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FLOW CYTOMETRIC ASSESSMENT OF EX VIVO *CARASSIUS AURATUS* RED BLOOD CELLS IN SUBLETHAL EXPOSURE TO HEAVY METALS

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ABSTRACT. Among major problems that directly concern the quality of life and health of people, two of them are in a direct dependence, namely environmental pollution and food quality. Setting maximum levels allowed toxic substances in surface waters, knowledge of action harmful to aquatic life and assessing the degree of risk that they have on the future quality fish food home have become more than a necessity. Internationally, increased food security and food safety is precisely the current concerns that expect quick results. In this general context, we aimed to test whether in vivo apoptosis of nucleated fish red blood cells can be simultaneously a sanogenesis test, assessing the conditions under which a fish product was obtained, respectively if the aquatic environment from which the fish product was contaminated with pollutants or not and how much the degree of pollution has an impact on product quality as a fish foodstuff. To verify our hypothesis we measured by flow cytometry and microscopy ex vivo nucleated red blood cells from the fish undergoing the sublethal heavy metal poisoning in laboratory and we tested fish from two areas with different degrees of pollution. The results we obtained show that in vivo, in the case of sub-lethal poisoning with heavy metals is observed an apoptotic phenomenon of erythrocytes. This apoptosis of fish red blood cells under the action of heavy metals as pollutants suggests that this phenomenon may be the basis of a simple test for assessing the conditions under which a product comes from a polluted fishery or unpolluted aquatic environment (test of sanogenesis).

Keywords: fish nucleated erythrocytes, apoptosis, flow cytometry, optic macroscopy, ESEM, ecotoxicology

INTRODUCTION

Biosphere pollution by technological civilization has not only consequences for the sustainability of plant and animal species, it compromises the very future of humanity by acting on natural resources, in particular on compliance of agricultural productivity in various ecosystems. Dispersion of toxic substances in the natural environment leads to more dangerous contamination of human food chains in which we are integrating in the top of the ecological pyramid. (Lagadic, 1997). Discharge of all waste resulting from human activities in the environment exposes us to a boomerang effect through food chains. Pollution of food or more specifically, human contamination of consumption is one of the most worrying environmental problems. Pesticides (Muirhead-Thomson, 1971, Fournier, 1974), heavy metals (Holden, 1973) use of antibiotics and even hormones contribute substantially to the contamination of food chains. Food additives, colours, stabilizers, emulsifiers not justify its presence and should be banned. In addition, aquatic food products are also becoming contaminated more. World Ocean is at present almost its entire polluted area (Peres, 1976) with various hydrocarbons contained in crude or degradation products resulting after action of biogeochemical factors. Only oil contamination, for example, is extremely high, about 5 million tons of crude oil contaminating ocean waters. This has directly consequences on phytoplankton, algae and the zooplankton. On the marine animals, contamination of fish and other marine animals has direct consequences for marine or oceanic origin foods. Scallops or fish collected from the neighbouring coast industrialized regions contain 5 to 10 times more oil than those from unpolluted areas. "Taste of oil", even if not a direct toxic fish, leads to economic loss and prevent them from commercialization (Ramade, 2000). But the biggest problem is related to determining the maximum tolerated dose for each pollutant and especially the combination of several micropollutants, the multiplicity of pollutants or their synergism, Where lapses in current knowledge are numerous and important. The contamination of the aquatic environment is traditionally presented in terms of concentrations of chemical contaminants in the environment. However, these concentrations do not give an estimate of the adverse effects on organisms, which involves almost mandatory today, the use of biological criteria obtained by bioassay. (Chapman & Long, 1983). Bioanalysis results of these bioassays or toxicity thresholds for determining potential candidate

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for being in the aquatic environment from accidental or chronic manner, as "biological quality" of a medium and its potential toxicity (Quiniou et al., 2005). The bioassays probably respond the best to the current need to determine sanogenesis origin products used as food fish.

