UDC 612:616-092 doi: https://doi.org/10.15407/ubj91.04.058

Carassius auratus AS A NOVEL MODEL FOR THE HYPERGLYCEMIA STUDY

H. I. FALFUSHYNSKA[1]✉, O. I. HORYN[1], L. L. GNATYSHYNA[1,2], B. B. BUYAK[1], N. I. RUSNAK[1], O. O. FEDORUK[1], O. B. STOLIAR[1]

[1]Ternopil Volodymyr Hnatiuk National Pedagogical University, Ukraine;
✉e-mail: falfushynska@tnpu.edu.ua;
[2]I. Horbachevsky Ternopil State Medical University, Ukraine

Received: 21 August 2018; **Accepted:** 13 December 2018

The aim of the present study was to create a suitable model of the glucose toxicity and elucidate the ability of zinc-binding proteins metallothioneins in the crucian carp Carassius auratus to reflect it. For that, fish was loaded by three waterborne concentrations of glucose (low (5.55 mM, LC), middle (55.5 mM, MC) or high (111 mM, HC)) for 21 days. The level of blood glucose, responses of metallothioneins, oxidative stress, DNA instability in the liver, as well as erythrocytes indices, cholinesterase activity in the brain and morphometric variables were evaluated. An increase in blood glucose levels (up to 3–5 times), glycated hemoglobin (HbA1c, only by the HC, by 55%), methemoglobin (by two times), oxyradicals (16-57%) and TBARS levels (up to 57%), frequency of the micronucleated erythrocytes, DNA fragmentation in hepatocytes, body mass and hepatosomatic indices and a decrease in metallothioneins concentration (40-74%), cholinesterase activity (~70%), total hemoglobin (by 18%) and red blood cells count (only after HC-treatment, by 47%) were detected. The lysosomal membrane stability, evaluated by the neutral red retention time, was affected by all studied concentrations of glucose (decreased by 58%). The most prominent changes were observed after the HC of glucose. CART analysis revealed the significant splitting parameters for studied group differentiation including HbA1c, lysosomal membrane stability and lipid peroxidation. We could consider the crucian carp is a useful model organism to perform DM studies and in the future, this fish model can help in mechanistic investigations and testing therapeutic interventions under glycemic states.

K e y w o r d s: glucose toxicity, crucian carp, metallothioneins, glycated hemoglobin, oxidative stress, lysosomal membrane stability, micronuclei.

The growing prevalence of diabetes has been increasing steadily all over the world at an alarming rate according to irrational nutrition, obesity, sedentary lifestyle and aging [1]. The global diabetes expansion in adults aged 20–79 years was estimated at 8.8% for 2015 and was predicted to rise to 10.4% by 2040 [2]. Developing countries face undiagnosed and uncontrolled hyperglycemia (approximately 50%), which has become the main cause of mortality and morbidity. It has been widely recognized that appropriate animal models that familiarly resemble the process of diet-induced type 2 diabetes are urgently needed for the understanding concepts cover biochemical mechanism implicated early in the development and progression of the disease and how the complications of diabetes develop. Zebrafish *Danio rerio* has similar signs of diabetes mellitus to human, such as glycation of hemoglobin, persistent hyperglycemia and impaired insulin response after water-borne glucose treatment [3-5]. Therefore, *Danio rerio* genetic analysis and morphological changes usually serve as biomarkers for the late consequences of hyperglycemia. However, the study of biochemical responses in this species is complicated due to the small size.

The persistent high glucose concentration in diabetes provokes glycosylation of amino groups of biopolymers and subsequently forms advanced

glycation end-products and pro-inflammatory molecules, oxidative stress and metabolic changes. It has been proven that oxidative stress is closely related to the mechanism of the oppression of insulin synthesis and secretion, which is the main causation of glucose toxicity and promotes cellular damage [6]. High glucose concentrations after in vitro exposure intensified lipid peroxidation, oppression of human erythrocyte enzymes, loss of glutathione and increase in the GSSG/GSH ratio [7]. Generation of ROS in diabetes seems to be directly linked to chronic hyperglycemia and should provoke chromosomal instability. Unfortunately, a little attention has been devoted to evaluating the effects of hyperglycemia and glucose toxicity in fish [8], despite fish farming species are fed with carbohydrate-rich diet as a low-cost food which allows to reaching desired mass within a short time, but provokes hyperglycemia [9].

Metallothioneins (MTs) are the family of multifunctional proteins which could serve for both detoxification and buffering of metal ions and scavenging of a wide range of free radicals including hydroxyl radical and peroxynitrite [10, 11]. Various agents, such as metals, organic xenobiotics, stress hormones and cytokines can affect MT gene expression and synthesis. MTs as the redox-sensitive adapter proteins could release Zn^{2+} from metal-thiolate clusters in response to the pro-oxidant milieu and oxidative damage [12], a status often found in the tissues of diabetic patients, obviously reflecting persistent hyperglycemia [13]. Due to the involving of Zn^{2+} in the insulin functionality, the Zn-binding activity of MTs could be important for the carbohydrate metabolism [14]. It has been expected that patients and diabetic animals with reduced MTs functional activity might be more susceptible to oxidative injury and hyperglycemia. In opposite Zn-induced MT synthesis in murine models prevented chemical streptozotocin-induced diabetes and significantly prevented diabetes-induced cardiomyopathy [15].

With the aim to create a model for the study of early biochemical responses to hyperglycemia in fish and their corrections, we develop a new model system that offers several advantages to study molecular and cellular events when hyperglycemia occurs. Fish crucian carp, *Carassius auratus*, Cyprinidae is a widely distributed European species pertain to farm fish cluster and characterized by unique carbohydrates metabolism. It has high liver glycogen concentrations above 1500 μmol g^{-1} liver and consequently extremely high anoxic-tolerance [16]. It has shown the similarity of response to different stressors to high vertebrate model [17, 18]. Therefore, the aim of this initial study was to investigate the fluctuation patterns of red blood cells, stress-responsive systems and cytotoxicity parameters in crucian carp in response to waterborne glucose treatment. Moreover, we investigated potential differences in the pattern of stress-induced hyperglycemia in a concentration-dependent manner.

Materials and Methods

Chemicals. All chemicals were purchased from Sigma Aldrich (St. Louis, USA) or Merck (Synbias, Kyiv, Ukraine), and were of the analytical or higher grade.

