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EXPERIMENTAL STUDY

Goldfish as a model for studying the effect of hypernatremia on blood plasma lipoproteins

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ABSTRACT

AIM: To evaluate the effect of hypernatremia on the organization of blood plasma high density lipoproteins (HDL) in goldfish; to compare the state of hypernatremia in fish and humans; to assess the possible risks and consequences of the effect of hypernatremia on human plasma lipoproteins.

METHODS: The fish were acclimated for 20 days at a critical salinity of 11.5 g/L; after that the salt water was gradually "desalinated". The concentration of Na^+ and the content of total water were determined in tissues, cells, and body fluids. The HDL organization was assessed by the number of apolipoprotein molecules per particle. The methods of flame spectrophotometry, electrophoresis and MALDI were used.

RESULTS: In fresh water, the state of normonatremia was maintained in the fish body; at critical water salinity, the state of hypernatremia. Against the background of hypernatremia, the initial signs of muscle and erythrocyte dehydration appeared in fish, the total water content in the plasma did not change, and HDL disintegrated into small particles, which, upon restoration of normonatremia, were combined into the original large forms.

CONCLUSION: In goldfish at the state of normonatremia, large forms of HDL are stable while at the state of hypernatremia, the small forms of HDL are stable. Under conditions of a hypertonic environment and plasma hypernatremia, the breakdown of HDL prevents the loss of water from the fish organism and reduces the threat of their dehydration. Human hypernatremia is characterized by plasma sodium levels comparable to that in goldfish, however accompanied by life-threatening metabolic changes. The results of this study may be useful for assessing the risks of HDL breakdown at hypernatremia and for the development of protocols for the treatment of pathological conditions in humans *(Fig. 4, Ref. 45)*. Text in PDF *www.elis.sk*

KEY WORDS: goldfish, blood plasma high density lipoproteins, hypernatremia.

Introduction

The sodium concentration in human plasma is normally maintained in the range 130–145 mmol/kg (1, 2, 3, 4, 5). In case of violations of ionic homeostasis, which are often found in clinical practice, the sodium content in the plasma changes beyond the normal range and manifests itself in the form of dysnatremia (6), often ending in death (7). With hyponatremia, the concentration of sodium in the plasma drops below 130 mmol/kg, while with hypernatremia it rises above 145 mmol/kg, reaching ~ 200 mmol/kg (6, 8, 9, 10, 11, 12, 13).

Proteins and, in particular, protein complexes may be the "targets" of increased Na^+ concentration. Their response to an increase in sodium levels may be manifested in a change in the profile of protein-protein interactions and the balance of dissociation-association processes. High-density lipoproteins (HDL) are also organized by the type of complexes – from proteins and lipids associated with each other. HDL serves as factors of complex defence of the organism (14) and is present in all vertebrates (15, 16). Proteins account for about 80% of the HDL surface, and apolipoproteins dominate; the "minor" fraction is represented by ~ 100 proteins, the majority of which are not associated with lipid transport (14, 17). Hypernatremia can provoke the breakdown of blood plasma HDL and a change in the activity of proteins in the composition of the particles.

The range of plasma sodium concentration in freshwater teleosts is comparable to that of mammals (18, 19). In the conditions of extremely high critical water salinity (CS) tolerated by fish, the sodium concentration in their plasma increases to ~190 – 206 mmol/kg (10, 18, 20). This level is comparable to the level of Na^+ in human plasma at the state of hypernatremia. The coincidence of the ranges of variation of sodium concentration in the plasma of humans and fish, on the one hand, and the presence in their plasma

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of potential "targets" of high sodium concentration in the form of HDL, on the other hand, give grounds for using fish as models for studying the effect of Na^+ on blood plasma HDL organization.

The goal of the present paper is to analyse the effect of hypernatremia in goldfish *Carassius auratus* on the organization and pattern of remodelling (reversible/irreversible) of blood plasma HDL.