In this general context, we aimed to test whether in vivo apoptosis of nucleated fish red blood cells can be simultaneously a sanogenesis test, assessing the conditions under which a fish product was obtained. To verify our hypothesis we measured by flow cytometry and microscopy ex vivo nucleated red blood cells from the fish undergoing the sublethal heavy metal poisoning in laboratory and we tested fish from two areas with different degrees of pollution.

MATERIALS AND METHODS

Chemicals

Fluorescein-conjugated, Annexin-V and Propidium Iodide were from PharMingen (San Diego, CA, USA).

Acute poisoning of fish with heavy metals and erythrocytes collection

For acute intoxication of fish were used two tanks with a capacity of 20 liters, using tap water with a pH 7.8, 70-80% oxygenated, water temperature 19-200C under laboratory conditions with natural light.. During intoxication the fish were not fed. A group of 5 juvenile fish of the species *Carassius auratus* were placed in an aquarium for intoxication and the control groups constituted from 5 fish were placed in the 2nd tank. Fish were purchased from authorized fisheries and before starting the experiment, there were weighed and measured. Erythrocytes from blood of fish species *Carassius auratus* were collected under ether anaesthesia on heparin, sedimented by centrifugation (1000 g, 4°C, 5 min) and resuspended in Dulbecco's phosphate buffered saline solution pH 7.4 (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4 and 1.5 mM KH2PO4.

Flow cytometric analysis

Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), using the CellQuest Pro software for acquisition and analysis. The light-scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale, a minimum of 10 000 cells being analysed in each condition. Erythrocyte size and density were assessed using forward and side-angle scatters (FSC versus SSC).

Cell death assays

Cell death was determined using an annexin-V-FITC/propidium iodide apoptosis kit. Annexin-V is a Ca2+- dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS) and is useful for identifying apoptotic cells with exposed PS. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells, since viable cells with intact membranes exclude PI whereas membranes of dead and damaged cells are permeable to PI. Cells that stain positive for annexin-V-FITC and negative for propidium iodide are undergoing apoptosis. Cells that stain positive for both annexin-V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both annexin-V-FITC and PI are alive and not undergoing measurable apoptosis.

The nucleated red blood cells (2x105), resuspended in 100 µl of 1x binding HEPES buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) were incubated with 10 µl annexin-V-FITC and 10 µl propidium iodide for 30 min. at room temperature in the dark. After adding 400 µl of 1X binding buffer, the suspension was analysed in the flow cytometer and gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (PI fluorescence). The light scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale. All studies were performed at least three times with three replicates each time.

Optical and scanning electron microscopic analysis.

Direct light microscopic analysis was performed using an inverted microscope equipped with the Olympus BX 43 Olympus VC-30 and the room visualization software CellSens Dimension. ESEM analysis was performed using scanning electron microscope Fei Quanta 250. Fish erythrocytes were fixed for 1 hour with a working solution of 2.7% glutaraldehyde in 0.1M phosphate buffer pH 7.4 and stored at 40C until examination. Erythrocyte pellet was mounted on a Millipore filter nylon 0,45 mm, then the examination room is closed and the examination was performed at a temperature of -3°C, relative humidity of 100% and a pressure of 910 Pa. Working with Spot 4:01 accelerating voltage of 15KV, using GSED detector (gaseous secondary electron detector). Examination time was between 10 to 30 minutes, order the examination in 1500 mag, 3000 mag, 6000 mag.

RESULTS AND DICUSSIONS

To understand the mechanisms underlying the process of cell death by heavy metals action, we applied simultaneous staining of erythrocytes with Annexin-V and propidium iodide for flow cytometric analyses and an optical and scanning electron microscopy were accompanied the research.

