Histomorphometric Study Regarding the Effect of Experimental Exposure of *Carassius gibelio* Species to Increasing Doses of Ethylenediaminetetraacetic Acid(EDTA)

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Abstract

The aim of this paper is to show the preliminary results regarding some histomorphometrical changes induced by chronic exposure of Prussian carp specimens to increasing doses of EDTA.

150 Prussian carps, 10-12 g of weight, collected from a local pond and acclimated for two weeks in laboratory conditions were housed in 60 L capacity glass aquariums (30 individuals/aquarium) and divided into four groups: a control group maintained in EDTA-free freshwater and others three groups receiving 0.05 g EDTA/L water, 0.1 g EDTA/L water and 0.15 g EDTA/L water respectively.

Tissue samples (gill, small intestine and ovary) were processed and microscopically examined at the end of a 21 days experimental period. A specific QuickPHOTO Micro 2.2 software has been used for the histomorphometric study.

Gill lamellae length, intestinal villi height and chorion thickness of ovaries follicles suffered EDTA dose-dependent histomorphometrical changes.

Keywords: EDTA, freshwater fish, gill, histomorphometric study, ovary, small intestine

1. Introduction

EDTA is used as a complexing agent in many industrial branches, agriculture, human and veterinary medicine [1-3]. Since it is used mainly in aqueous medium, its environment release is via wastewaters. Compared to other chelating agents, EDTA has two important characteristics: its relatively low biodegradability in aquatic systems (Novak et al., 1997, cited by Oviedo and Rodriguez, 2003) [3] and its strong complexing ability to heavy metals [4].

The interaction mechanisms of EDTA with living organisms are not sufficiently clarified and the range of their potential risks is not known. The effects of EDTA differ according to the type of organism studied, the concentration of EDTA and the metal analyzed. Thus, Dufkova et al. 1984, cited by Oviedo and Rodriguez, 2003 [3] studying the interaction of EDTA with photosynthetic organisms and found that EDTA is toxic, since it inhibits cellular division, chlorophyll synthesis and algal biomass production. Tubbing et al. [5] demonstrated with river microalgae in which photosynthesis is inhibited at low concentrations of EDTA chelated with copper (II) (5-10 mM) and unchelated EDTA. As stated previously, this is also evident in the work of Vassil et al. [6]. Acute toxicity test in *Daphnia magna* found that Cd(II)-EDTA and Cu(II)-EDTA complexes were more toxic than their respective free metals [7].

Since previous studies on tissue damage caused by EDTA in fish are very little, we plan to expand them in order to highlight the histomorphometric

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changes induced by chronic exposure of carp specimens to increasing doses of EDTA.

Fresh fish samples were collected from a local pond and gravimetrically selected. Thus, individuals of 10-12 g weight were acclimated for two weeks in laboratory conditions removing those with suspicions on their health. Then acclimated fish were distributed in glass aquariums equipped with aeration system and a 60 L capacity (30 fish/aquarium), resulting four experimental groups: a control group maintained in EDTA-free freshwater and others three groups receiving 0.05 g EDTA/L water, 0.1 g EDTA/L water and 0.15 g EDTA/L water respectively. Routinely, doses of EDTA applied in aquaculture range from 10 mg/L in shrimp larval rearing prior for stocking of nauplii to 1-5 ppm for remove organic substances in the water [8]. Fish were fed twice a day with commercial dry pellets.

Physico-chemical indicators of water-dissolved oxygen, temperature, NO_2^-, NO_3^-, pH, hardness were daily measured (water temperature and dissolved oxygen with a movable oxygen-meter with water resisting microprocessor Hanna HI 9145; pH, NO_2^-, NO_3^-, pH, hardness of water with a Germany termatest kit).

Fragments of gill, small intestine and ovary were removed at the end of a 21 days experimental period after fish euthanasia with an overdose of the anesthetic tricaine methanosulfonate (MS-222) (>250 mg/L) [9]. The material was subjected to histological analysis, being fixed in a formalin solution-10%, dehydrated, clarified, impregnated and embedded in histological paraffin, according to standard routine methods in the Histology laboratory [10]. Five-micrometer-thick sections were obtained and stained by Mallory's trichrome method. Gill, intestinal and ovary metrics were estimated for 10 specimens from each field population.