Materials and methods

The object and experiment design

The object of the study were the female goldfish *Carassius auratus* L. (Cypriniformes; Cyprinidae), caught in September in the Rybinsk Reservoir. For the experiment, 40 females with gonads at the III stage of maturity and body length of 15–22 cm were selected. The fish were placed in two aquaria (aq) with fresh water (FW), at a density of 10 fish per 100 litres of water. The water temperature was maintained at 10.0–11.0 °C. All the aquaria were artificially aerated. During the experiments, the fish were not fed. A control group kept in fresh water ("FW" group, 20 fish) was placed in a freshwater aquarium (aq1). The remaining fish were placed into the aq2 with FW; and three days later, NaCl was added at a rate of 1.0 grams per kilogram of water per day. After 12 days in aq2, a critical salinity (CS) level of 11.5 g/kg was reached. The fish were kept in these conditions for 20 days ("CS" fish group). After that, biological materials were sampled from 10 specimens, in parallel with the biological materials from the control group (sampling 1). The rest of the fish (aq2) were subjected for 12 days to gradual desalination of water by the replacement of salt water with portions of FW. Upon reaching the complete "desalination" of water, the fish were kept in FW for another 14 days ("FW_{rev}" fish group); then the biomaterials were sampled in parallel with the control group (sampling 2).

Analysed biological samples

The following samples were analysed: Body fluids – blood plasma, cerebrospinal fluid (CSF); tissues – liver, skeletal muscles, brain; cells - erythrocytes.

Methods

Blood was collected from the caudal vein. To obtain plasma, individual blood samples were collected in tubes with a 1 % heparinoid solution and centrifuged at room temperature for 15 minutes at 1400 g.

The sampling protocol for the tissues of liver, skeletal muscles, brain, erythrocytes and CSF was described in detail in (21).

The content of total water (*tw*) in samples was determined according to protocol described in (22).

The concentrations of Na^+ (mmol/kg) in biological samples and in aquarium water were measured using Flapho-4 flame spectrophotometer (Carl Zeiss, Jena, Germany). Then, the difference between these concentrations was calculated (ΔNa^+).

The protein concentration in plasma was measured using the microbiuret test (23).

Disk-electrophoresis (disk-E) in PAG, 2D-E in 5–40 % PAG and 12.5 % SDS-PAG (24), were used to separate blood plasma proteins. The polymeric forms of human serum albumin (67, 134, 201, 268, 335 kDa), ovalbumin (45, 90, 135 kDa) and myoglobin (16, 32, 64 kDa) (Serva, Germany) and PageRulerTM Prestained Protein Ladder Plus (11, 17, 28, 36, 55, 72, 95, 130, 250 kDa) (Fermentas, USA) were used as markers of molecular weight (Mr).

Mr values for apolipoproteins (ApoA-I, Apo-14) and HDL; ApoA-I/Apo-14 *molar ratio*; the number of Apo molecules in 1 HDL particle –were calculated using ONE –Dscan, Ver 1.31 (Scan-analytic Inc.) software package.

For protein identification, the MALDI mass spectrometry was used. The mass spectra (*ms*) of trypsin-digested proteins were obtained using a MALDI-TOF/TOF mass-spectrometer (UltrafleXtreme Bruker Daltonics, Germany). To analyse the *ms*, FlexAnalysis 3.3 software (Bruker Daltonics, Germany) was used. The proteins were identified using the MASCOT search software ("peptide fingerprint" option; www.matrixscience.com). The search was carried out in the NCBI and/or EST vertebrates DB. Candidate proteins were considered to be reliably identified at *score* > 83 ($p < 0.05$).

Results are presented as the means and the standard error of the means (±SEM). A comparative analysis of the obtained data was carried out using the Mann-Whitney test for unpaired statistics, with $p \leq 0.05$ as the threshold for statistical significance.

Results

Organization of goldfish blood plasma HDL

On the plasma protein electropherogram, HDL is either adhered tightly to transferrin (AAK92216.1; transferrin precursor (*Carassius gibelio*); 73939 Da; *score* 203) or "covered" (Fig. 1). Two proteins adjoined the HDL from the anode side: serine proteinase inhibitors serpins (P32759.1; Full=Alpha-1-antitrypsin homolog; Flags: Precursor (*Cyprinus carpio*); 41873 Da; *score* 82) and "warm temperature acclimation related 65 kDa protein" (BAP90357.1; warm temperature acclimation related 65 kDa protein-1 (*Carassius carassius*); 50773 Da; *score* 145) (Fig. 1).