Detection of erythrocytes death by Annexin V-FITC and propidium iodide double-labelling

Our research attempted to verify the hypothesis that in vivo environmental pollutants which come in contact to the level of fish gills with nucleated erythrocytes will induce an apoptosis phenomenon of erythrocytes that is quantifiable with Anexin-V-FIC/propidium iodide. Multiparametric flow cytometric analysis

discriminates and quantifies viable, apoptotic and necrotic cells.

In Figure 1, (acute Mercury poisoning), Figure 2 (acute Copper poisoning), Figure 3 (acute Cadmium poisoning), Figure 4 (acute Lead poisoning) are presented assessments of the discriminatory apoptosis /necrosis phenomenon for all individuals of *Carassius auratus* after different time of intoxication and at various concentrations.

Analyzing the percentages of viable cells (lower left quadrant), cells in apoptosis (lower right quadrant) and late apoptosis or necrosis of the post-apoptotic (upper right quadrant), it appears that the polluting effect of apoptosis induction is extremely powerful in the case of 1 mM concentration. Individu no.1 and 2 show all red blood cells in apoptosis, for no.4 and 5 for the phenomenon is more advanced and the erythrocytes can be found in late stage apoptosis, when there already permeabilization of the cell membrane, which enables propidium iodide to bind to the core. Noteworthy is great resistance of the individual no. 3 to which a significant percentage of red blood cells are still viable (approximately 75%).At a lower poisoning by 0.01mm Hg, the phenomenon is slower but is present in all the cases with the exception of individual no. 4, which indicate the presence of viable red blood cells at 6H, while after 24h the red cells of all individuals poisoned are in the late stage of apoptosis.