Experimental exposures. The experiments were carried out in December of 2017. Adult crucian carp Carassius auratus (15-18 cm long and 180-240 g weight) were collected from the fish farm which is located in the pristine site (49°49′ N, 25°23′ E). Fish were transported to the laboratory in 60 L cages with aerated native water (dissolved oxygen concentration was 8.7 ± 0.5 mg·l^{-1}). 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). After seven days of preliminary acclimation, fish were randomly distributed into four groups (10 individuals per group). One group was exposed to the aquarium water without any addition and was considered control (C). Other groups were exposed to 5.55 mM (LG), 55 mM (MG) and 111 mM (HG) glucose during 21 days. The selected concentrations were in the range proposed by Capiotti et al. [3] for water-borne glucose exposure of *Danio rerio.*

The water quality parameters were: temperature 17 ± 1 °C, pH 7.3 ± 0.2, $CaCO_3$ 86.8 ± 1.0 mg·l^{-1}, dissolved oxygen concentration 8.7 ± 0.5 mg·l^{-1}, ammonia (NH_3/NH_4^+) and nitrite concentrations below 0.1 mg·l^{-1}. There was no mortality of fish during the performed experiments.

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. For serum preparation, whole blood

was allowed to clot and centrifuged for 10 min at 1500 g. Glucose concentration in blood serum was determined with a diagnostic kit (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/length (kg/m^2). For each biochemical analysis, 8 samples were used. The tissue samples were kept at –40 °C until analyses. Hepatic and brain tissues were homogenized (1:10 w:v) in 0.1 M pH 7.4 phosphate buffer containing 100 mM KCl, 1 mM EDTA and 0.1 mM PMSF to inhibit proteolysis. Homogenization was carried out at 4 °C using 12–15 strokes of a motor-driven Teflon Potter-Elvehjem homogenizer and centrifuged at 6,000 g for 10 min at 4 °C. Protein concentration in the supernatant was measured by the method of Lowry et al. [19] using bovine serum albumin as a standard.

Quantification of stress-related parameters. Metallothioneins (MTs) were determined in the liver. The level of MT-related thiols (MT-SH) was measured after ethanol/chloroform extraction with DTNB as described by Viarengo et al. [20] 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.

Oxyradical 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 [21]. 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) [22]. A molar extinction coefficient of $1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ was used.

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 [23] using Hoescht 33342 dye. To reduce the possible interference with traces of SDS, the assay was carried out in the presence of 0.4 M NaCl, 4 mM sodium cholate, and 0.1 M Tris (pH 9). Probe fluorescence signal was detected by using f-max fluorescence plate-reader (excitation = 360 nm, emission = 450 nm).

Nuclear lesions were determined by the frequency of the erythrocytes with micronuclei (MN). The suspension of erythrocytes, obtained from each fish, was spread on a slide, transferred to a lightproof humidity chamber for 15 min to allow cells to attach. Cells were then fixed in methanol/acetic acid (3/1), stained with 5% Giemsa and mount in Canada balsam. The stained slides were analyzed under the light microscope (Olympus BX40) at a final 1000 × magnification. In total, 2000 cells were scored in each specimen studied [24]. A frequency of micronuclei was assessed and expressed per 1,000 cells.

Stability of the lysosomal membranes was determined by the Neutral Red Retention (NRR) assay [25]. The lysosomal fraction was separated from liver tissue according to Nazar et al. [26]. Lysosomes were monitored under oil immersion at 1,000 x magnification. Observations for NRR were recorded at 5 min intervals. The NRR time was estimated as the time at which 50% of lysosomes released the accumulated neutral red dye (ET50).

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. [27]. Enzyme activity was calculated using a molar extinction coefficient of $13.6 \cdot 103$ $M^{-1} \cdot cm^{-1}$ and standardized to the soluble protein content.

Statistical analysis. For all studied parameters and all experimental treatment groups, the sample size was eight. The data are presented as means ± standard deviation (SD) unless otherwise indicated. Data were tested for normality and homogeneity of variance by using Kolmogorov-Smirnoff and Levene's tests, respectively. Whenever possible, data were normalized by Box-Cox common transforming method. 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 results showed (Fig. 1) that the immersion in all glucose solution tested

(Fig. 1, *A*) promoted up to a 3–5 times increase in blood glucose levels tenuously depending on studied concentration ($F_{(3; 23)}$ = 5.4, $P < 0.05$) when compare to the control group. Also, glucose treatment was able to cause an increase in glycated hemoglobin (HbA1c), but only by the highest acute concentration (by 55%) and methemoglobin with LG group exception (by two times), while the total hemoglobin was decreased by all studied concentrations (by 18%). Red blood cells (RBC) count remained stable only in LG group. At medium concentration loading, RBC increased (by 53%), but at high concentration they dramatically, two-fold, decreased.

Morphometrical indices. So as to know that treatment with glucose was causing overweight and fatty liver disease which are highly prevalent in type 2 diabetes mellitus, we determined the BMI and HIS indices and obtained results demonstrated that the treatment with glucose 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. 2).

Stress-relating responses. The evaluation of the oxidative stress indices in the crucian carp liver revealed quite similar, concentration-dependent pattern in oxyradicals formation and TBARS levels