In the fish of "FW" and "FW_{rev}" groups, HDL were located on the electrophoregram in the area with Rf 0.45–0.63; in the

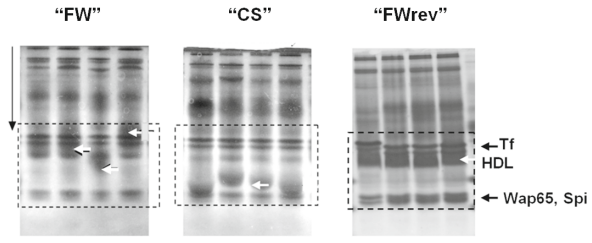

Fig. 1. Disk– E of goldfish plasma proteins from "FW", "CS" and "FW_{rev}" groups. The low molecular protein fractions, including transferrin (Tf), HDL, warm temperature acclimation related 65 kDa protein (Wap65) and serine proteinase inhibitors (Spi) are highlighted with a dotted line. Vertical arrow on the left indicates the direction of movement of proteins. Small horizontal white arrows indicate the position of HDL; the black arrows, the positions of Tf, Wap65 and Spi.

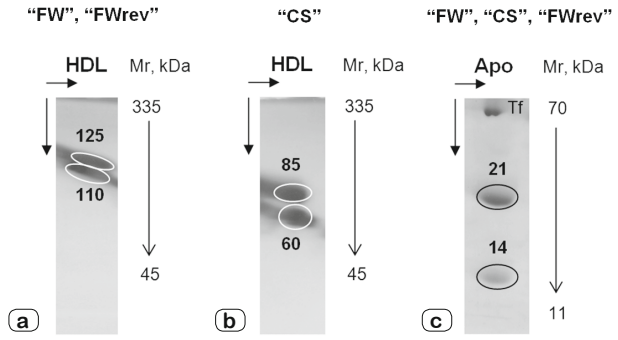

Fig. 2. Fractionating of goldfish plasma HDL in 5–40 % PAGE (a, b) and Apo in HDL composition in SDS-PAGE (c) from "FW", "CS" and "FW_{rev}" groups (fragments of electrophoregrams): 125 and 110 kDa HDLs (a); 85 and 60 kDa HDLs (b); 21 kDa ApoA-I and 14 kDa Apo-14 (c). White ovals mark HDL "spots" and dark ovals mark Apo "spots". To the right of electropherograms there are a Mr scales.

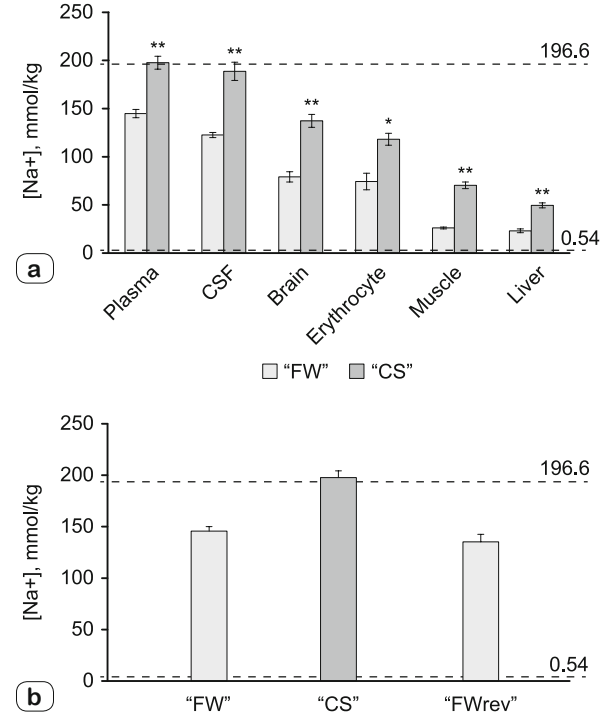

Fig. 3. Na^+ concentration in biological samples of goldfish in fresh water (no fill) and critical salinity (dark fill) conditions (a), and its changes in blood plasma in the fish of "FW", "CS" and "FW_{rev}" groups (b). The dashed lines and the values 0.54 and 196.6 (mmol/kg) indicate the concentrations of Na^+ in fresh water and CS, respectively. Differences are statistically significant at $p<0.05$ (*), at $p<0.01$ (**).