Microscopic examination was performed using a CX 41Olympus light microscope equipped with a digital camera and a specific QuickPHOTO Micro 2.2 software has been used for the histomorphometric study.

Statistical analysis was performed by using the SPPS IBM 22 software. Data were reported as Mean ± SD at a significant level of $p< 0.05$.

Testing differences between means was realized by ANOVA completed with post-hoc Tukey test.

3. Results and discussion

Gills are the primary site of respiratory gas exchange in most fishes. Oxygen acquisition from the environment involves diffusion along a concentration gradient from the ventilator water stream, across the gill epithelium and into the blood [11].

The gills of fishes are the most susceptible and vulnerable to the action of contaminants due to their function (they considered multifunctional organs [12], interfering in respiratory gases transport (large area and minimum distance of diffusion between dissolved oxygen and blood capillary for gas exchange streamline), electrolyte balance regulation, osmoregulation and nitrogenous waste excretion.

Gills form over 50 percent of the total surface area of the animal [13] that makes it sensitive to change in the composition of the environment and they can be considered as an important indicator of waterborne toxicants. Indeed, gill filaments and lamellae provide a very large surface area for direct and continuous contact of the fish with water contaminants.

The descriptive statistics composed by mean, standard deviation, coefficient of variation, minimum and maximum values, of the histomorphometrical measures is presented in Table 1. It is interesting to note that the different measurements obtained presented similar variability, as indicated by the coefficient of variation. The same table shows the values of absolute and relative differences on the length of primary gill lamellae between the groups compared.

Length of primary gill lamellae shows significant differences whether the control group C is compared with all other experimental groups (EDTA1, EDTA2, EDTA3), or if the last ones are compared with each other (Figure 1).

The length of primary gill lamella belonging to individuals of the control group is approximately 20% ($p<0.001$) higher than that of EDTA1 group, increases by approximately 33% ($p<0.001$) than that of the experimental group EDTA2 and by about 59% ($p<0.001$) respectively as compared to that of the experimental group EDTA3.

Compared with each other, the significant differences between experimental groups EDTA1, EDA2 and EDTA3 maintained namely: length of primary gill lamella measured in EDTA1 group is about 15% ($p<0.05$) higher than that of EDTA2 group and approx. 49% ($p<0.001$) relative to the same parameter measured in individuals EDTA3 group. Considered parameter is about 39% ($p<0.001$) higher for EDTA2 group in detriment of EDTA3 group.

Table 1. Descriptive statistics, differences and statistical significance on histomorphometrical measures of primary gill lamellae length (µm)

Descriptive statistics						
Groups	n	X	SD	Cv	Min.	Max.
C	10	3032.30	385.33	12.70	2499.00	3670.00
EDTA1	10	2410.90	252.77	10.48	2055.00	2835.00
EDTA2	10	2032.20	258.99	12.74	1749.00	2462.00
EDTA3	10	1230.80	70.99	5.76	1098.00	1349.00
Differences and statistical significance (Tukey test)						
Multiple comparisons		Absolute differences		Relative differences %		Statistical significance
C-EDTA1		621.40*		-20.49		***
C-EDTA2		1000.10*		-32.98		***
C-EDTA3		1801.50*		-59.41		***
EDTA1-EDTA2		378.70*		-15.70		*
EDTA1-EDTA3		1180.10*		-48.94		***
EDTA2-EDTA3		801.40*		-39.43		***

*The mean difference is significant at the 0.05 level
***The mean difference is significant at the 0.001 level