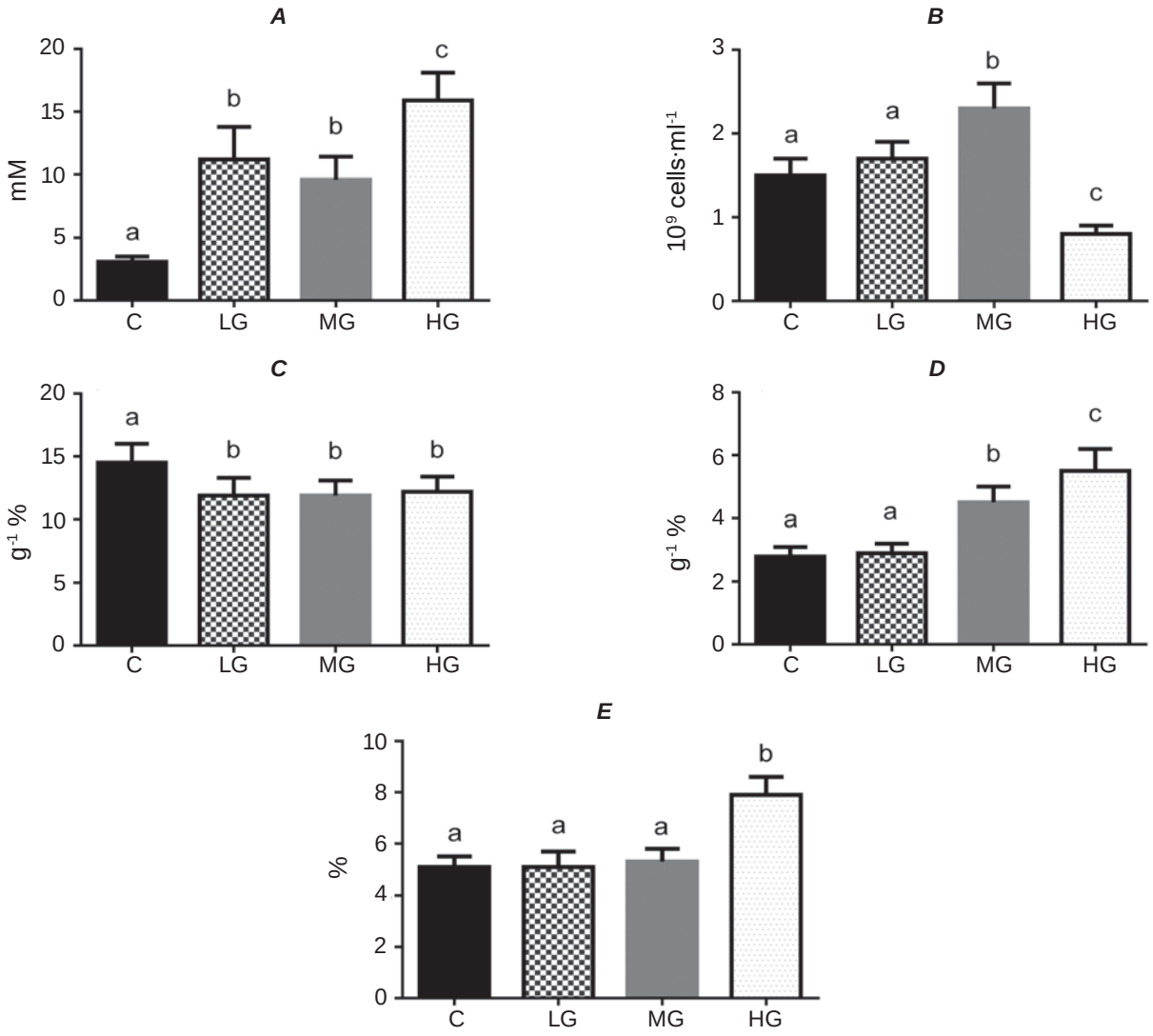

Fig. 1. Effects of experimental exposures to glucose in low (LG, 5.55 mM), medium (MG, 55 mM) and high (HG, 111 mM) concentration on blood parameters of fish Carassius auratus. ***A*** *– glucose concentration,* ***B –*** *red blood cells count;* ***C*** *– hemoglobin concentrations;* ***D*** *– methemoglobin concentrations;* ***E*** *– 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)*

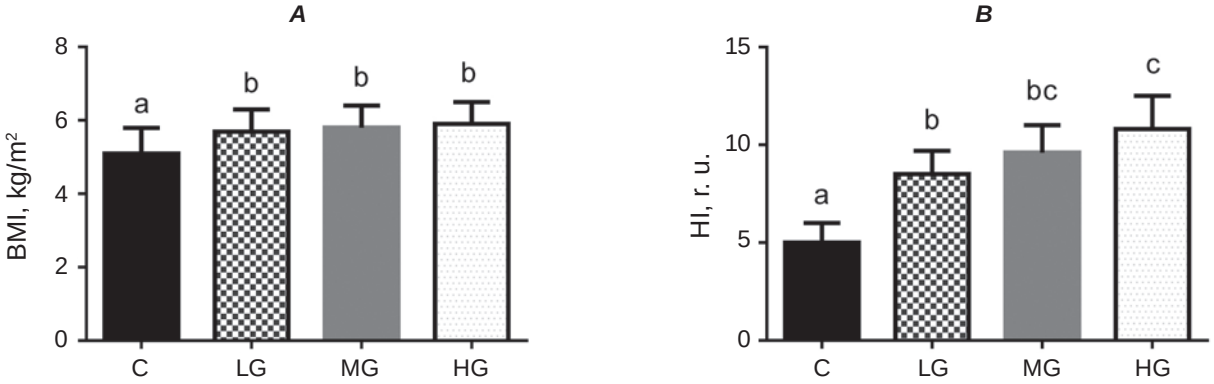

Fig. 2. Effects of experimental exposures to glucose on morphometrical indices of fish Carassius auratus. ***A*** *– body mass index (BMI);* ***B*** *– hepatosomatic index (HI). Data are presented as means ± SD, n = 8*

(Fig. 3). The elevated glucose concentration increased linearly the level of lipid peroxidation, as well as oxyradical level with one exception, namely the lower TBARS concentration in the LG group.

The MTs concentration in the crucian carp liver was decreased in each treated group, particularly by lower glucose concentration (by 74%).

Toxicity of exposures. The animals loaded with glucose had a higher frequency of the erythrocytes with micronuclei and higher DNA fragmentation in hepatocytes when compared with control (Fig. 4), but no dependence on the concentration was disclosed. The lysosomal membrane stability was also affected by all studied concentrations of glucose (decreased by 58%).

Besides, the loading by glucose led to the ChE activity inhibition (Fig. 4), that was not related to concentration ($F = 182.1$, $P < 0.001$).

Data integration. The PCA analysis with the NIPALS algorithm of the biological traits reveals concentration-specific distinctions in the biomarker response to glucose (Fig. 5, *A*). Principal component analysis showed that 72.5% of the variation in the studied traits was explained by the first two principal components (PC 1 and 2) (Fig. 5, *A*). C and LG groups had similar loadings on Factor 1 and 2 opposite to the loadings of the two heavily glucose treated groups, namely MG- and HG. Control group reflected by MTs, NRR, Hb and ChE activity, and they all were explained by PC1 and had high loadings (≥0.6), the LG group was conjoint only to RBC count. No particular parameter was associated with the MG group, whereas HG group was associated with a set of markers, among them DNA fragmentation, methemoglobin, HbA1c and TBARS.

