In vivo Experiments (*Carassius gibelio Bloch*) on Copper Homeostasis Alteration After Lead Intoxication and Natural Biologic-active Principles Treatments

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Minerals are involved in the most metabolic pathways and are necessary for living organisms in different concentrations. When the concentration of these biominerals is unbalanced due to different factors, the metabolic processes are disturbed and the organism tries to find recovery solutions. Cu is one mineral indispensable for living organism, also for fishes, and its concentration it could be drastically reduced by the presence of high concentration of some heavy metals – like Pb, due to antagonistic effect. Our research evaluates the Pb toxic potential on Carassius gibelio Bloch on Cu distribution in different tissues and two natural solutions for potentiation of antagonistic effect of Pb on Cu. We worked on four different fish groups: control group (C); and for three experimental groups we add 75 ppm Pb – as $Pb(NO_3)_2$ x ½H_2O into the water from aquarium. To potent the Pb toxicity we add into the grounded fish feed 2% lyophilized garlic to E3 group and 2% chlorella to E4 group. Every group had 30 fishes in separate aquarium, the fishes were fed every 2 times a day and had 12h alternate light and dark. After 21 days of experiment the fishes were euthanized with cloves oil and the tissue samples were collected (brain, gill, gonads, intestine, kidney, liver, striated muscle – epaxial myotomes, cardiac muscle, and skin). The samples were analytical prepared for AAS in order to determinate the Cu concentration in all tissue samples. The results presented the best protection of garlic against antagonistic effect of Pb on Cu in brain and testicles, and the lowest protection in muscle-striatal; while chlorella best protection was observed in heart muscle, brain, kidney and liver, and lowest protection in muscle-striatal.

Keywords: copper, lead, Carassius gibelio Bloch, garlic, chlorella.

For living organism, and especially for aquatic ecosystems, lead is considered an important environmental metal, being present everywhere. Lead is a heavy metal, known as a promoter of silent long-term environmental disease, especially to children, because lead has no physiological role in living organisms [1,2]. Generally, heavy metals such as lead, cadmium, copper, nickel, mercury and others are dense metals or metalloids (with a higher density of 5kg/dm^3), usually associated with soil and water pollution – with high impact on the living organisms [3-7].

Lead exposure can be related to air, water, food or dust contamination. The sources of lead intoxication includes: fields of turf fibers; candies ingredients (chili powder, tamarid) or candies wrapper materials (ink of paper or plastic wrapping); lead from paint or plastic from toys or toys jewelry (if put into the mouth or swallowed); contaminated tap water; some traditional medicines (originally from India, Hispanic culture, Middle Eastern or West Asian) contaminated with lead during grinding or coloring, or even from the package or wrapping the product; employees of mines, copy centers and paint factors [1,8].

The diagnosis of lead intoxication is made by blood lead quantitative methods. In US, Centers for Disease Control set up maximum limit for blood lead as 10μg/dL (or 10μg/100g) for adults and 5μg/dL (or 5μg/100g) for children [9,10]. Heavy metals contamination influence also the mother, fetus and new born living organism, both in terrestrial (soil food, feed contamination, smoking) and aquatic (water, waste) environment [11,12].

One very efficient therapeutic approach is creating favor environment for chelation (meaning coordination bonds between the metal and a polydentate ligand – organic molecule), which ameliorate the toxicity of heavy metals in organs tissue (kidney, liver, brain, hearth, lung, and others).

Natural chelating compounds are found in animal and plant products, with very good experimental results in detoxification pathways [13-16]. The best natural plant components with chelating action include: dietary fibers; natural polymers (polysaccharides alginate, citrus pectin alginate products, poly(g-glutamic acid), chlorella); sulphur-containing peptides (alliums – garlic, brassicas – broccoli); coriander (*Coriandrum sativum* leaves, known in Spain as cilantro) [17].

The synthetic chelation agents are also used for detoxification of heavy metals, and most common chemicals with chelation effect are EDTA (Ethylenediaminetetraacetic acid), DTPA (diethylene-triaminepentaacetic acid or pentetic acid, EDDHA (ethylene-diamine-N-N'-bis(2-hydroxy-phenylacetic acid)), DMPS (2,3-Dimercapto-1-propane-sulfonic acid), DMSA (Dimercaptosuccinic acid) [18].

The effects of initial heavy metal contamination can be evaluated in tissue and cellular samples before important behavior alteration or exterior appearance can be observed in fish. Thus, our experiment aim was to evaluate tissue copper homeostasis after lead toxicity in *Carassius gibelio Bloch* (known also as silver Prussian, Prussian carp or Gibel carp) – a fish belonging to *Cyprinidae* family, and also to evaluate the detoxifying potential of lyophilized garlic and chlorella.

