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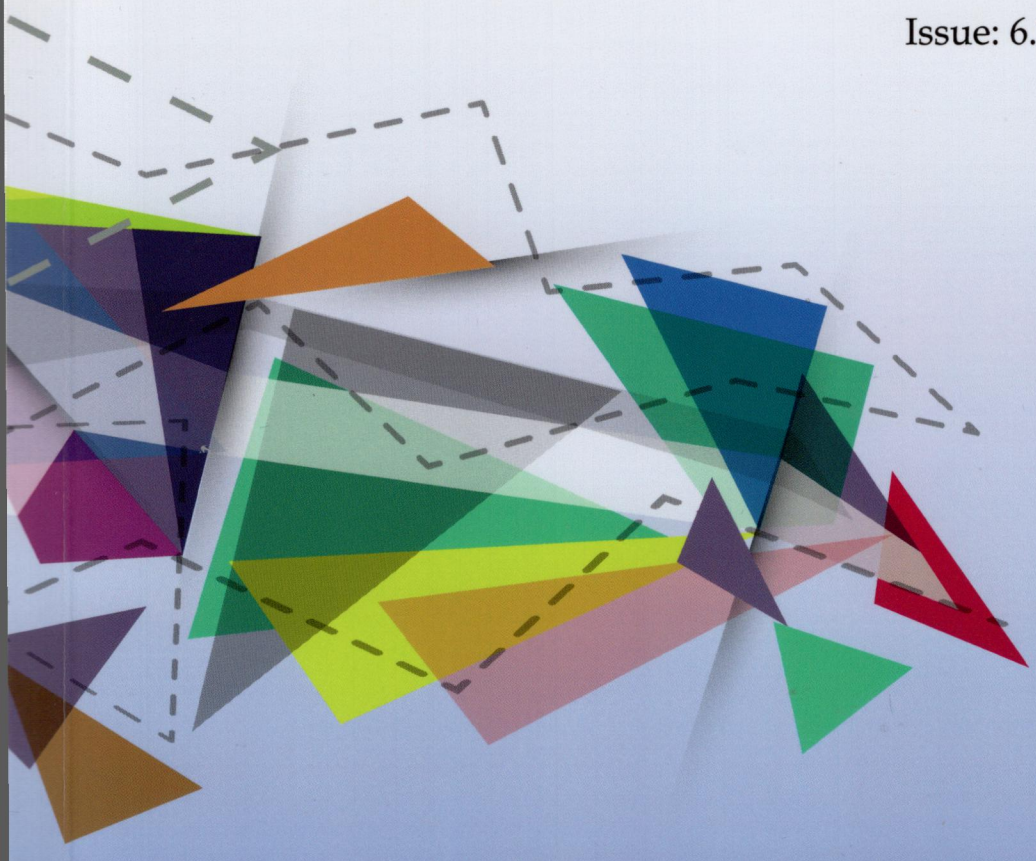

MICRO AND NANO TECHNOLOGIES

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WATERBORNE SUCROSE IMPAIRS HEALTH STATUS OF FARMING FISH *CARASSIUS AURATUS GIBELIO*

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ABSTRACT

Glucose toxicity is a well-established being that has been proven in higher vertebrates. The goal of this the present study was to elucidate the response of farming fish gibel carp *Carassius auratus gibelio* to waterborne sucrose in low (5.55 mM, LS) and high (55.5 mM, HS) concentrations for 21 days mimicking carbohydrate-enrich diet. An increase in blood glucose levels, body mass and hepatosomatic indices were the common sign for both concentrations of sucrose. The treatment with low concentration of sucrose provoked prominent manifestations of oxidative stress traits, namely enhancement of reactive oxygen species (ROS), lipid peroxidation and lipofuscin in the liver. In contrast, exposure of fish to HS induced the oppression of stress response: the decreasing the levels of ROS and lipid peroxidation, accompanied by the elevation of DNA strand break, depletion of cholinesterase activity and enhancing of glycated hemoglobin. Treatment of carp with HS caused 50% mortality for 21 days. Substantially that hyperglycemia in LS and HS groups were accompanied by significant upregulation of stress-related proteins metallothioneins in the liver. Overall, gibel carp represents the prospective model for study hyperglycemia mechanisms and consequences in vertebrates. The appropriate level of carbohydrate in the diet of the farming fish is essential.