CART analysis utilized to identify the primer splitting parameter for each group resulted in a tree with three splits and four terminal nodes (Fig. 5, *B*). The first branch on the tree distinguished untreated (C group) and glucose-treated (HG) animals based on the higher level of the HbA1c in this group. On the next separating stage, the splitting indices were

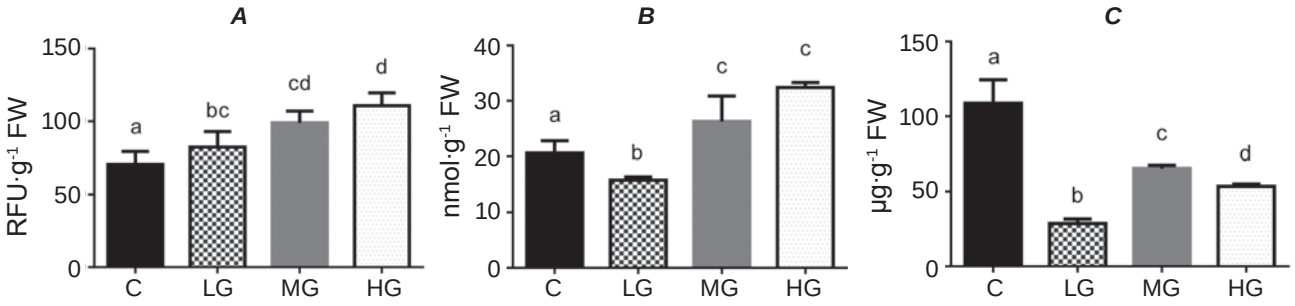

Fig. 3. Effects of experimental exposure to glucose on oxidative stress parameters and metallothioneins concentrations in the liver of fish Carassius auratus. ***A*** *– oxyradicals formation;* ***B*** *– TBA-reactive substance concentrations;* ***C*** *– metallothioneins concentrations. Data are presented as means ± SD, n = 8. RFU·g⁻¹ FW – relative fluorescence units/fresh weight, g*

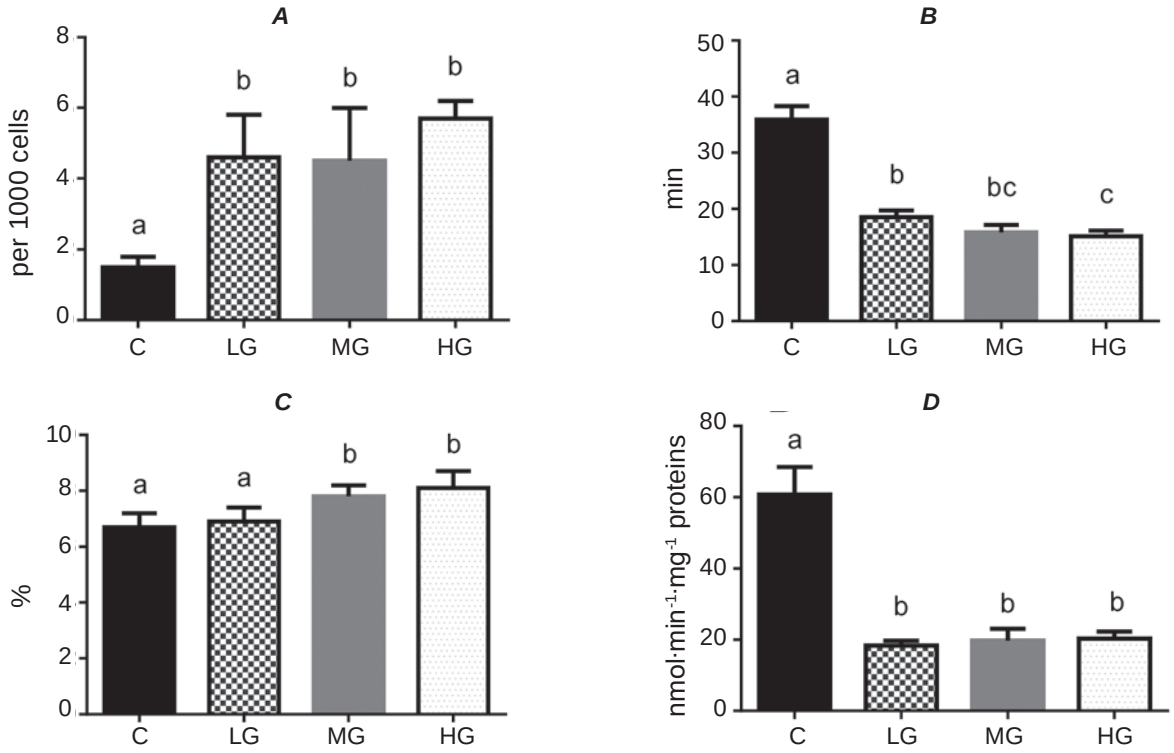

*Fig. 4. Cytotoxicity markers in the blood (**A**), liver (**B**, **C**) and brain (**D**) of crucian carp, exposed to glucose in different concentrations. **A** – micronuclei frequency, **B** – neutral red retention time, **C** – DNA strand break, **D** – cholinesterase activity. Data are presented as means ± SD, n = 8*

NRR and TBARS for C/LG and MG/LG groups correspondingly.

Our study has revealed some remarkable peculiarities of crucian carp response to water-borne glucose treatment. Unfortunately, not much is known about glucose tolerance in low vertebrate, and therefore, we could not collate our results with a similar one in fish. There is the reason why we have emphasized the information concerning high vertebrates and human.

We have justified the association between an RBC count and impairment of glucose tolerance in crucian carp. The RBCs are unambiguously determined as the first cells which are tackled with excess amount of glucose and affected in diabetes, before progression of other diabetic complications [28, 29]. The opposite responses of RBC count in HG and MG groups could be explained by different grade of stress response to excess amount of glucose. Obviously, at low and medium glucose concentration, fish switch adaptive mechanisms on and try to tackle deleterious effects of glucose on RBC (among them increased membrane rigidity and decreased cellular deformability) and possible tissue hypoxia [28, 29] and elevate the RBC count. But after exceed of adaptation limit, increased glycosylation of RBC membrane proteins and decreased mean corpuscular hemoglobin concentration are appeared [30, 31]. Some pieces of evidence support this hypothesis. It has been shown that red cell count is increased in the diabetes precursor states of IGT/IFG. Also, there is a clear relation between hematocrit and blood viscosity and subsequent development of type 2 diabetes in a population-based, middle-aged cohort including men and women [32]. But severe diabetes both in animal and human are accompanied by the lower level of RBC [33] and anaemia [34].