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Experimental part

Materials and Methods

For our experiment the ecological accessibility and living organism representativeness were the most important criteria for choosing the living test organism. Thus, we focused on *Carassius gibelio Bloch* – a fish species part of *Cyprinidae* family, *Pisces* class. It is a freshwater or brackish water (more saline then fresh water) and benthopelagic fish which can tolerate pollution and low oxygen concentration [19-22].

Our experiment consisted on four different groups – each group was formed of 30 fishes, with 22-25 g body weight, accommodated in 60 L aquariums for each group. The aquariums were equipped with aeration facilities and the experimental fishes were acclimated for 2 weeks before the experiment to remove suspicions related to the health status of the fish.

Thus, the fourth groups were experimentally distributed in one control group; one experimental lead group (E1 – administration of lead); one experimental lead+garlic group (E2 – administration of lead and lyophilized garlic); one experimental lead+chlorella group (E3 – administration of lead+chlorella).

During the accommodation and experimental time, the fishes were fed 2 times a day with a commercial granular product, 12 hours light and 12 hours dark regime was assured, and we assured special attention to some quality indices of water such as: temperature, pH, dissolved oxygen, water hardness, nitrites, nitrates; thus avoiding that water quality indices could possible to potentiate the toxic action of the lead, garlic or chorella.

The heavy metal – lead, was administered as 75 ppm concentration – such as $Pb(NO_3)_2$ x ½ H_2O, to all three experimental groups, and was well distributed and circulated in the aquarium water (lead concentration was adapted to data presented by Veena and Abedi with their collaborators as $LC50_{Pb}$).

During the experiments we tested the lead intoxication, and we also tried to protect the fishes by adding 2% lyophilized grounded garlic (*Allium sativum*) – one experimental group and 2% chlorella (*Chlorella pyrenoidosa*) – the second experimental group, incorporated in the feed grounded granular products. The feed with garlic and chlorella was dosed by setting the quantity of daily feed / fish [23].

After 21 days of experiment, at the finish of the experiment, we collected the biological tissue samples of brain, gill, gonads (of both genders), intestine, kidney, liver, striated muscle-epaxial myotomes, cardiac muscle, and skin, after prior euthanasia (using cloves oil) from all fishes using in experiment (control group and experimental E1, E2, E3 groups).

The tissue samples were analytical processed and copper concentration of all samples was evaluated using atomic absorption spectrometer (VARIAN AAS). The statistical analysis using the ANOVA without replication test was performed, taking into account two factors: the tissue and the adopted treatment.

Results and discussions

Exposure of fish (*Carassius gibelio Bloch*) to $LC50_{Pb}$ concentrations for 21 consecutive days caused its accumulation in the tissues. Bioaccumulation of lead had an organ-specific distribution – respectively the heart, as it can easily noticed in the control group. In addition, the administration of 75 ppm lead for 21 days resulted in the tissue mobilization of some minerals known as essential for the different biological structures and for certain metabolic functions in the living organisms.

In our experiment, high levels of Cu in the tissues belonging to individuals of the control group were measured for heart (8.09 ppm), liver (4.27 ppm) and intestine (4.27 ppm). Chronic poisoning with lead drastically reduces the concentration of copper in all animal tissues exposed to its action (pÂ0.001) (fig. 1), but the most affected being the muscles (only 0.15 ppm) and ovary (only 0.12 ppm).

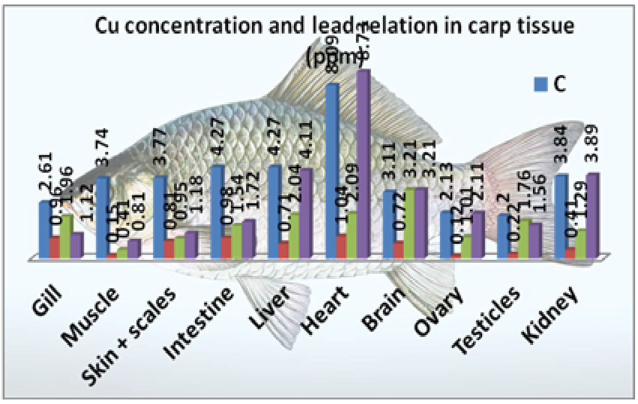

Fig. 1. Copper (ppm) distribution in tissue samples after lead, garlic and chlorella administration in *Carassius gibelio* fishes

In tissues sampled from fishes that were feed with garlic or chlorella in granulated formula, the copper tissue levels presented significant variations in experimental groups compared to control.

Lead in high concentration in aquatic environment manifest antagonistic effect on copper in all tissues collected from our experimental fishes compared to control. The highest decreased in copper concentration was registered in cardiac muscle (96%) and ovary (94.3%); followed by kidney (89.3%), testicles (89%), heart (87.1%) and liver (83.3%); moderate decreases was found in skin and scales (78.5%), intestine (77%), and brain (76.8%); and the lowest diminish of copper concentration was observed in gill (63.3%). In a previous work we found a similar depletion action of lead on Zn and Fe from fish tissues [24].