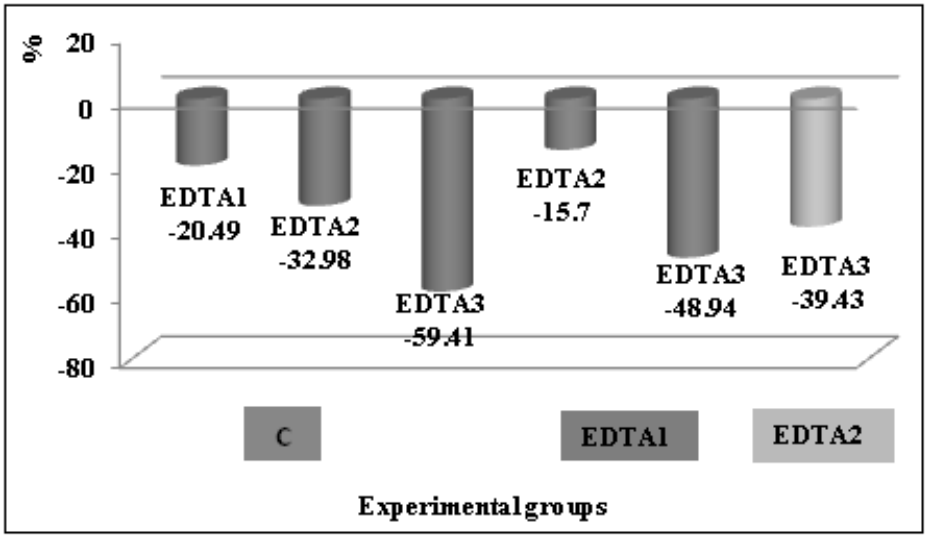

Figure 1. Graphic representation of the relative differences (%) on the length of the primary gill lamellae between experimental groups

There are significant differences between experimental groups for length of secondary gill lamellae as well except where EDTA1 is compared with EDTA2 and EDTA2 with EDTA3 respectively (Table 2).

The relative differences recorded between the control group and the other experimental groups starts at 18% ($p<0.001$) (C-EDTA1) and reach 26% ($p<0.001$) (C-EDTA3).

Although present, the differences between EDTA1 and EDTA 2 and EDTA 2 and EDTA3 respectively, they are insignificant ($p>0.05$) (Figure 2). All these histomorphometric alteration lead to a total decreasing in the diffusive conductance of the gills to respiratory gases, compromising their function.

Table 2. Descriptive statistics, differences and statistical significance on histomorphometrical measures of secondary gill lamellae length (μm)

Descriptive statistics						
Groups	n	X	SD	Cv	Min.	Max.
C	10	96.10	4.20	4.37	91.00	102.00
EDTA1	10	78.70	4.00	5.08	71.00	84.00
EDTA2	10	73.90	4.70	6.35	68.00	80.00
EDTA3	10	71.10	4.95	6.96	64.00	81.00
Differences and statistical significance (Tukey test)						
Multiple comparisons		Absolute differences		Relative differences %		Statistical significance
C-EDTA1		17.40*		-18.10		***
C-EDTA2		22.20*		-23.10		***
C-EDTA3		25.00*		-26.01		***
EDTA1-EDTA2		4.80*		-6.09		is
EDTA1-EDTA3		7.60*		-9.65		*
EDTA2-EDTA3		2.80*		-3.93		is

*The mean difference is significant at the 0.05 level
***The mean difference is significant at the 0.001 level
is-insignificant difference

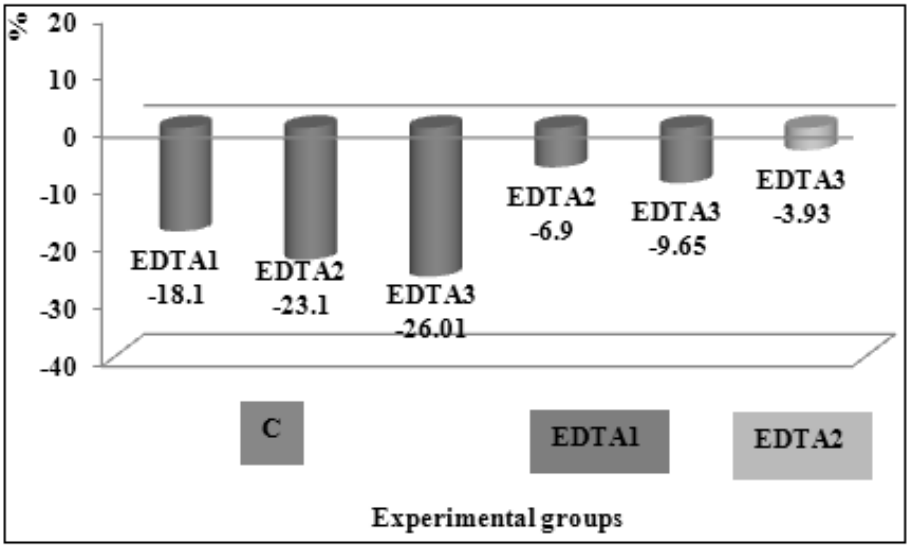

Figure 2. Graphic representation of the relative differences (%) on the length of the secondary gill lamellae between experimental groups