Levels of glycated hemoglobin have been reported in a wide range of high vertebrate animals [35, 36], and recently in some low vertebrate, among them sharks, some of Cyprinidae fish and frog. HbA1c is a valuable indicator of long term glycaemia in diabetes mellitus and is considered to be the "gold standard" for the evaluation of mean glycemic control in human. However, some mismatches between HbA1c and mean blood glucose as well as red blood indices have been noticed [37] and they should evoke from alterations in RBC survival. In

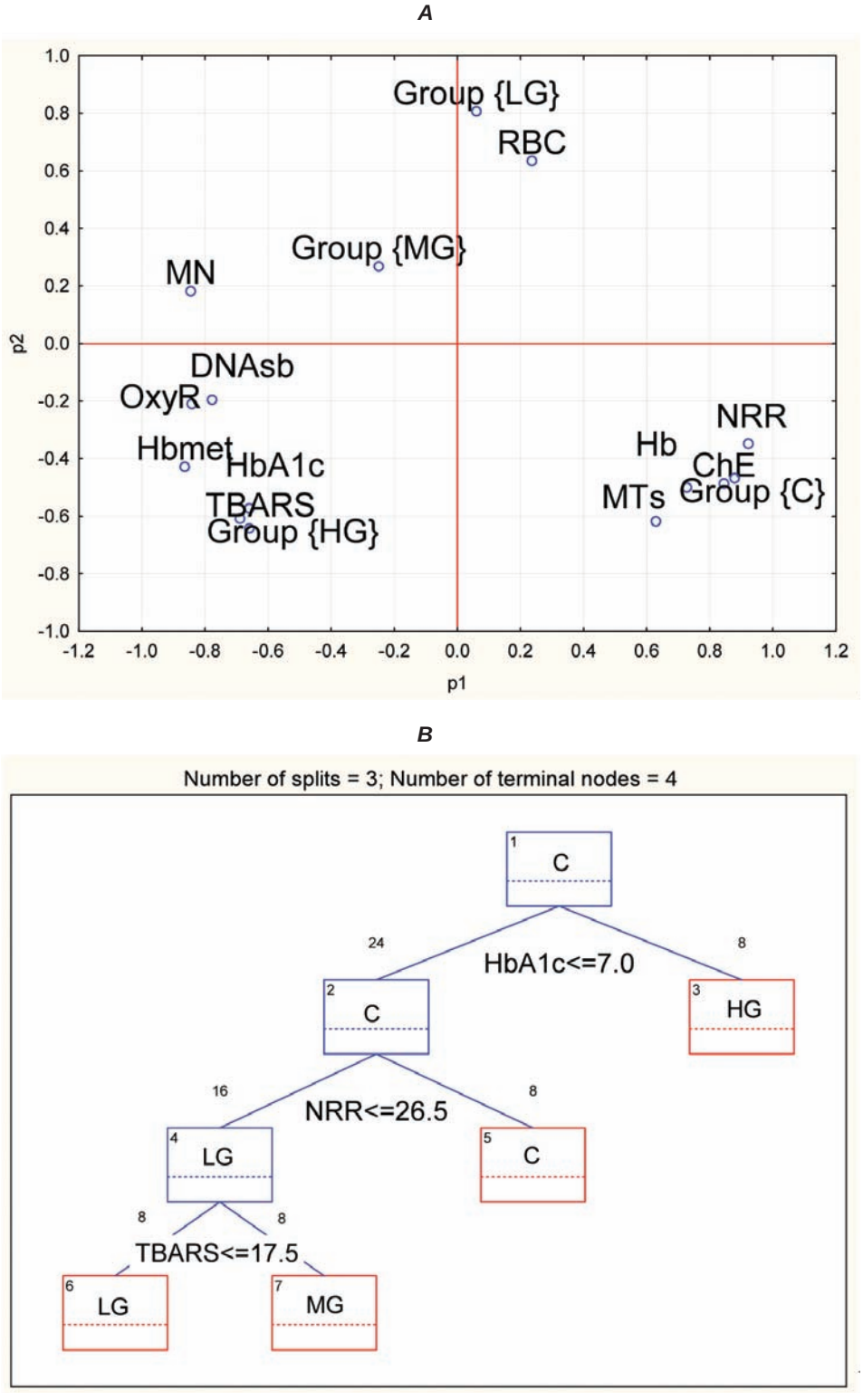

Fig. 5. Integrative analysis of the studied biological traits in crucian carp Carassius auratus from different treatment groups. ***A*** *– results of the principal component analysis with NIPALs algorithm;* ***B*** *– results of the classification and regression tree analysis*

the present study, water-borne glucose exposure significantly increased the HbA1c in crucian carp blood only after the highest concentration. Meanwhile, we have had evidence of hyperglycemia and signs of impaired glucose tolerance in LG and particularly in MG group which related to lysosomal membrane destabilization and micronuclei appearance, which were in the same range with HG group. Obviously, HbA1c is not suitable for the early-warning diagnosis of hyperglycemia in crucian carp, but it is valuable for justifying the severe hyperglycemia and glucotoxicity. HbA1c is the very parameter which has split MG and HG groups in CART analysis (Fig. 5).

Lysosomes are incorporated in numerous actions, including autophagy, cell death, endocytosis, phagocytosis etc. Many of these activities are governed by the function of acid hydrolase enzymes within the lysosome that can degrade biopolymers, lipids or cellular debris. Hexosaminidase A is a lysosomal enzyme that converts GM2 ganglioside to G3M by removing an N-acetyl- glucosamine residue and takes part in the regulation of insulin sensitivity [38]. There is evidence that lysosomal functions are altered in diabetes mellitus in high vertebrate and human [39]. It has been disclosed differential expression in lysosomal-related genes in the hippocampi of a db/db mouse model of type 2 diabetes [40]. Also the expression of aspartyl lysosomal protease namely cathepsin D increased in serum and leucocytes of type 2 diabetes patients [39] and in both the cortex and hippocampus in T2D db/db mice [41]. Our findings, related to the remarkable increase in lysosomal membrane permeability after induced hyperglycemia, are consistent with reported above records for human and high vertebrate, and as far as we know, it is the first attempt at Cyprinidae fish.