"CS" group they shifted toward the anode to the region with Rf 0.63-0.72 (Fig. 1).

The values of the total protein concentration in the plasma of fish from the "FW", "CS" and "FW_{rev}" groups were 4.9±0.4, 5.3 ±0.8 and 4.6±0.4 g%, respectively. The relative contents of HDL in the plasma of fish from these groups were 27.4±6.3, 29.6±3.8 and 26.8±5.1 % respectively.

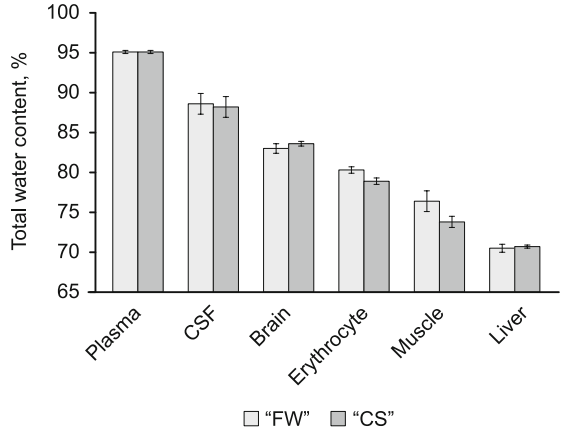

Fig. 4. Content of total water in tissue, cells and fluids samples of goldfish from "FW" (no fill) and "CS" (dark fill) groups.

In the fish of "FW" and "FW_{rev}" groups, native HDL were represented by two types of particles with Mr ~125 and ~110 kDa (Fig. 2a). In denaturing PAGE, they disintegrated into ~21 and ~14 kDa proteins, identified as the ApoA-I (APOA1_DANRE; Apolipoprotein A-I OS=Danio rerio GN=apoa1 PE=2 SV=1; 30237 Da; *score* 257) and Apo-14 (AAW82445.1; 14 kDa apolipoprotein (*Carassius gibelio*); 15771 Da; *score* 104) respectively (Fig. 2c).

In the "CS" group, native HDLs were represented by ~85 and 60 kDa particles (Fig. 2b). Under denaturing conditions, these particles also disintegrated into ~21 and 14 kDa proteins, identified as apolipoproteins ApoA-I (APOA1_DANRE; Apolipoprotein A-I OS=Danio rerio GN=apoa1 PE=2 SV=1; 30237 Da; *score* 257) and "14 kDa apolipoproteins" (Apo-14) (AAW82445.1; 14 kDa apolipoprotein (*Carassius gibelio*); 15771 Da; *score* 104), respectively (Fig. 2c).

Analysis of the HDL organization parameters made it possible to distinguish two discrete types of these particles. Thus, in the fish of "FW" and "FW_{rev}" groups, the plasma contains only large HDL (125, 110 kDa); in the "CS" group, only small HDLs (85, 60 kDa). In all particles, the *molar ratio* of apolipoproteins was close to equimolar (~1:1), except for 85 kDa particles, in which it was ~ 3:2. The number of Apo molecules in HDL particles was different. One 125 kDa particle contains 3-4 molecules of each Apo (6–8 molecules in total); one 110 kDa particle – 3 molecules of each Apo (6 in total); one 85 kDa particle – 3 ApoA-I and 2 Apo-14 molecules (5 in total), and the 60 kDa particle – 2 molecules of each Apo (4 in total).

The breakdown of large particles into small forms was reversible, since the original large forms were found again in the blood of fish transferred to FW after 20 days of acclimation to CS.

Parameters of water-salt homeostasis

The concentration of Na^+ in the biological samples of the fish in CS conditions was higher than in FW (Fig. 3a). The Na^+ concentration values in the plasma were 144.8 ± 4.3 mmol/kg in "FW" group fish and 197.6 ± 6.7 mmol/kg in "CS". In the "FW_{rev}" group, the Na^+ content did not differ from the control values (Fig. 3b).