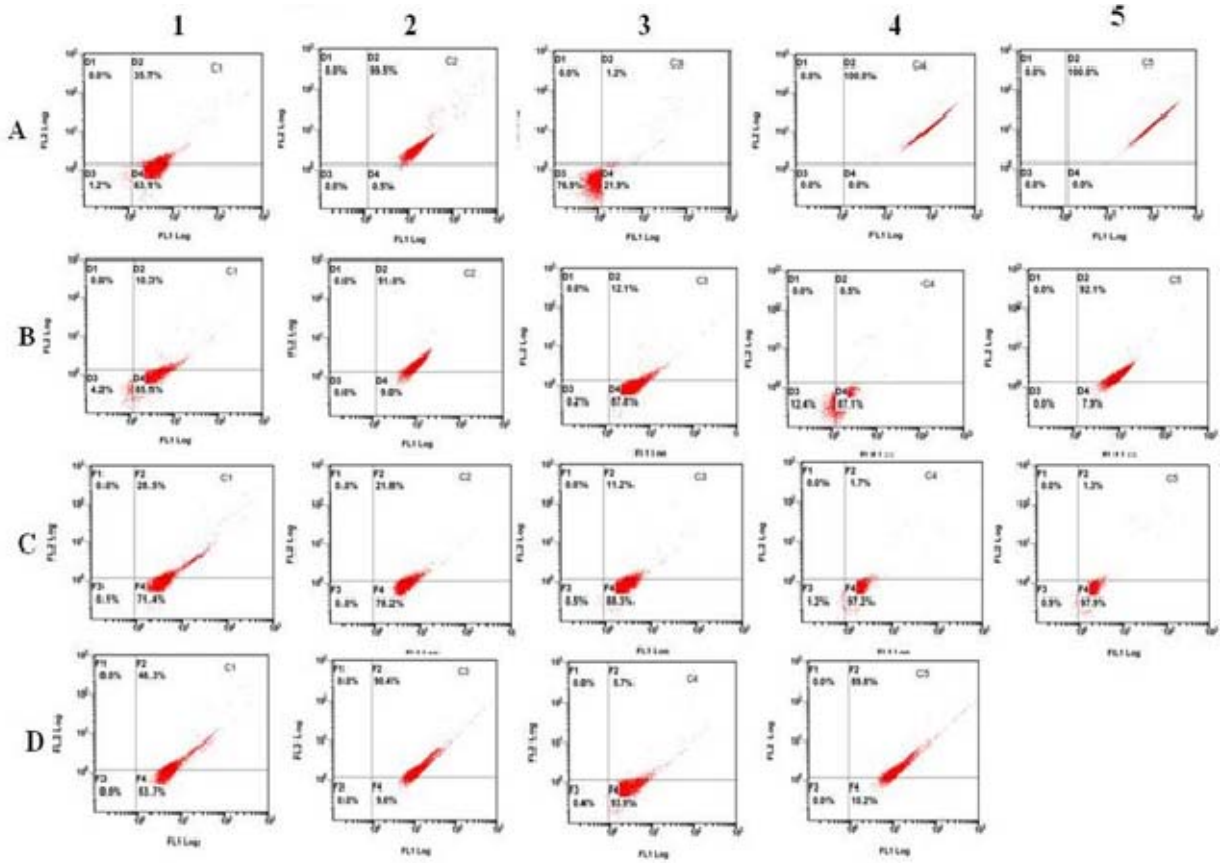

Fig. 1. Comparative flow cytometric analysis by technique quadrant of viable, apoptotic and necrotic cells by double staining with Annexin-V-FITC (FL1) and propidium iodide (FL2) for nucleated red blood cells collected from acute poisoning fish with Hg at various concentrations. Poisoning: **A**: 1 mM Hg after 1 h, **B**: 0.01mM Hg after 6H, C: 0.01 mM Hg after 24h, D: 0.01mM Hg after 48h of intoxication. The number 1 to 5 represents the 5 individuals *Carassius auratus* evaluated.