Metallothioneins (MTs) are a group of small intracellular metal-binding and cysteine-enriched proteins. Functionally, MTs predominantly act as the metal-buffering and metal-detoxification proteins, but also they should scavenge reactive oxygen species and protect cells and tissues from oxidative damage [10, 11]. They are transcriptionally induced by various transition metals, steroid hormones, cytokines, interleukins, prooxidants according to three responsible elements in the promoter of the MT genes namely MRE, GRE and ARE [10]. It has recently been noted that the MTs are required for normal physiological function of pancreatic β-cells [15]. Moreover, MTs are capable of preventing diabetes development in a wide range of diabetic models, both for type 1 and type 2 diabetes [15] and, as a potent antioxidant, MTs have been proven to protect humans and animals against diabetic complications, among them diabetic nephropathy and cardiomyopathy and subsequent other pathogenesis [42].

Our results justified that the MTs level has decreased in all studied groups, consistent with an increase in blood glucose ($r = –0.75$, $P < 0.001$) and reactive oxygen species ($r = –0.40$, $P < 0.05$). The bivariate relationship is expressed by the regression equation: MT = 73.5 – 6.75×Glucose* + 0.63×Oxyradicals* ($R^2 = 0.6$, $F_{(2,29)} = 24.27$, $P < 0.000$), *the indicator makes a plausible contribution to the statistical model. Thus, an increase in the content of MTs at higher glucose concentrations compared with low concentrations can be regarded as overlaying the effects of hyperglycemia and enhancing peroxidation processes in the cell. Our results concerning hepatic MTs down-regulation in crucian carp after 21 days of water-borne glucose treatment, are in a good correlation with results presented by Bellomo et al. [43]. They also have shown that Mt-1 and Mt-2 mRNA in mouse pancreatic β-cells significantly decreased after 16.7 mM glucose, but the mechanism remained concealed [43]. The possible mechanisms of MTs down-regulation in crucian carp will be further elucidated in future studies.

High glucose is known to cause oxidative stress [44]. It was confirmed in our model study by the evaluation of oxyradicals level and TBARS. Meanwhile, TBARS has been determined according to CART (Fig. 5) as a powerful parameter to distinguish low- and media-glucose treatment of crucian carp. Just as some findings had proved that MTs can attenuate oxidative stress which is arising in hyperglycemia, our results pointed out that crucian carp MTs were unfit in the present model due to significant decreasing. In sum, all these changes together resulted in the cytotoxicity.

Micronuclei (MN) assay, as a biomarker of effects, is very useful for evaluating the effects of a wide range of mutagenic and carcinogenic factors, among them toxic chemicals (both inorganic and organic), ionizing radiation, non-point environmental pollution and dietary on chromosomal stability. This assay has been successfully applied either for vertebrate and invertebrate animals or human [17, 18, 45-47]. MN represent fast genetic damage arising from both aneugenic and clastogenic mechanisms [45]. It is now well-established that MN mainly originates from acentric chromosome fragments, acentric chro-

matid fragments or whole chromosomes that fail to be included in the daughter nuclei [45]. It was shown that in type 2 diabetes patients, the frequency of MN in peripheral blood lymphocytes increased significantly with the duration of diabetes [48] and it was in good agreement with elevated glycosylation of haemoglobin [49]. Our outcomes indicated that after water-borne glucose treatment the frequency of MN in crucian carp erythrocytes increased significantly, but wasn't dependent on the applied concentration. This could testify that genomic instability in crucian carp caused by hyperglycemia is not affected by the worsening of the pathology. Obviously, the lower repair ability of DNA double-strand breaks, which we have seen in the crucian carp hepatocytes, leads to chromosome aberrations or deletion mutations through unrepaired and misrepaired DNA double-strand breaks, and should be linked to a higher yield of micronuclei and the severe injury of animals. Moreover, parameters of oxidative injury conjoin with micronuclei formation and DNA fragmentation in PCA (Fig. 5) which proves the oxidative nature of genomic instability in crucian carp after acute glucose treatment.

It has been established that diabetes mellitus and hyperglycemia in high vertebrate affect the neural system and can impair cognitive functions. Some explorations have shown the mutual relation between hyperglycaemia and malfunction on neurotransmission systems, among them cholinergic [50]. It has been proven an increase in acetylcholinesterase (AChE) mRNA levels and AChE activity in murine and Danio rerio fish diabetes models [4, 51]. One putative mechanism for glucose neurotoxicity is oxidative stress, which is step forth by glucose through a combination of free radical expression and diminished free-radical scavenging [52]. Cell damage from oxidative and nitrosative stress triggers DNA lesions, which then activates the nuclear enzyme poly(ADPribose) polymerase. This could give rise to cell dysfunction and death, as has been shown in Schwann cells [52]. Nevertheless, we have shown contradicting ChE response pattern to reported in murine and zebra fish, ChE inhibition in glucose-treated crucian carp, as the well-known sign of neurotoxicity, was in a good correlation with oxidative stress and genotoxicity according to PCA (Fig. 5).

Summarizing, in this study we found concentration-specific changes in crucian carp blood glucose, morphometrical indices and molecular stress-response systems due to exposure to glucose. We infer from our study that in general the glucose treatment impaired glucose tolerance in cyprinidae fish and was able to affect their red blood cells, provoke oxidative stress, down-regulate metallothioneins and cause cyto- and genotoxicity. The high glucose toxicity additionally related to increasing of hemoglobin glycation (HbA1c) and erythropenia. In conclusion, our study provides valuable data about the response of alternative model of diabetes mellitus namely crucian carp to long-term elevated glucose levels. Despite the distinction from reported human type 2 diabetes model responses of metallothioneins and cholinesterase, these sensitive indices merit deeper investigation as probable markers of glucose overloading in fish. Crucian carp is suggested to be a valuable model for future study to target and test therapeutic interventions.

Acknowledgements. This work has been supported by the Ministry of Education and Science of Ukraine (Projects # 133B) and Alexander von Humboldt Foundation.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

Carassius auratus ЯК НОВА МОДЕЛЬ ДЛЯ ДОСЛІДЖЕННЯ ГІПЕРГЛІКЕМІЧНИХ СТАНІВ

Г. І. Фальфушинська[1]✉, О. І. Горин[1], Л. Л. Гнатишина[1,2], Б. Б. Буяк[1], Н. І. Руснак[1], О. О. Федорук[1], О. Б. Столяр[1]

[1]Тернопільський національний педагогічний університет імені Володимира Гнатюка, Україна;
✉e-mail: falfushynska@tnpu.edu.ua;
[2]Тернопільський державний медичний університет імені І. Я. Горбачевського, Україна

