidin in a dose of 1mg/kg body weight administered by intraperitoneal injections and kept in water with the same concentration of Roundup®. Exposure of *Pelophylax ridibundus* adults to Roundup® herbicide action has the effect of installation of pathological changes in the liver tissues characterized by vacuolation of hepatocytes and nuclear pycnosis, perisinusoidal fibrosis, dilatation of Disse space and sinusoid capillaries, presence of cellular infiltrates. We also observed increasing the hepatosomatic index, the number of white blood cells (WBC), serum cholesterol and triglycerides values, and decreasing the number of red blood cells (RBC) and glycaemia values. Malvidin administration as an adjunct, counteracts the toxic action, describing itself as normal liver parenchyma in animals intoxicated with Roundup® herbicide, without lesion, and a return to normal values of the studied markers.

Rezumat

Acest studiu investighează efectul protector al malvidinei asupra unor markeri hematologici, biochimici şi histopatologici la broasca-de-lac (*Pelophylax ridibundus*) expusă unor concentraţii subletale de erbicid Roundup®. Animalele utilizate au fost împărţite în 3 loturi: un lot martor, un lot de animale tratate cu erbicidul Roundup® în concentraţie de 0,138mL/L administrat în apă timp de 3 săptămâni şi un lot tratat cu aceeaşi concentraţie de toxic la care s-a administrat malvidină în doză de 1mg/kg greutate corporală

prin injecţii intraperitoneale. Expunerea adulţilor de *Pelophylax ridibundus* la concentraţii subletale de erbicid Roundup® este urmată de instalarea unor modificări patologice hepatice caracterizate prin vacuolizarea hepatocitelor, fibroză perisinusoidală, dilatarea spaţiilor Disse şi a capilarelor sinusoide, prezenţa infiltratelor leucocitare. De asemenea, a fost observată creşterea valorii indicelui hepatosomatic, a numărului de leucocite, a colesterolului şi trigliceridelor precum şi o scădere a numărului de eritrocite şi a glicemiei. Administrarea malvidinei ca adjuvant contracarează acţiunea toxicului, descriindu-se prezenţa unui parenchim hepatic normal la animalele intoxicate cu erbicidul Roundup®, fără leziuni precum şi o revenire a valorilor indicilor studiaţi spre valorile normale.

Keywords: malvidin, Roundup®, liver, red blood cells, white blood cells, cholesterol, triglycerides, glycaemia

Introduction

Pesticides are applied throughout the world often with unintended consequences on ecological communities and in some region pesticides are associated with declining amphibians. One of the widely used agrochemical products is Roundup® a non-selective postemergent herbicide with systemic action. It contains glyphosate 360 g/L and controls perfectly all species of annual and perennial weeds. The commercial product contains, in addition to glyphosate, a cationic surfactant called polyoxyethylene (POEA) which gives the product toxicological properties different from those of glyphosate.

On the other hand, recent studies indicated that some vegetables extract can reduce the production of reactive oxygen species (ROS) and increase the resistance of plasma lipoprotein to oxidation that may contribute in preventing disease [15].

Anthocyanins are flavonoids widely distributed in the human diet through fruit, vegetable, and grain products and are of considerable nutritional interest as it has been suggested that they may play an important role in the promotion of health and disease prevention [11]. Interest in anthocyanins and their biological effects has increased significantly during the past decade and numerous studies have shown that they display a wide range of biological activities [12,17] including antioxidant [4], anti-inflammatory [13,22], antimicrobial [17], anti-carcinogenic activities [8,9] and neuroprotective effects [5,10,21].

The aim of this study was to demonstrate the possible protective role of malvidin (an anthocyanin) obtained from the epicarp of red grape berries (*Vitis vinifera*) on hematological, biochemical and histopathological parameters in marsh frog (*Pelophylax ridibundus*) exposed to sublethal dose of Roundup® erbicide.