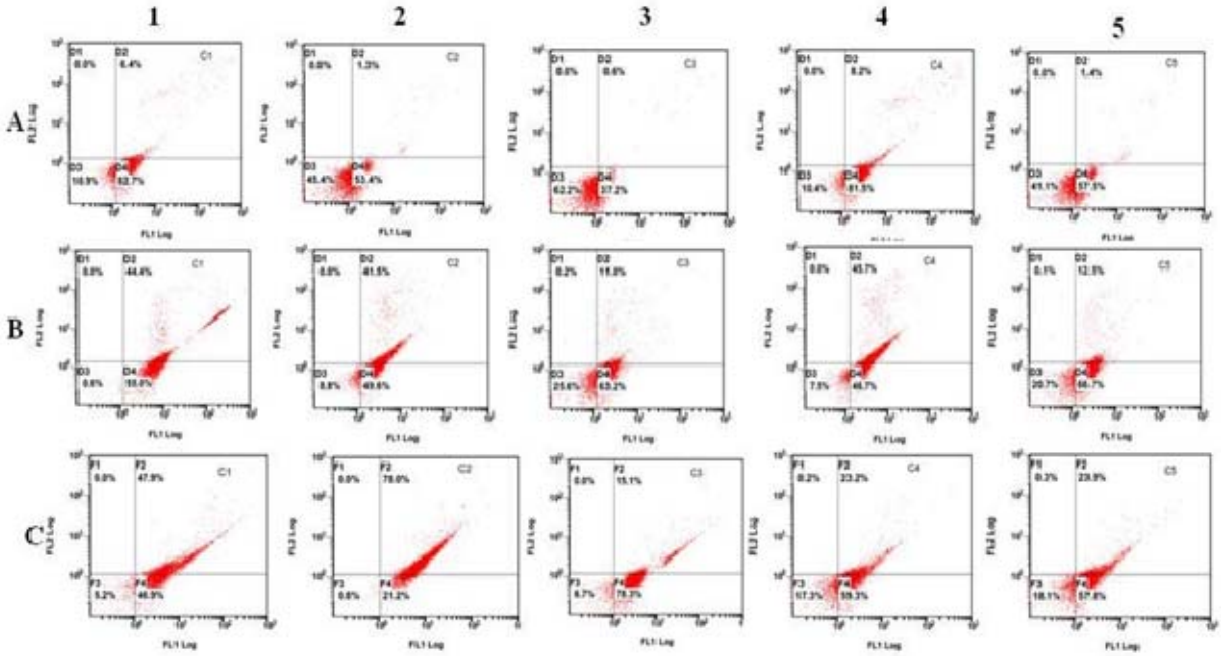

Fig. 2. Comparative flow cytometric analysis by technique quadrant of viable, apoptotic and necrotic cells by double staining with Annexin-V-FITC (FL1) and propidium iodide (FL2) for nucleated red blood cells collected from acute poisoning fish with Cu at various concentrations. Poisoning: **A**: 1 mM Cu after 6 h, **B**: 0.01mM Cu after 6H, C:0.01mM Cu after 24h of intoxication. The number 1 to 5 represents the 5 individuals *Carassius auratus* evaluated.

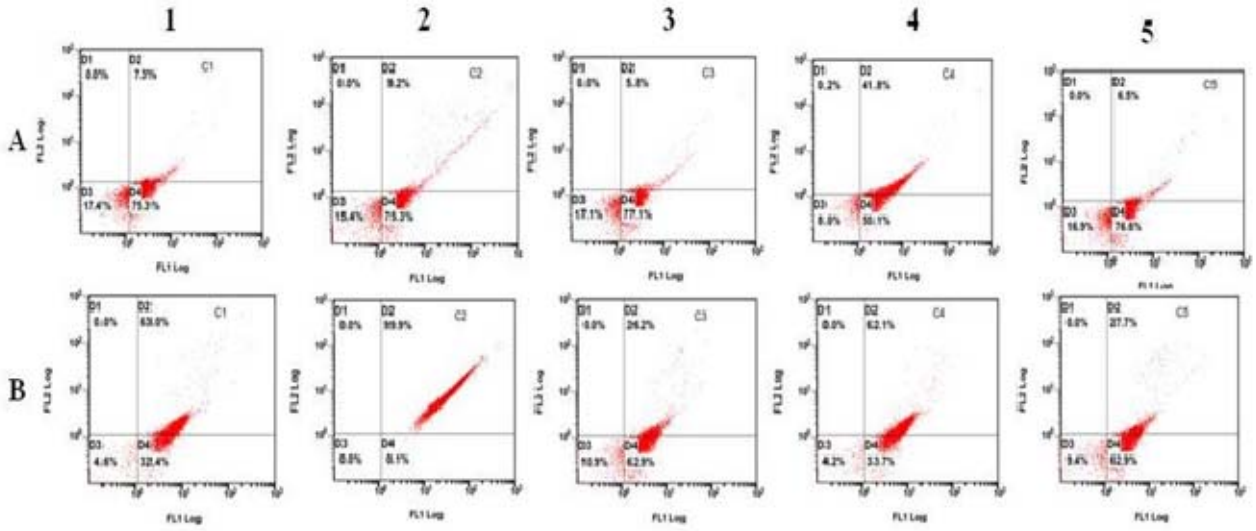

Fig. 3. Comparative flow cytometric analysis by technique quadrant of viable, apoptotic and necrotic cells by double staining with Annexin-V-FITC (FL1) and propidium iodide (FL2) for nucleated red blood cells collected from acute poisoning fish with Cd at various concentrations. Poisoning: **A**: 1mM Cd after 6 h, **B**: 1mM Cd after 6 h of intoxication. The number 1 to 5 represents the 5 individuals *Carassius auratus* evaluated.