Keywords: gibel carp, hyperglycemia, metallothioneins, oxidative stress, cytotoxicity

INTRODUCTION

Carbohydrates are one of the lowest price component of diet both for people and animals, but their utilization by fish varies and remains unclear. No dietary requirement for carbohydrate has been demonstrated in fish. Meanwhile the glucose turnover rate in fish, particularly in carnivorous species is much lower than in mammals [1], [2]. Glycolysis is the predominant mechanism of glucose metabolism in fish. Enrichment of diet with carbohydrate was improved the glycolytic pathway in the livers of fish [3]. In general, glucose stimulates of insulin secretion in fish in a weak manner and should cause glucose intolerance [3]. It has been shown that overdigestion of carbohydrates in rainbow trout results in prolonged postprandial hyperglycemia and impaired growth [1].

High level of glucose under diabetes mellitus should trigger oxidative stress, which is closely linked to inhibition of synthesis and insulin secretion [4]. In particular, human erythrocytes which were immersed in high-concentrated glucose showed an increase in

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lipid peroxidation and a decrease in the activity of glutathione S-transferase and glutathione reductase [5]. Meanwhile the effect of hyperglycemia in fish is unclear, despite a carbohydrate-enriched diet, a cheap source of energy, that allows to gain body mass within short period of time, but provokes hyperglycemia [6].

Therefore, the aim of our study was to investigate the effects of sucrose on morphometrical and biochemical markers of gibel carp Carassius auratus gibelio, as a widespread, important omnivorous industrial species. The battery of biomarkers namely body mass index and biochemical panel level, glycated haemoglobin, key parameters of oxidative stress, metallothioneins, geno- and neurotoxicity were evaluated under the effects of moderate and acute concentration of waterborne sucrose.

METHODS AND MATERIALS

Experimental design

Adult gibel carp *Carassius auratus* gibelio (15-18 cm long and 180-240 g weight) were collected from the fish farm which is located in the Ukrainian reference site (49°49' N, 25°23' E). Fish were transported to the laboratory in 60 L cages with aerated native water. Experiments were performed in accordance with the national and institutional guidelines for the protection of animal welfare and approval of the Committee on the Bio-Ethics at Ternopil V. Hnatiuk National Pedagogical University (No 2/5.09.2017).

Fish were acclimated in aerated, softened tap water and fed throughout the experiment with commercial food (21% of protein, Aquarius, Ukraine). One group was exposed to the aquarium water without any addition and was considered control (C). Other groups were exposed to 5.55 mM (LS) and 55 mM (HS) sucrose for 21 days.

After the exposure, fish were anesthetized by clove oil, the whole blood was collected from the heart, and plasma was immediately separated by centrifugation of the heparinized blood at 10,000 ×g for 10 min. Glucose concentration in blood serum was determined with diagnostic kit (*Erba*-Lachema, Ukraine) according to the manufacturer's instructions. Glycated haemoglobin was determined by spectrophotometrical method using test kit (GHB 100, *Erba*-Lachema, Ukraine) according to the manufacturer's instructions. The liver and brain were removed after killing of fish, drained with filter paper and weighed. The Hepatosomatic Index (HSI) was calculated as the ratio: drained mass of liver/total body mass × 100. Also, Body Mass Index (BMI) was calculated as the ratio: total body mass in kg/(length in meters)^2. For each biochemical analysis, 8 samples were used. Protein concentration in the supernatant was measured by the method of Lowry et al. (1951), with using bovine serum albumin as a standard. All protocols were used for analytical measurements represent in detailed in [7].

Quantification of stress-related parameters

Metallothioneins (MTs) were determined in liver. The level of MT-related thiols (MT-SH) was measured after ethanol/chloroform extraction with DTNB as described by Viarengo et al. (1999) and calculated by assuming the relationship: 1 mol MT-SH = 20 mol GSH and expressed as µg of MTs per gram of fresh weighted (FW) tissues.

Reactive oxygen species (ROS) formation in tissue 1/10 w/v homogenates was determined by a signal of non-fluorescent dye dihydrorhodamine which is converted to the fluorescent derivative rhodamine-123 in a reaction with the reactive oxygen species

(Viarengo et al., 1997). Probe fluorescence signal was detected by using fluorescence plate-reader [excitation (ex.) = 485 nm, emission (em.) = 538 nm] immediately, and in 20 min.