Materials and Methods

Animals

The study was performed with the approval of the local Committee of Bioethics according to the Romanian law 205/2004 art.7, 18, 22 and regulation number 143/400/2002 for care and use of animals for research purposes. A total number of ten healthy adult frogs (male and female) were used in the study. The animals were captured in spring (April-May) from the surrounding areas of the city Piteşti (South Romania) and were kept in laboratory conditions in aquaterrarios filled with tap water at a constant temperature (22±2°C), humidity (55%) and 12 h light-dark conditions for five days to test their health and accommodate them for the experiment. The water was changed daily to avoid the accumulation of toxic substances and the animals were fed *ad libitum*.

Preparation of vegetable extract

The vegetable extract was obtained from the peels (epicarp) of red grape berries (*Vitis vinifera*). For the separation and purification of anthocyanins have been applied several methods based on solid-liquid, liquid-liquid extraction, liquid chromatography with reversed phase (with C18 stationary phase, liquid chromatography with stationary phase in ion exchanges. The separation quality was characterized by HPLC chromatography and UV-VIS spectrophotometry, spectra being compared with those of standards.

Experimental design

After acclimatization for one week in the laboratory, the frogs were separated in three lots. Each lot comprised 6 animals: (1) lot of control individuals, containing animals which were administered saline solution 6.5‰ by intraperitoneal shots (one shot at two days in a scheme of 3 weeks); (2) a second lot containing animals which were administered saline solution 6.5‰ by intraperitoneal shots (one shot at two days in a scheme of 3 weeks) and kept in water with Roundup® in a dose of 0.138mL/L administered in water for 3 weeks; (3) a third lot containing animals which were subjected to malvidin in a dose of 0.1mg/g body weight administered by intraperitoneal shots (one shot at two days in a scheme of 3 weeks) and kept in water with Roundup® in a dose of 0.138mL/L.

The administered dosage of toxic and extract was not lethal as none of the subjects died through the experiment.

Tissue preparation

After 3 weeks of treatment, the frogs were killed by decapitation after chloroform anesthesia and fragments from the liver were quickly removed. Tissues samples were fixed in 8% neutral formalin for poikilothermes for 24h and were processed using a graded ethanol series and embedded in paraffin. Paraffin sections were cut in 5μm-thick slices using a rotary microtome and stained with: hematoxylin (H) as a general screening method, Sirius red [7] for collagen stain (fibrosis) and Perl's method for ferrous iron. The sections were viewed and photographed using an Olympus microscope with an attached camera.

Blood specimens were withdrawn from the frogs by cardiac puncture after chloroform anesthesia. The values of operational factors were determined by using standard automated method: number of erythrocytes and leukocytes was microscopically determined with a Thoma cells numbering chamber [16]; the glycaemia, cholesterol and triglycerides level has been determinate using an Accutrend GCT.

Statistical Analysis

Values are given as arithmetic means ± standard error of the mean (S.E.M.). The data was statistically analyzed by using multiple comparison tests (LSD - SPSS/PC program version 10.0 for Windows).

Results and Discussion

Control liver tissues

No histopathological changes were observed in the liver of the control frogs. The structural details of the liver of control *Pelophylax ridibundus* are shown in Figure 1a.

Treated liver tissues

Hepatotoxic effects of Roundup® herbicide are manifested by congestion of intrahepatic blood vessels, including dilated sinusoid capillaries blocked by the presence of hyaline erythrocytes with inflammatory elements (Figures 1b, d). Blocking of sinusoids causes the blood flow from the artery and hepatic vein to centrilobular vein to be low, therefore they expand to ensure proper blood flow in the centrilobular vein [14]. Also in response to the toxic action of glyphosate, sinusoidal

capillaries have an incipient perisinusoidal fibrosis (Figures 1c, d) along with the presence of leukocyte infiltration (Figures 1d).