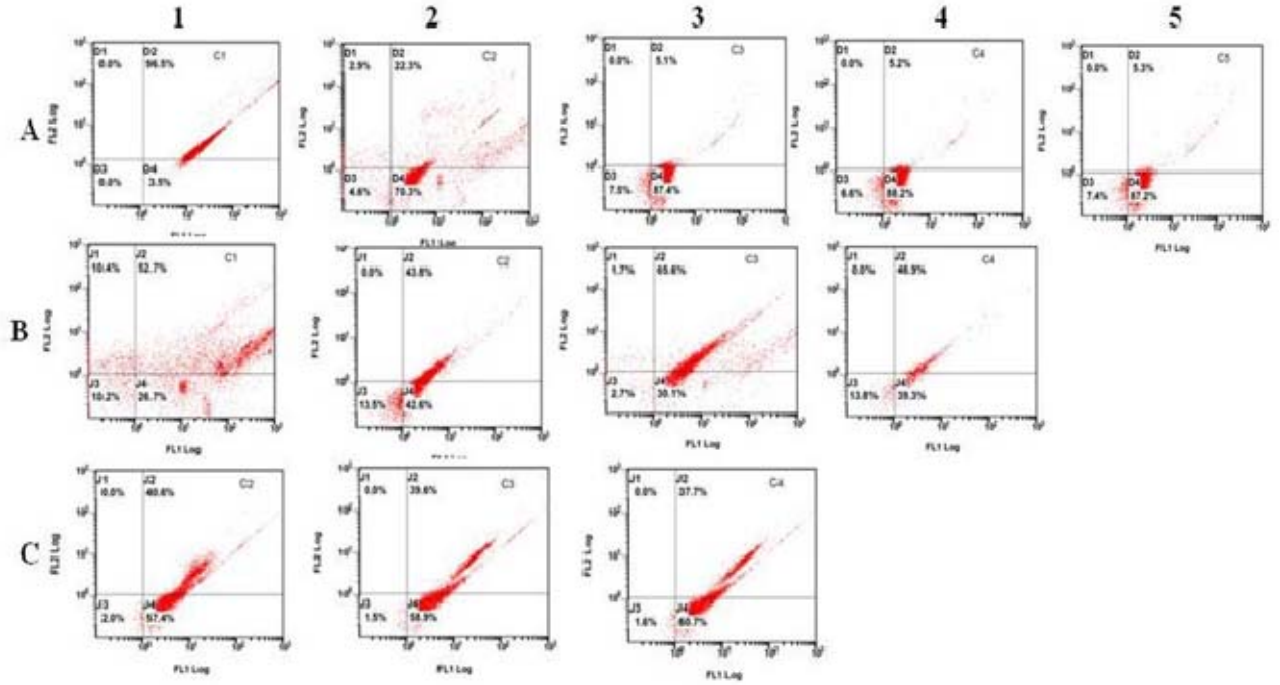

Fig.4. Comparative flow cytometric analysis by technique quadrant of viable, apoptotic and necrotic cells by double staining with Annexin-V-FITC (FL1) and propidium iodide (FL2) for nucleated red blood cells collected from acute poisoning fish with Pb at various concentrations. Poisoning: **A**: 1mM Pb after 6h, **B**: 1mM Pb after 24h, C: 1mM Pb after 48h of intoxication. The number 1 to 5 represents the 5 individuals *Carassius auratus* evaluated.

Massive acute intoxication with Copper 1mM still indicates to 6h the presence of viable erythrocytes which signifies that Copper compared with Hg a much lower toxic potential.

After 6 h of intoxication with 0.01 mM Copper is observed the persistence of viable cells in the individuals no. 3 and 5 while to 24 hours after intoxication the phenomenon of apoptosis is present in all the cases.

After 6h of 1mM cadmium intoxication, induced apoptosis is a moderate manner but at 24h the phenomenon of apoptosis is total. The intoxication with 1mM Pb show at 6h and 24h, in the both cases, a phenomenon of pointing that which indicates a high toxicity of Hg.

Optical and scanning electron microscopic analysis of nucleated red blood cells collected from acute poisoning fish with heavy metals.