Gastrointestinal tract is a major route of entry into the fish body of a wide variety of toxic substances coming from diet or the aquatic environment in which they live. According to Ferrando et al., (2006) [14] knowledge about intestinal morphological alterations, both at macroscopic and microscopic level can be used as a rapid, easy to detect, and cost-effective biomarker for a preliminary assessment of field contamination.

The results of histomorphometric analysis are given in Table 3.

Exposure to environmental action of EDTA had to led to significant tissue alterations in the gut of all specimens subjected to EDTA exposure.

For instance, administration of EDTA in water affects the height of the intestinal villi (Figure 3) and consequently their absorption capacity in specimens from all three experimental groups. Thus, this one recorded relative differences with 22% lower ($p<0.05$) in specimens of EDTA1 group, with 34% lower ($p<0.001$) in those of EDTA2 group and with nearly 42% ($p<0.001$) lower in EDTA3 group than the control group. Relative differences between the experimental group EDTA1-EDTA2 ($p>0.05$) and EDTA2-EDTA3 ($p>0.05$) were statistically insignificant.

Table 3. Descriptive statistics, differences and statistical significance on histomorphometrical measures of intestinal villi height (µm)

Descriptive statistics						
Groups	n	X	SD	Cv	Min.	Max.
C	10	377.00	38.64	10.24	301.00	409.00
EDTA1	10	292.60	80.61	27.54	186.00	473.00
EDTA2	10	247.00	23.24	9.40	217.00	280.00
EDTA3	10	218.70	38.62	17.65	178.00	287.00
Differences and statistical significance (Tukey test)						
Multiple comparisons		Absolute differences		Relative differences %		Statistical significance
C-EDTA1		84.40*		-22.38		*
C-EDTA2		130.00*		-34.48		***
C-EDTA3		158.30*		-41.90		***
EDTA1-EDTA2		45.60		-15.58		is
EDTA1-EDTA3		73.90*		-25.25		*
EDTA2-EDTA3		28.30		-11.45		is

*The mean difference is significant at the 0.05 level
***The mean difference is significant at the 0.001 level
is-insignificant difference

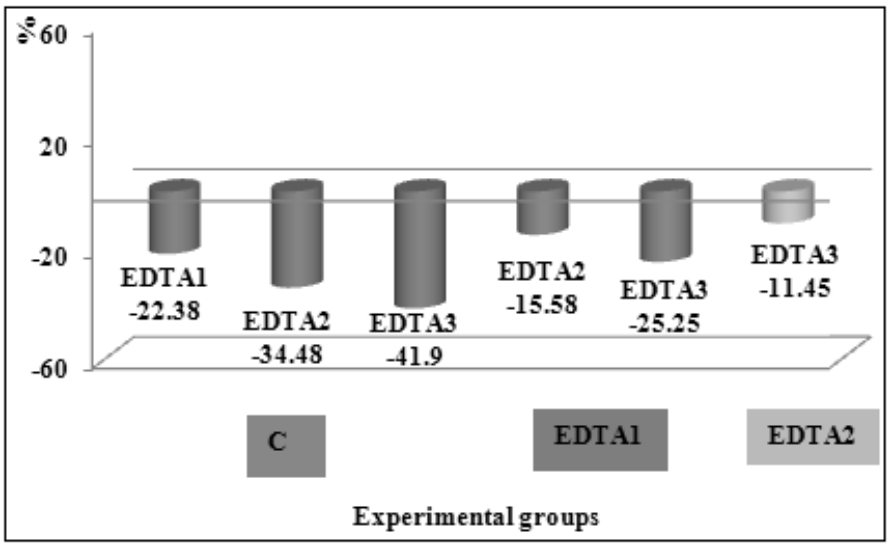

Figure 3. Graphic representation of the relative differences (%) on the height of the intestinal villi between experimental groups

Presence of atretic cortical alveolar oocytes with thickened chorion is the major characteristic of follicles obtained from ovary samples of EDTA1-EDTA3 groups. Effect of EDTA on chorion thickness of females specimens has manifested in a dose-dependent manner, which is proved by statistical processing of measurements performed on samples obtained from them (Table 4).