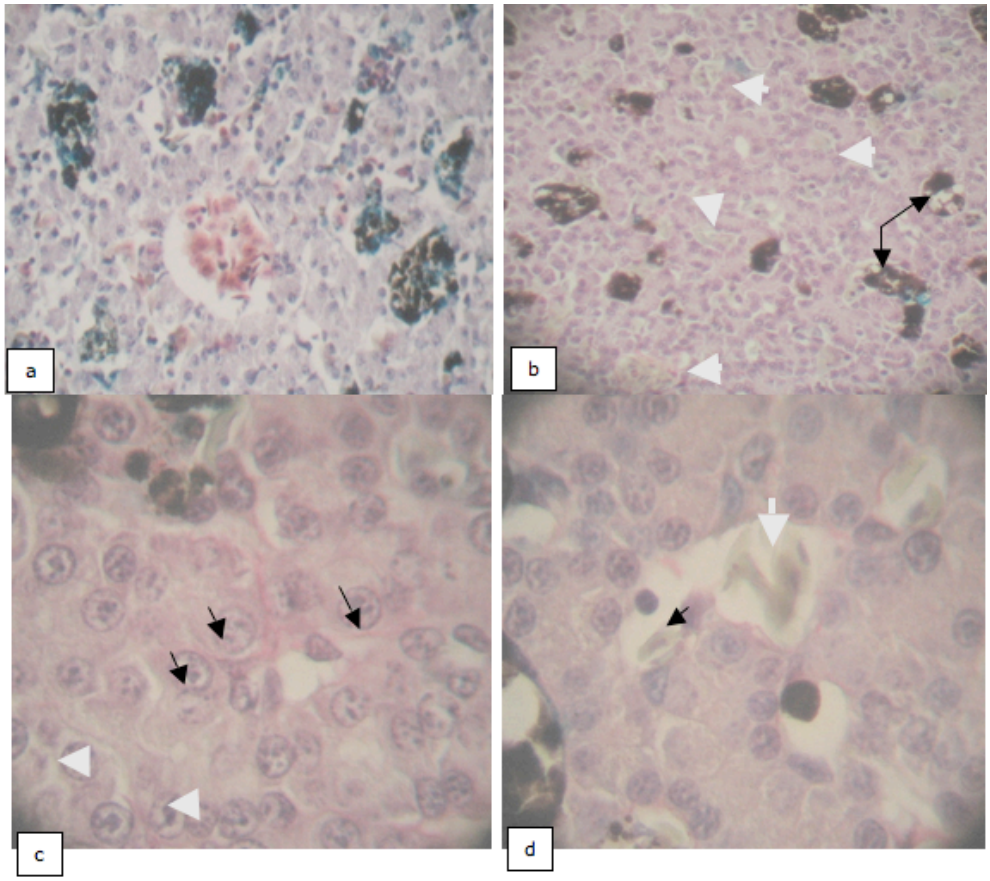

Figure 1

a- The liver tissue of control showing typical parenchymatous appearance: centrolobular vein, lipofuscin deposits. 100×. b,c, d - Liver of frogs treated with Roundup® herbicide: b- deposits of disassimilation pigment (melanin, ceroid and very little positive Perls material) – black arrow; dilated sinusoid capillaries (white arrows). 100×. c- hepatocytes with hypertrophied polyploid nuclei (black arrow); hepatocytes with small pyknotic nuclei (white arrow). d- leukocyte infiltration (black arrow); incipient perisinusoidal fibrosis (white arrows). 400×. Perl's staining, H-Sirius red.

The liver parenchyma has few deposits of disassimilation pigment represented mainly by melanin, ceroid and very little positive Perls material (Figure 1b). Hepatocytes have hypertrophied polyploid nuclei (Figure 1c) in addition to hepatocytes with small pyknotic nuclei in the process of

degeneration. The increase in hepatocyte volume due to hypertrophied nuclei was recorded in species *Clarias gariepinus* [14] based on the treatment with glyphosate herbicide.

The toxic also initiates the vacuolation of hepatocytes (Figure 1c), which may indicate an imbalance between the rate of synthesis processes of parenchyma cells and the rate of elimination process in their circulatory system [6]. The presence of these hyaline droplets in the cytoplasm of hepatocytes was identified in the liver of *Piaractus brachypomus* species with multifocal areas of necrosis [20]. Appearance of fat deposits in hepatocytes is a defense mechanism against xenobiotic lipophilic substances which are retained at this level and thus reduce their bioavailability [3,19].

Liver histopathological effects of glyphosate administered to juvenile African cats (*Clarias gariepinus*) consist of vacuolation of hepatocytes, intrahepatic blood vessel congestion, infiltration of lymphocytes, hepatocytes with pyknotic nuclei and even their necrosis, hemorrhage [1].