As a complementary step for correlating data, erythrocyte samples were analyzed by optical and scanning microscopy. The representative images are shown in the following figures, Fig 5, Fig. 6, Fig. 7 and Fig.8. In all cases of acute intoxication found same morphological changes occurring to direct action of pollutants on erythrocytes in vitro. In particular is observed the appearance of cell nuclei excluded, appearance of crown shape in almost all cases and even hexagonal shape.

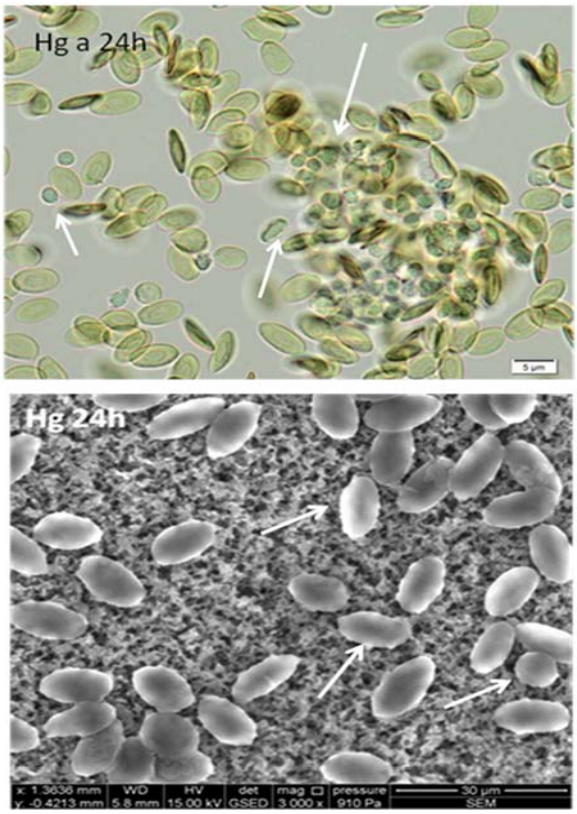

Fig. 5. Optical and ESEM microscopic analysis of *Carassius auratus* nucleated red blood cells after 24 h intoxication with Hg 0.01 mM for evidence of changes induced morphology.

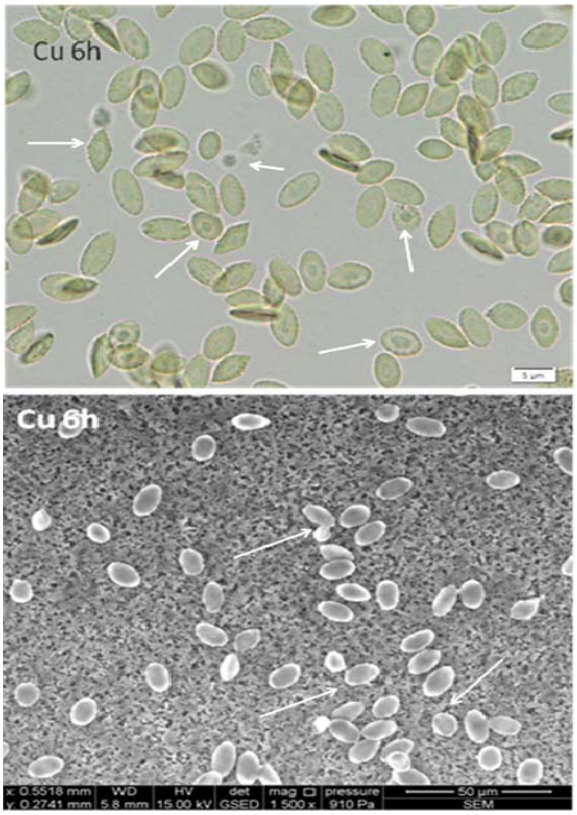

Fig. 6. Optical and ESEM microscopic analysis of *Carassius auratus* nucleated red blood cells after 6 h intoxication with Cu 1 mM for evidence of changes induced morphology.