Lipid peroxidation (LPO) was determined in the soluble fraction of liver tissue homogenate by the production of thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979). A molar extinction coefficient of $1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ was used.

Lipofuscin concentration in liver tissue was determined using chloroform:methanol (2:1, v/v) extraction of the homogenate. Probe fluorescence signal was detected by using a *f*-max fluorescence plate-reader [excitation = 350 nm, emission = 450 nm] immediately. Standardization was done with a freshly prepared solution of quinine sulphate (1 µg·mL^{-1} of 0.1N H_2SO_4) (Manibabu and Patnaik, 1997).

Assays of cytotoxicity

DNA damage was evaluated by the levels of protein-free DNA strand breaks in the liver by the alkaline DNA precipitation assay (Olive, 1988) using Hoescht 33342 dye. Probe fluorescence signal was detected by using *f*-max fluorescence plate-reader (excitation = 360 nm, emission = 450 nm).

Cholinesterase (ChE, EC 3.1.1.7) activity was determined in the brain as the acetylthiocholine-cleaving ChE activity at 25°C according to the colorimetric method of Ellman et al. (1961). Enzyme activity was calculated using a molar extinction coefficient of $13.6 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ and standardized to the soluble protein content.

Statistical analysis

For all studied traits and all experimental treatment groups, sample size was eight. The data are presented as means ± standard deviation (SD) unless indicated otherwise. Data were tested for normality and homogeneity of variance by using Kolmogorov-Smirnoff and Levene's tests, respectively. For the data that were not normally distributed, non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney U-test) were performed. The classification tree based on all studied traits was built using Classification and Regression Tree (CART) software using raw (non-transformed) data. All statistical calculations were performed with Statistica v. 12.0 and Excel for Windows-2013. Differences were considered significant if the probability of Type I error was less than 0.05.

RESULTS AND DISCUSSION

Blood parameters

The obtained results have disclosed (Fig.1) that the immersion of gibel carp in all sucrose solution tested (Fig. 1A) performed up to 3 times increase in blood glucose levels when compared to the correspondent control. Also sucrose exposure was capable to cause a significant increase in glycated hemoglobin (HbA1c) in HS group.

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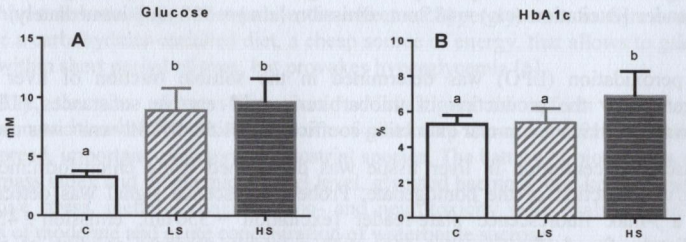

Figure 1. Effects of experimental exposures to sucrose in low (LS, 5.55 mM), and high (HS, 55.5 mM) concentration on blood parameters of gibel carp *Carassius auratus.* A – glucose concentration, B - glycated hemoglobin (HbA1c) concentrations. Data are presented as means ± SD. N=8. Here and on the Figures 2-4, the columns that share the same letters indicate the values that are not significantly different ($P > 0.05$).

Oxidative stress parameters

The results of the evaluation of oxidative stress manifestations (Fig. 2) indicate that in LS group the content of TBARS (by 46%), reactive oxygen species (by 79%) and lipofuscin (by 235%) increased when compare to control, whereas in HS group the opposite changes occurred – the TBARS (by 32%) and ROS (by 81%) decreased, while lipofuscin increased, however, in a smaller range than in LS group (by 85%). Reducing the level of oxidative damage products in HS group was accompanied by mortality at 50% for 21 days, and therefore the concentration of 55.5 mM can be considered as LC_{50}. The content of MTs in experimental groups was higher than in control animals and didn't depend on the sucrose concentration.