Malvidin administration as an adjunct, counteracts the toxic action, describing itself as normal liver parenchyma in animals intoxicated with Roundup® herbicide, without lesion changes (Figure 2).

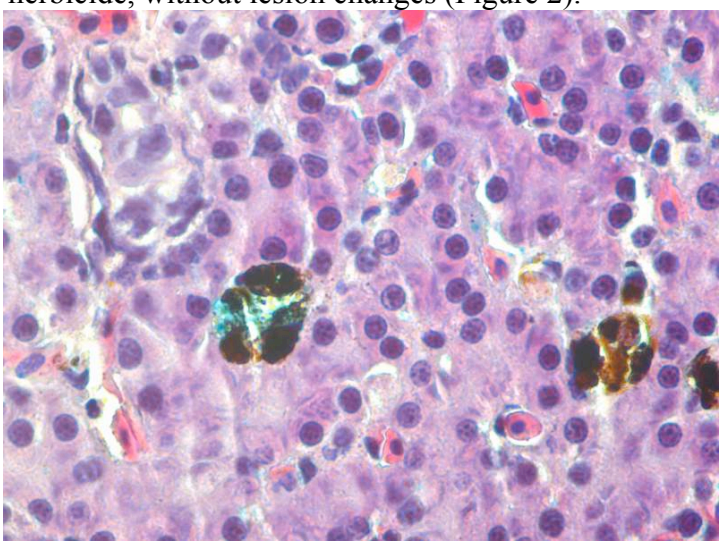

Figure 2
Normal liver parenchyma after maldivin administration (as an adjunct). 100×. Perl's staining, H-Sirius red.

It was suggested that a dietary intake of anthocyanin-rich foods in rats may contribute to overall antioxidant status, particularly in individuals habitually consuming a diet deficient in vitamin E [18].

Hematological and biochemical profile

Hematological and biochemical parameters can be used as standard laboratory tests to determine histopathological effects and metabolic disorders caused by the action of xenobiotics [2]. Roundup® herbicide administration is followed by changing these parameters, exemplified by increasing the hepatosomatic index, the number of leukocytes, serum cholesterol and triglycerides, and decreasing the number of erythrocyte and glucose (Table I). For animals intoxicated with the same concentration of toxic but which received malvidin through intraperitoneal injections, there is a return to normal values of the indices studied.

Table I

Mean ± S.E.M values of RBC, WBC, glycaemia, cholesterol, triglycerides and IHS

Parameters		
RBC (number of erytrocytes/dL blood)	Lot I	481111.1±25017.76[a]
	Lot II	355922.2±1446.9611[b]
	Lot III	456166.7±2039.2393[a]
	Significance	***
WBC (number of leukocytes/dL blood)	Lot I	5163.33±200.9439[b]
	Lot II	6502.22±26.0411[a]
	Lot III	5258.33±16.1741[b]
	Significance	***
Glycaemia (mg/dL blood)	Lot I	22.72±0.6558[a]
	Lot II	15.33±0.3234[c]
	Lot III	20.2778±0.2658[b]
	Significance	***
Cholesterol (mg/dL blood)	Lot I	140.22±1.3249[c]
	Lot II	165.38±0.3040[a]
	Lot III	147.11±0.8889[b]
	Significance	***
Triglycerides (mg/dL blood)	Lot I	59.94±1.6617[c]
	Lot II	75.22±0.2977[a]
	Lot III	64.50±0.7287[b]
	Significance	***
IHS (hepatosomatic index)	Lot I	3.04±4.535[c]
	Lot II	3.78±2.122[a]
	Lot III	3.33±3.61[b]
	Significance	***

$p<0.05$*, $p<0.01$**, $p<0.001$***, a, b, c: Different letters within same column showed significant differences.

Conclusion

Exposure of *Pelophylax ridibundus* adults to Roundup® herbicide action has the effect of installation of pathological and functional changes in the liver tissues, suggesting that they may be target organ of the herbicide toxic effect. Also we observed an increase of the hepatosomatic index, the number of WBC, serum cholesterol and triglycerides values, and decrease in the number of RBC and glycaemia values. Malvidin administration, as protective substance, counteracts the toxic action in poisoned animals and demonstreated remarkable antioxidant properties.

Acknowledgements

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