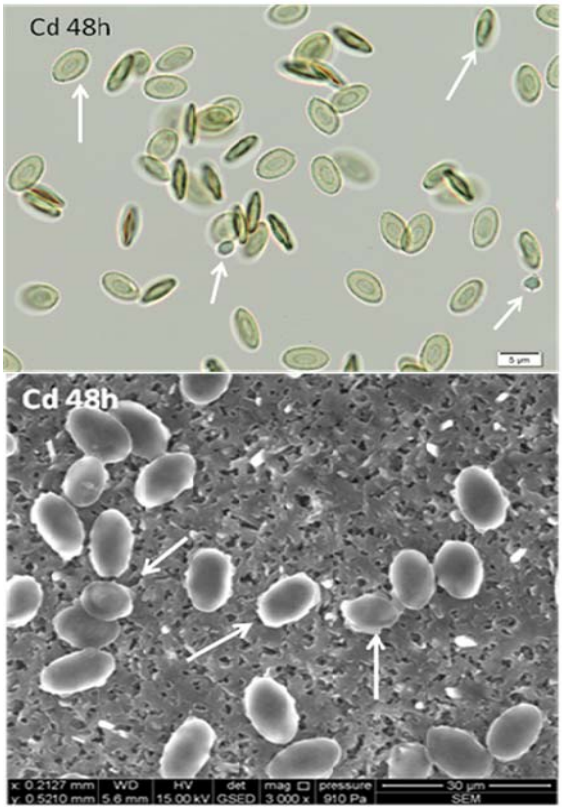

Fig. 7. Optical and ESEM microscopic analysis of *Carassius auratus* nucleated red blood cells after 6 h intoxication with Cd 1 mM for evidence of changes induced morphology.

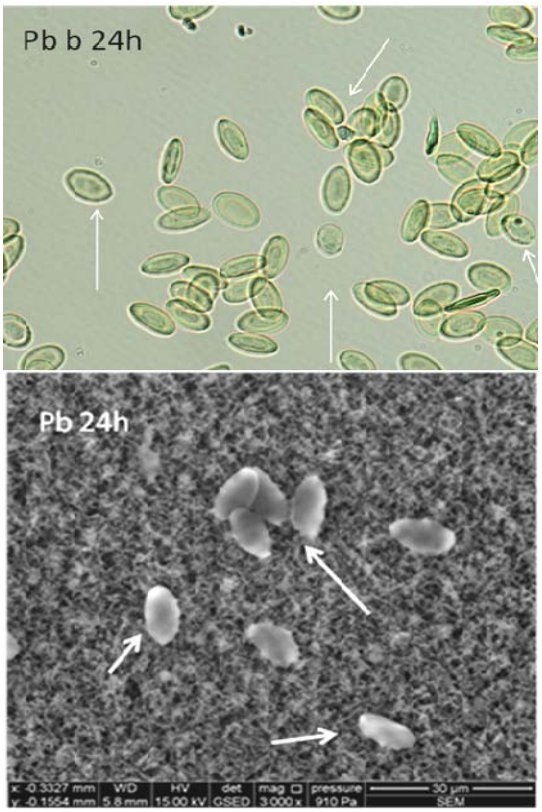

Fig. 8. Optical and ESEM microscopic analysis of *Carassius auratus* nucleated red blood cells after 24 h intoxication with Pb 1 mM for evidence of changes induced morphology.

CONCLUSIONS

In our paper, from this series of experiments, we can conclude that studied heavy metals have seriously deleterious effect on nucleated erythrocytes in a dose-dependent in vivo, and consequently the erythrocyte is extremely sensible and vulnerable.

The results we obtained show that in vivo, in the case of sub-lethal poisoning with heavy metals is observed an apoptotic phenomenon of erythrocytes. In vivo apoptosis of fish red blood cells under the action of heavy metals as pollutants suggests that this phenomenon may be the basis of a simple test for assessing the conditions under which a product comes from a polluted fishery or unpolluted aquatic environment (test of sanogenesis).

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