Метою роботи було створення альтернативної моделі для дослідження гіперглікемічних станів карася (*Carassius auratus)* та з'ясування можливості використання протеїнів металотіонеїнів як показників цієї моделі. Вивчали вплив на *Carassius auratus* трьох концентрацій глюкози: низька (5,55 мМ, НК), середня (55,5 мМ, СК) або висока (111 мМ, ВК) протягом 21 доби. Визначали рівень глюкози крові, стан металотіонеїнів, показники окисного стресу, фрагментації ДНК в печінці, а також показники еритроцитів,

холінестерази в мозку та морфометричні показники. Показано, що під час експерименту в риб збільшувався рівень глюкози в крові (у 3-5 разів), глікозильованого гемоглобіну (HbA1c, тільки за впливу ВК, на 55%), метгемоглобіну (у два рази), оксирадикалів (16-57%) і рівень ТБК-АП (до 57%), частота присутності мікроядер в еритроцитах, фрагментація ДНК у гепатоцитах, індекс маси тіла та гепатосоматичний індекс, а також зменшувалась концентрація металотіонеїнів (40-74%), холінестеразної активності (~70%), загального гемоглобіну (на 18%) і кількості еритроцитів (тільки за впливу ВК, на 47%). Стабільність лізосомальних мембран зменшувалася у всіх експериментальних групах (~58%). Найпомітніші зміни спостерігалися за впливу високої концентрації глюкози. Побудова класифікаційного дерева (CART аналіз) дозволила виявити набір найвагоміших показників для диференціації груп, до якого належать HbA1c, стабільність лізосомальних мембран та пероксидне окислення ліпідів. Зроблено припущення, що карась може бути зручною моделлю для дослідження діабету і тестування препаратів за глікемічних станів.

Ключові слова: токсичність глюкози, карась, металотіонеїни, глікозильований гемоглобін, окисний стрес, стабільність лізосомальних мембран, мікроядра.

References

1. Olokoba AB, Obateru OA, Olokoba LB. Type 2 diabetes mellitus: a review of current trends. *Oman Med J*. 2012; 27(4): 269-273.
2. Ogurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, Cavan D, Shaw JE, Makaroff LE. IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes Res Clin Pract*. 2017; 128: 40-50.
3. Capiotti KM, Antonioli R Jr, Kist LW, Bogo MR, Bonan CD, Da Silva RS. Persistent impaired glucose metabolism in a zebrafish hyperglycemia model. *Comp Biochem Physiol B Biochem Mol Biol*. 2014; 171: 58-65.
4. Capiotti KM, De Moraes DA, Menezes FP, Kist LW, Bogo MR, Da Silva RS. Hyperglycemia induces memory impairment linked to increased acetylcholinesterase activity in zebrafish (*Danio rerio*). *Behav Brain Res.* 2014; 274: 319-325.
5. Carnovali M, Luzi L, Banfi G, Mariotti M. Chronic hyperglycemia affects bone metabolism in adult zebrafish scale model. *Endocrine*. 2016; 54(3): 808-817.
6. Kawahito S, Kitahata H, Oshita S. Problems associated with glucose toxicity: role of hyperglycemia-induced oxidative stress. *World J Gastroenterol*. 2009; 15(33): 4137-4142.
7. Viskupicova J, Blaskovic D, Galiniak S, Soszyński M, Bartosz G, Horakova L, Sadowska-Bartosz I. Effect of high glucose concentrations on human erythrocytes *in vitro*. *Redox Biol*. 2015; 5: 381-387.
8. Palmer TN, Ryman BE. Studies on oral glucose intolerance in fish. *J Fish Biol*. 1972; 4(2): 311-319.
9. Kamalam BS, Medale F, Panserat S. Utilisation of dietary carbohydrates in farmed fishes: New insights on influencing factors, biological limitations and future strategies. *Aquaculture.* 2017; 467(20): 3-27.
10. Takahashi S. Positive and negative regulators of the metallothionein gene (review). *Mol Med Rep*. 2015; 12(1): 795-799.
11. Krężel A, Maret W. The Functions of metamorphic metallothioneins in zinc and copper metabolism. *Int J Mol Sci*. 2017;18(6). pii: E1237.
12. Bellomo E, Hogstrand C, Maret W. Redox and zinc signalling pathways converging on protein tyrosine phosphatases. *Free Radic Biol Med*. 2014; 75(Suppl 1): S9.
13. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science*. 2005; 307(5708): 384-387.
14. Chabosseau P, Rutter GA. Zinc and diabetes. *Arch Biochem Biophys*. 2016; 611: 79-85.
15. Cai L. Metallothionein as an adaptive protein prevents diabetes and its toxicity. *Nonlinearity Biol Toxicol Med*. 2004; 2(2): 89-103.
16. Moon TW, Foster GD. Tissue carbohydrate metabolism, gluconeogenesis and hormonal and environmental influences. *Biochem Mol Biol Fish*. 1995; 4: 65-100.
17. Falfushynska HI, Gnatyshyna LL, Stoliar OB, Nam YK. Various responses to copper and manganese exposure of *Carassius auratus* gibelio from two populations. *Comp Biochem Physiol C Toxicol Pharmacol*. 2011; 154(3): 242-253.
18. Falfushynska H, Gnatyshyna L, Turta O, Stoliar O, Mitina N, Zaichenko A, Stoika R. Responses of

hepatic metallothioneins and apoptotic activity in *Carassius auratus* gibelio witness a release of cobalt and zinc from waterborne nanoscale composites. *Comp Biochem Physiol C Toxicol Pharmacol*. 2014; 160: 66-74.