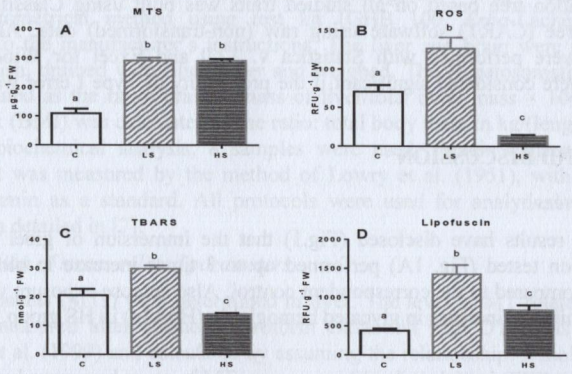

Figure 2. Oxidative stress parameters in the liver tissue of gibel carp after the effect of sucrose

Morphometrical indices and signs of cytotoxicity

So as to know that treatment with glucose causes overweight and fatty liver disease which are highly prevalent in type 2 diabetes mellitus, we determined the BMI and HSI indices and obtained results demonstrated that the treatment with sucrose was able to increase total mass of animal and, particularly in concentration-dependent manner ($F_{(3; 23)} = 12.3$, $p<0.01$) mass of liver (Fig. 3).

Treatment of gibel carp with sucrose led to appearance of cytotoxicity signs namely higher level of DNA fragmentation in HS group (by 25%) and oppression of cholinesterase activity in the LS group when compared with the control (by 42%) (Fig. 3).

We have built a binary classification tree with CART algorithm which simulates a natural process of thinking in differential diagnostics. Application of this approach has allowed to establish the most robust biochemical indices that permitted to distinguish experimental groups after sucrose action. The determining role in the differentiation of the groups belongs to the lipofuscin and hepatosomatic index (Fig. 4).

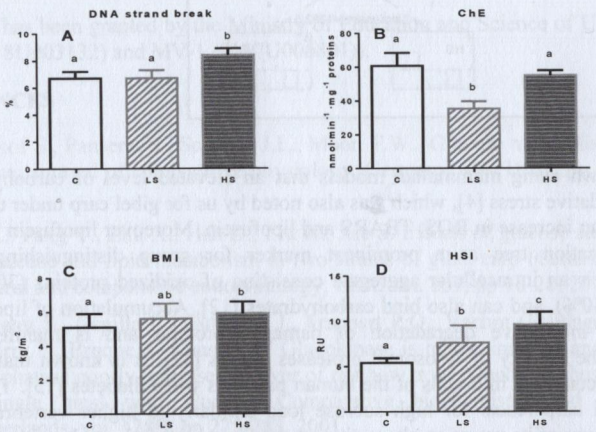

Fig. 3. Signs of cytotoxicity (A-B) and morphometrical indices (C-D) of gibel carp after the effect of sucrose

MTs are multifunctional, cytosolic, thermostable proteins with high content of thiols (up to 30%) and transition metals [8]. There are three regulatory elements in the promoter of the MT gene, including metal (MRE), antioxidant (ARE) and glucocorticoid-sensitive (GRE) that control their inclusion in the deposition and detoxification of metals, sequestration of ROS, immune response, proliferation and differentiation cells, etc. (s been proven [9]. Moreover, MTs in humans and animals prevent the development of type I / II diabetes, and, as potential antioxidants, play a tread effect on its complications, in particular nephropathy and cardiomyopathy [10]. The defect in the *MT2A* A-5G gene in the Japanese population of Nagoya was consistent with the increased risk and incidence of type 2 diabetes [11]. According to

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our results, sucrose caused two-fold up-regulation of MTs. Moreover, MTs level varied concordantly with the studied oxidative stress indices, particularly with ROS, which was confirmed by regression analysis: MTs = 126.28 – 2.24×TBARS – 1.17×ROS* + 0.27×Lipofuscin*, R^2= 0.97, p<0.0001, * – the index makes a plausible contribution to the mathematical model. Therefore, MTs of gibel carp under hyperglycemic states act as radicals scavenger, reduce manifestations of oxidative damage and can serve as an informative indicator for early detection and progression of diabetes.

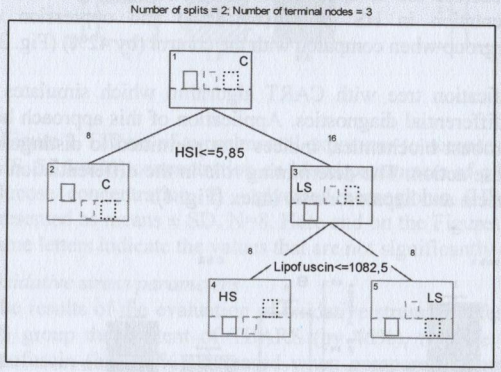

Fig. 4. Classification tree of all studied biological traits after the effect of low (LS) and high (HS) concentration of sucrose