In EDTA3 group which has been exposed to a dose of 0.15 g/L EDTA in water, the chorion thickness increases by about 138% compared to the control group ($p<0.001$), with about 129% vs. EDTA1 group and with approximately 118% vs. EDTA2 group (Figure 4). Between the control group and the experimental groups EDTA1 and EDTA2, as well as between experimental group EDTA1 and EDTA2 relative differences of chorion thickness are not significant ($p>0.05$) (Figure 4).

Table 4. Descriptive statistics, differences and statistical significance on histomorphometrical measures of ovary chorion thickness (µm)

Descriptive statistics						
Groups	n	X	SD	Cv	Min.	Max.
C	10	10	15.50	2.71	17.48	11.00
EDTA1	10	10	16.1	2.18	13.54	12.00
EDTA2	10	10	16.90	2.99	17.69	11.00
EDTA3	10	10	36.90	6.78	18.37	27.00
Differences and statistical significance (Tukey test)						
Multiple comparisons		Absolute differences		Relative differences %		Statistical significance
C-EDTA1		-0.60		3.87		is
C-EDTA2		-1.40		9.03		is
C-EDTA3		-21.40*		138.06		***
EDTA1-EDTA2		-0.80		4.96		is
EDTA1-EDTA3		-20.80*		129.19		***
EDTA2-EDTA3		-20.00*		118.34		***

*The mean difference is significant at the 0.05 level
***The mean difference is significant at the 0.001 level
is-insignificant difference

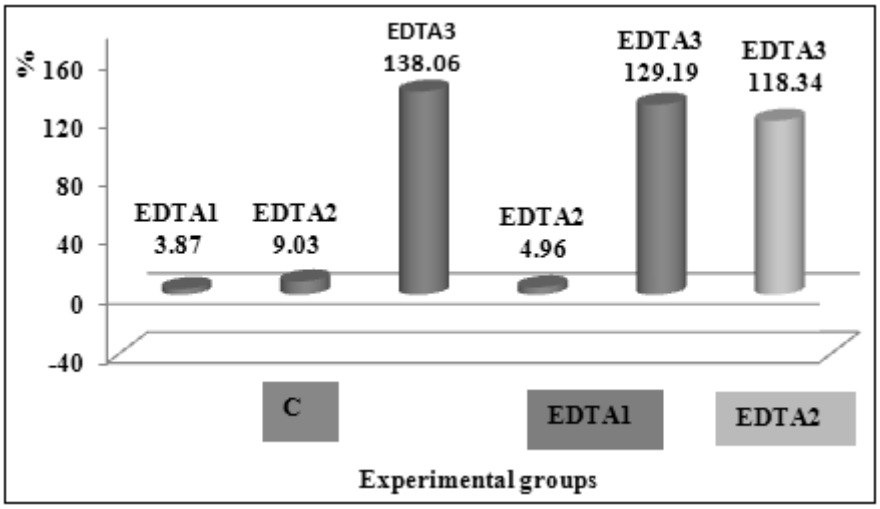

Figure 4. Graphic representation of the relative differences (%) on the thickness of the oocytes chorion between experimental groups

4. Conclusions

In conclusion, we can state that:
1. EDTA, which is a chemical that reaches in aquatic ecosystems from various anthropogenic sources can affect the structure and function of aquatic organisms tissue;
2. The progressive histomorphometrical alterations of the gill and gut tissue at the varied dosage of EDTA is due to the his takeover from aqueous medium through the lamella surface or intestinal mucosa;
3. EDTA has a direct impact on female gonad with implications for the future reproductive capacity of adult females.

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