The Na^+ contents in the aquarium water were 0.03 g/L (0.54 mmol/kg) in FW; and 11.5 g/L (196.6 mmol/kg) in CS; consequently, the ΔNa^+ values were ~ 144 mmol/kg for goldfish in FW and ~1 mmol/kg for goldfish in CS (Fig. 3b).

Comparison of the "FW" and "CS" groups in parameter of *tw* content in plasma, CSF, brain, muscles, erythrocytes, and liver did not reveal significant differences (Fig. 4). In the CS conditions, the *tw* content in muscles and erythrocytes was slightly lower than in the control; in muscles it was 80.3±0.4 % (FW) and 78.9±0.4 % (CS), in erythrocytes – 76.4±1.3 % and 73.8±0.7 %, respectively (the differences between the "FW" and "CS" groups are statistically insignificant) (Fig.4).

Discussion

Comparison of water-salt homeostasis parameters in the body of goldfish in CS conditions and in humans at hypernatremia

The critical salinity studied in the present paper is within the habitable salinity range of water for the freshwater teleosts. The living of goldfish in saline estuarine sections of rivers and the absence of mortality in experiments on fish acclimation to CS evidence the effectiveness of the homeostatic mechanisms of fish in CS conditions (21, 25, 26). This was also confirmed by the results of the present study. Water salinity is a factor in the regulation of the metabolism in aquatic poikilotherms. Its rise induces a stress response in the body of stenohaline freshwater teleosts, manifested in an increase in the titer of circulating cortisol, which adapts the energy metabolism of fish to high salinity (27, 28). Goldfish tolerates water salinity to 6.0 g/L without pronounced signs of stress (29). At water salinity in the range of 8.0–10.0 g/L and up to the upper limit of tolerable salinity (CS), the concentration of Na^+ in the plasma of goldfish and other freshwater Teleostei rises from ~ 150 to 200 mmol/kg, the level of cortisol in the blood is elevated, signs of muscle dehydration appear, along with a decreased locomotor activity and growth rates, decreased urine volume, and increased water consumption (18, 19, 20, 21, 27, 28, 29, 30, 31, 32, 33).

The results of the present study are consistent with these data. At the Na^+ level in plasma close to 200 mmol/kg, in the muscles and erythrocytes of goldfish, a tendency towards a decrease in the total water content was noted (the differences are statistically insignificant), which indicates the development of initial signs of tissue (cellular) dehydration. The hyperionic status of the goldfish plasma relative to CS was maintained at an insignificant level ($(\Delta Na^+) \approx 1$ mmol/kg) compared to that in the fish of control group ($(\Delta Na^+) \approx 144$ mmol/kg). Nevertheless, this value (~ 1 mmol/kg) supported the minimal inflow of water into the body, termination of which would lead to death. In such conditions, we did not find a decrease in the plasma protein concentration and total water content in the goldfish blood. This indirectly indicates the stability of its protein and water homeostasis.

In humans, the state of hypernatremia is considered to be at the plasma Na^+ concentration of more than 145 mmol/kg. This may occur in infants and the elderly people with an inadequate water replenishment as well as in the patients with neurological impairment of the thirst control mechanism (34, 35). Hypernatremia may develop with the use of saline 0.9 % solution (154 mEq/L Na^+) (13) and in rare cases of forced long-term use of seawater. Unlike freshwater Teleostei, which adapt to elevated plasma Na^+ levels to 200 mmol/kg (20), even minimal changes in this parameter in humans lead to dangerous consequences at the cellular level (11, 36). The concentration body Na^+ is under the control of many hormones: vasopressin, cortisol, aldosterone, renin, angiotensin II, urotensin II, natriuretic peptides, dopamine, insulin, apelin, etc. and its deviations from the norm are regarded as manifestations of endocrine pathology (37). Rapid correction of hypernatremia may lead to cerebral oedema (35). With prolonged dysnatremia, adaptation processes take place in the cells to balance the osmolality of the intracellular fluid with the extracellular fluid. However, the accompanying metabolic changes are dangerous due to the risk of developing hyperosmotic encephalopathy, osmotic demyelization and rhabdomyolysis, at which the death rate reaches 50 % (38).