It was shown using mammalian models that an elevated level of carbohydrates can initiate oxidative stress [4], which was also noted by us for gibel carp under the effect of sucrose as an increase in ROS, TBARS and lipofuscin. Moreover lipofuscin was choose by classification tree as a prominent marker for group distinguishing (Fig. 4). Lipofuscin is an intracellular aggregate consisting of oxidized proteins (30-70%) and lipids (20-50%), and can also bind carbohydrates [12]. Accumulation of lipofuscin is a marker of ineffective degradation of damaged proteins and is manifested when inhibiting the activity of lysosomal proteases occurs [12]. It is known that lipofuscin granules accumulate in β-cells of the human pancreas when diabetes [13]. Thus, highly likely gibel carp reacts for high sucrose load similarly to higher vertebrates, which proves in favor of its use as an alternative model for mechanistic study of diabetes.

It is known that hyperglycemic states in higher vertebrates were accompanied by lesions of the nervous system and weakening of cognitive functions. It has been shown that the expression and activity of acetylcholinesterase were enhanced in type 2 diabetes in mice [14]. One of the possible mechanisms of neurotoxicity of carbohydrates is oxidative stress indirectly determined by increasing the level of active forms of oxygen and nitrogen and weakening of the ability to neutralize them [14]. We have proven an inhibition of cholinesterase in LS group, which was in consistent with the development of oxidative stress. Similar response was registered for murine and fish diabetes models [7], [15].

CONCLUSION

Thus, the immersion of gibel carp in sucrose-enrich milieu determined the appearance of signs of oxidative damage and neurotoxicity and/or uncompensated suppression of stress-sensitive systems after acute effect, which was accompanied by an increase in DNA fragmentation, 50% mortality of individuals, glycated hemoglobin as well as body mass and hepatosomatic indices. The determining role in the differentiation of the groups belongs to the lipofuscin and hepatosomatic index according to the results of CART analysis. Substantially that hyperglycemia caused significant upregulation of stress-related proteins metallothioneins in the liver. Metallothioneins seems to play antioxidant functions under hyperglycemia in gibel carp via partial sequestration of reactive oxygen species. The response of the *C. auratus gibelio* to hyperglycemic states was similar to that of the higher vertebrates, which proves in favor of its use as an alternative model for the mechanistic study of diabetes. The appropriate level of carbohydrate in the diet of the farming fish is essential.

ACKNOWLEDGEMENTS

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DIETARY PROTEIN LEVEL AFFECTS COMPENSATORY GROWTH RESPONSE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) UNDER CYCLIC FEEDING

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ABSTRACT

This study was conducted to determine the effects of various levels of dietary protein and short-term starvation periods on compensatory growth, body compositions and organ indices of rainbow trout (*Oncorhynchus mykiss*) during 46 days. Fish were fed ad libitum twice a day or starved as follows: two control groups, one group was feed daily with extruded pellets containing 41% crude protein (D41) and the other control group was feed daily with commercial pellets containing 50% crude protein (D50); two groups fasted for 2 days and then reefed with commercial pellets with 41% (D2/41), respectively 50% crude protein (D2/50) until either their relative feed intake differed by less than 10% from control groups; two groups fasted for 4 days and then reefed with commercial pellets with 41% (D4/41), respectively 50% crude protein (D4/50) until either their relative feed intake differed by less than 10% of control groups. At the end of the trial, the rainbow trout starved for two or four days has shown only partial compensation of growth. This was due to the fact that their weight and length differed significantly ($p<0.05$) from those from the control group ($p<0.05$). Regarding the body composition, it was observed an increase in water content, respectively a decrease of lipid content, fish body trying to limit the loss of body mass during starvation periods. However, it can be concluded that application of short periods of starvation (2 or 4 days) followed by refeeding with a diet containing 41% crude protein can generate lower production costs since we did not register significant differences ($p>0.05$) between the technological indicators.

Keywords: compensatory growth, starvation, protein level, rainbow trout

INTRODUCTION

Compensatory growth or catch-up growth is a phase of accelerated growth, greater than normal or control growth rates associated with adequate refeeding of animals, following a period of weight loss caused by under nutrition [1], [2]. In aquaculture, compensatory growth can be seen as a capacity of fish to recover after the exposure of unfavorable conditions such as low temperature, low oxygen or

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