The maximum effectiveness in counteracting lead antagonism against copper was observed in chlorella E3 group, in the heart samples (8.73 ppm compared to 8.09 ppm in control), brain (3.21 ppm compared to 3.11 ppm in control); kidney (3.89 ppm compared to 3.84 ppm in control); ovary (2.11 ppm compared to 2.13 ppm in control); and liver (4.11 ppm compared to 4.27 ppm in control). Spirulina, another microalga, recommended as an alternative source of feed in aquaculture, has manifested the same high potency in counteracting of lead toxicity in Prussian carp [25-28].

Statistical analysis was used to evaluate for all determined data to present if the modification in copper concentration are significant or not in our experiment (table 1).

Also, taking in consideration biologic-active components from garlic as protective against lead intoxication and copper distribution (E2 group), the best response was observed in brain (3.21 ppm compared to 3.11 in control) and testicle (1.76 ppm compared to 2.00 ppm in control).

Lead interferes with copper for binding to metallothionein – a cysteine rich protein, which is responsible for the homeostatic control of zinc, copper and selenium because it is physiological binding these elements. Also, heavy metals (lead, cadmium, mercury, silver, arsenic) can bind metallothionein due to its chemical similarity to essential elements. It seems that metallothionein could have heavy metal toxicity protection, being involved in the metabolism of zinc and copper which are responsible for oxidative stress protection.

Table 1
STATISTICAL INDEXES AND STATISTICAL SIGNIFICANCE FOR THE COPPER TISSUE LEVEL IN EXPERIMENTAL FISHES

Tissue	*Number*	*Sum*	*Average*	*Variance*	*SD*
Cu (ppm) wet tissue					
Gill	4	6.65	1.66	0.59	0,66
Muscle	4	5.11	1.27	2.76	1.44
Skin + scales	4	6.71	1.67	1.96	1.21
Intestine	4	8.51	2.12	2.13	1.26
Liver	4	11.13	2.78	2.94	1.48
Heart	4	19,.95	4.98	15.87	3.45
Brain	4	10.25	2.56	1.51	1.06
Ovary	4	5.37	1.34	0.93	0.83
Testicole	4	5.54	1.38	0.63	0.69
Kidney	4	9.43	2.35	3.15	1.53
Group					
C	10	37.83	3.78	2.97	1.63
Pb	10	6.12	0.61	0.12	1.67
Pb + garlic	10	16.26	1.62	0.60	1.77
Pb + chlorella	10	28.44	2.84	5.64	1.89
Source of variance					*p*
Between tissues					p<0.001
Between treatments					p<0.05

Most likely, lead disturbs the copper metabolism by reducing the ceruloplasmin-protein concentration, responsible for its transport to the posthepatic circulation. Ceruloplasmin is an enzyme from ferroxidase class, very important in activity of blood copper carrying protein, participating directly in copper homeostasis. Also, the ceruloplasmin quantification is necessary to establish if a patient has Wilson disease (a disease specific for copper storage), Menkes diseases (Kinky hair disease), copper deficiency or aceruloplaminemia (total copper deficiency).

Lead and copper act as antagonistic elements, fact demonstrated in our experiment for all tissues samples. Regarding these data, administration of garlic or chlorella reduces lead toxic potential against copper distribution, fact observed in all analysis of tissue samples.

Physical and chemical water characteristics, especially depending on the season, can modify the distribution of the pollutants and the biominerals in fish tissues [29]. Lead, cadmium and copper exposure caused high dose-dependent glutathione-S-transferase increased activity in gills and liver sampled from European eel (*Anguilla Anguilla*). Also heavy metals exposure caused peroxidative damages with irreversible oxidative stress, with possible severe consequences even in neuronal cell membrane [30].

Associated toxic heavy metals (such as mixture of cobalt, chromium and lead) act significant, more toxic compared to individual metals toxicity in fish from freshwater [31].

Conclusions

Copper distribution in tissues from *Carassius gibelio* was analyzed by atomic absorption spectroscopy, after lead intoxication and garlic and chlorella protection. This experimental research demonstrates that lead in aquatic environment manifest antagonistic effect on copper in tissues from *Carassius gibelio*.

The biologic active principles from garlic and chlorella diminish the antagonistic effect on lead on copper. Copper concentration from fish tissues is drastically decreased after lead intoxication in almost all analyzed samples.

Lyophilized garlic administrated into the feed of fish manifest very good protection of cooper distribution in brain, and good protection of cooper in testicles. Chlorella mixed in grounded feed and administrated to fishes presented very good protection on copper distribution in hearth muscle, brain and kidney, and good protection of cooper in liver, ovary and testicles.

The lowest protection effect of garlic and chlorella was registered in muscles sampled from *Carassius gibelio Bloch*.

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