The coordination of changes in the water salinity and the organization of HDL in goldfish

The present study revealed that changes in water salinity caused changes in the water-salt balance in goldfish. These changes are manifested in alterations in the concentration of Na^+ in cells/tissues/body fluids and in the form of minor changes in the content of total water in muscles and erythrocytes.

In the fish plasma, the sodium concentration varied in the following sequence: 144.8±4.3 mmol/kg (FW) → 197.6±6.7 mmol/kg (CS) → 132.6±8.3 mmol/kg (FW). Against the background of these changes, remodelling of blood plasma HDL took place in the following order: 125/110 kDa HDL ("FW") → 85/60 kDa HDL ("CS") → 125/110 kDa HDL ("FW_{rev}"). The corresponding dynamics of the number of Apo molecules in single HDL particle may be represented as 6–8 (FW) → 4-5 (CS) → 6-8 (FW). These facts indicate the reversible nature of HDL remodelling. The calculated values of the number of Apo molecules in the composition of single HDL particle in goldfish plasma do not contradict the human HDL models (39, 40, 41). Finding of HDL remodelling in goldfish at similar changes in plasma sodium levels in humans suggests the possibility of breakdown and restoration of lipoprotein particles in human blood during hypernatremia.

Conclusions

The osmotic activity of HDL and their high titer in the plasma of freshwater Teleostei suggest a significant contribution of this group of lipoproteins to the creation of colloidal osmotic pressure in fish plasma (42). The breakdown of HDL into small forms leads to an increase in the total amount of osmotically active particles in plasma, while the restoration of large HDL from small forms leads to a decrease in this amount. Such dynamics becomes important in CS conditions, when the organism of goldfish is in a hypertonic environment and already has the initial signs of tissue dehydration. Considering the practically unchanged level of total protein and the relative content of HDL in fish plasma during the experiment, as well as the dynamics of changes in the number

of Apo molecules in a single particle, it may be assumed with a high degree of probability that HDL decay was accompanied by an increase in the number of particles per unit volume of plasma by factor of almost 1.5. An increase in the number of osmotically active particles in the plasma of fish from the "CS" group contributes to the retention of water in the body and the maintenance of osmotic homeostasis in the critical salinity zone, in which, due to plasma hypernatremia, the inflow of water into the body is extremely limited. The breakdown of HDL, contributing to an increase in plasma osmolality, may facilitate additional "pumping" of water into the body.

The analysis of published data revealed that there is no information on the breakdown of HDL particles in human plasma at hypernatremia. Nevertheless, the possibility of their decay is indirectly indicated by data on the decay of other human plasma proteins under the influence of an increased level of Na^+. Thus, *in vitro*, at the concentration of Na^+ in the solution of only 150 mmol/kg, the decomposition of the tetrameric human plasma of alpha-2-macroglobulin into dimers (in ~ 5% of the tetramer pool) was registered and the breakdown was accompanied by a change in the functional profile of the protein (43). In this example, the concentration of Na^+ in the solution (150 mmol/kg) does not exceed its titer in the physiological solution used in clinical practice (154 mEq/L) (13).

Consequently, there is a risk of disintegration of protein complexes and lipoprotein structures of the plasma in the patients infused with such a solution. The importance of studying the HDL response to hypernatremia is determined by the fact that these particles are not only lipid carriers, but also a part of the body's complex defence (14, 17). The presence in their composition of dozens of "minor" proteins involved in various biochemical processes (44), in the event of HDL degradation, may lead to undesirable functional consequences. The results of the HDL response to hypernatremia revealed in fish may be useful in the development of protocols for the use of infusion solutions in clinical practice, as well as at using synthetic HDL particles (rHDL) (45).

Considering that, the pathogenetic mechanisms responsible for the development of hypernatremia and its effects in humans are not yet fully clear and that hypernatremia is considered a potentially life-threatening condition (38), it seems promising to use fish as model objects for studying its effects.

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