19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193(1): 265-275.
20. Viarengo A, Ponzano E, Dondero F, Fabbri R. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. *Mar Environ Res.* 1997; 44(1): 69-84.
21. Viarengo A, Burlando B, Cavaletto M, Marchi B, Ponzano E, Blasco J. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. *Am J Physiol*. 1999; 277(6): R1612-R1619.
22. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979; 95(2): 351-358.
23. Olive PL. DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. *Environ Mol Mutagen*. 1988; 11(4): 487-495.
24. Falfushynska HI, Gnatyshyna LL, Stoliar OB. Population-related molecular responses on the effect of pesticides in Carassius auratus gibelio. *Comp Biochem Physiol C Toxicol Pharmacol*. 2012; 155(2): 396-406.
25. Moore MN, Lowe D, Köhler A. Biological effects of contaminants: measurement of lysosomal membrane stability. *ICES Tech. Mar. Environ. Sci*. 2004; 36: 31.
26. Nazar ML, Rodrigues LEA, Nascimento I. The lysosomal stability as a biomarker for the determination of pollution in aquatic environments. *Braz Arch Biol Technol*. 2008; 51(5): 1071-1077.
27. Ellman GL, Courtney KD, Andres VJr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*. 1961; 7(2): 88-95.
28. Graham JJ, Ryall RG, Wise PH. Glycosylated haemoglobin and relative polycythaemia in diabetes mellitus. *Diabetologia*. 1980; 18(3): 205-207.
29. Agrawal R, Bhatnagar R, Smart T, Richards C, Pavesio CE, Shima DT, Jones P. Assessment of red blood cell deformability by optical tweezers in diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2015; 56(7): 5183.
30. Stookey JD, Burg M, Sellmeyer DE, Greenleaf JE, Arieff A, Van Hove L, Gardner C, King JC. A proposed method for assessing plasma hypertonicity *in vivo*. *Eur J Clin Nutr*. 2007; 61(1): 143-146.
31. Oyedemi SO, Adewusi EA, Aiyegoro OA, Akinpelu DA. Antidiabetic and haematological effect of aqueous extract of stem bark of *Afzelia africana* (Smith) on streptozotocin-induced diabetic Wistar rats. *Asian Pac J Trop Biomed*. 2011; 1(5): 353-358.
32. Tamariz LJ, Young JH, Pankow JS, Yeh HC, Schmidt MI, Astor B, Brancati FL. Blood viscosity and hematocrit as risk factors for type 2 diabetes mellitus: the atherosclerosis risk in communities (ARIC) study. *Am J Epidemiol*. 2008; 168(10): 1153-1160.
33. Mahmoud AM. Hematological alterations in diabetic rats - Role of adipocytokines and effect of citrus flavonoids. *EXCLI J*. 2013; 12: 647-657.
34. Bosman DR, Winkler AS, Marsden JT, Macdougall IC, Watkins PJ. Anemia with erythropoietin deficiency occurs early in diabetic nephropathy. *Diabetes Care*. 2001; 24(3): 495-499.
35. Richter NA. Percentage of glycosylated hemoglobin and serum concentration of glucose in the blood of Japanese macaques and in three exotic ruminant species. *Am J Vet Res*. 1986; 47(8): 1783-1784.
36. Alayash AI, el-Hassan AM, Omer R, Bonaventura J. Glycosylated haemoglobin: an indicator of long-term blood glucose in domestic sheep and goats. *Comp Biochem Physiol A Comp Physiol*. 1988; 90(2): 229-231.
37. Unnikrishnan R, Anjana RM, Jayashri R, Mohan V. Unexpectedly low HbA1c levels in two diabetes patients following dapsone use. *Indian J Endocrinol Metab*. 2012; 16(4): 658-659.
38. Yamashita T, Hashiramoto A, Haluzik M, Mizukami H, Beck S, Norton A, Kono M, Tsuji S, Daniotti JL, Werth N, Sandhoff R, Sandhoff K, Proia RL. Enhanced insulin sensitivity in mice lacking ganglioside GM3. *Proc Natl Acad Sci USA*. 2003; 100(6): 3445-3449.
39. Tiribuzi R, Orlacchio A, Crispoltoni L, Maiotti M, Zampolini M, De Angeliz M, Mecocci P, Cecchetti R, Bernardi G, Datti A, Martino S,

Orlacchio A. Lysosomal β-galactosidase and β-hexosaminidase activities correlate with clinical stages of dementia associated with Alzheimer's disease and type 2 diabetes mellitus. *J Alzheimers Dis.* 2011; 24(4): 785-797.
40. Sims-Robinson C, Zhao S, Hur J, Feldman EL. Central nervous system endoplasmic reticulum stress in a murine model of type 2 diabetes. *Diabetologia*. 2012; 55(8): 2276-2284.
41. Sims-Robinson C, Bakeman A, Rosko A, Glasser R, Feldman EL. The role of oxidized cholesterol in diabetes-induced lysosomal dysfunction in the brain. *Mol Neurobiol*. 2016; 53(4): 2287-2296.
42. Tachibana H, Ogawa D, Sogawa N, Asanuma M, Miyazaki I, Terami N, Hatanaka T, Horiguchi CS, Nakatsuka A, Eguchi J, Wada J, Yamada H, Takei K, Makino H. Metallothionein deficiency exacerbates diabetic nephropathy in streptozotocin-induced diabetic mice. *Am J Physiol Renal Physiol*. 2014; 306(1): F105-F115.
43. Bellomo EA, Meur G, Rutter GA. Glucose regulates free cytosolic Zn^{2+} concentration, Slc39 (ZiP), and metallothionein gene expression in primary pancreatic islet β-cells. *J Biol Chem*. 2011; 286(29): 25778-25789.
44. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res.* 2010; 107(9): 1058-1070.
45. Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis.* 2011; 26(1): 125-132.
46. Falfushynska HI, Gnatyshyna LL, Stoliar OB. In situ exposure history modulates the molecular responses to carbamate fungicide Tattoo in bivalve mollusk. *Ecotoxicology.* 2013; 22(3): 433-445.
47. Falfushynska H, Gnatyshyna L, Horyn O, Shulgai A, Stoliar O. A calcium channel blocker nifedipine distorts the effects of nano-zinc oxide on metal metabolism in the marsh frog *Pelophylax ridibundus. Saudi J Biol Sci*. 2017; 4: 67-74.
48. Salimi M, Broumand B, Mozdarani H. Association of elevated frequency of micronuclei in peripheral blood lymphocytes of type 2 diabetes patients with nephropathy complications. *Mutagenesis.* 2016; 31(6): 627-633.
49. Shettigar SK, Shailaja C, Kulkarni RK. Elevated micronuclei frequency in type 2 diabetes with high glycosylated hemoglobin. *Diabetes Res Clin Pract*. 2012; 95(2): 246-250.
50. Sherin A, Anu J, Peeyush KT, Smijin S, Anitha M, Roshni BT, Paulose CS. Cholinergic and GABAergic receptor functional deficit in the hippocampus of insulin-induced hypoglycemic and streptozotocin-induced diabetic rats. *Neuroscience*. 2012; 202: 69-76.
51. Kuhad A, Chopra K. Lycopene ameliorates thermal hyperalgesia and cold allodynia in STZ-induced diabetic rat. *Indian J Exp Biol*. 2008; 46(2): 108-111.
52. Tomlinson DR, Gardiner NJ. Glucose neurotoxicity. *Nat Rev Neurosci*. 2008; 9(1): 36-45.