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BIOPHYSICS AND BIOCHEMISTRY

High-Carbohydrate Diets Affect Accumulation of Lipofuscin-Like Pigment in the Kidneys of Mice and Rats: Autofluorescence Confocal Microscopy Analysis

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The accumulation of lipofuscin-like granules in liver, kidneys, and spleen cells in mice and rats of different lines receiving 30% sugar solutions (fructose, glucose, their mixture, and sucrose) in addition to balanced semisynthetic diet for 62 or 122 days was studied by the method of laser scanning confocal microscopy. The granules were detected by their autofluorescence at maximum λ_{em}=570-600 nm and λ_{ex}=488 nm. In the kidneys of rats receiving glucose and, especially, the mixture of glucose and fructose, significant accumulation of lipofuscin-like granules was found that was absent in the control group animals receiving water. Intensive accumulation of the granules was observed in the kidneys of all groups of mice receiving sugars (except for glucose). Lipofuscin-like granules were located in the cytoplasm of epithelial cells of the distal and proximal convoluted tubules. In the liver of rats and mice, the signs of accumulation of lipofuscin-like granules were absent or minimal. In rat spleen, lipofuscin-like granules were found in the red pulp in all groups, but their accumulation significantly increased in animals receiving the diet enriched with glucose and sucrose.

Key Words: *high-carbohydrate diets; aging; lipofuscin; rats; confocal microscopy*

Adequate *in vivo* models and a complex of informative biomarkers are required for the development of new methods of dietary and pharmacological correction of metabolism disorders in patients with obesity and metabolic syndrome (MetS). In experiments on rodents (mice and rats of different lines), MetS can be modeled by substitution of drinking water with 20-30% sugar solutions (usually sucrose or fructose) [15]. This diet induces the development of a complex of pathological changes typical of MetS: insulin resistance, increased fat weight, BP elevation, changes in the plasma levels of glucose, peptide hormones, and lipoproteins, and liver steatosis. Previous studies [12] showed that excessive sugar consumption led to accelerated aging accompanied by lipofuscin accumulation in various tissues. Laser scanning confocal microscopy is a highly informative methods for analysis of biological objects that combines high resolution and the possibility of using various selective fluorescent labels for analysis of cell structures. Along with the use of exogenous fluorescent probes, laser scanning confocal microscopy allows analyzing autofluorescence of specific pigment inclusions in the cells of various organs.

Here we assessed informativity of confocal microscopy for analyzing the content of lipofuscin-like granules (LLG) in tissues and organs of rodents receiving high carbohydrate diet in *in vivo* model of MetS.

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MATERIALS AND METHODS

The experiments were performed on female and male outbred Wistar rats, female and male inbred Dark Agouti rats, female and male inbred C57Bl/6J mice, female inbred BALB/c mice, female inbred ICR-1 mice, and female tetrahybride DBCB mice. Tetrahybride mice were bred by crossing 4 various inbred mouse lines (DBA/2J, BALB/c, CBA/lac, and C57Bl/6J). The animals were kept in groups, 2-4 animals per group, in plastic polycarbonate cages with sawdust bed at 20-22°C and 12/12 light/dark cycle.

Three experimental series were performed. The animals of each species, line, and sex were divided into 5 (for the experiment No. 1) or 2 (for the experiments Nos. 2 and 3) groups (8 animals per group). In each experiment, group 1 (control) received balanced pelleted semisynthetic AIN93M ration with modifications and other (treatment) groups received similar ration, but drinking water was substituted with 30% water solutions of various sugars and their mixtures. The protocol of investigations and experimental conditions are presented in the Table 1.

The animals were decapitated under ether narcosis on days 120-122 (experiments Nos. 1 and 2) or 62 (experiment No. 3) of the experiment. For histological analysis, specimens of organs (liver, kidneys, and spleen; 5×5×5 mm) were fixed for 3 days in 3.7% formaldehyde (Sigma-Aldrich) in 0.1 M Na-phosphate buffer (pH 7.0) in deionized water immediately after sampling. Then they were washed with running tap water for 24 h, dehydrated in increasing concentrations of alcohols (70°, 80°, 90°, 96°, and 100°), ethanol:chloroform (1:1) mixture, and chloroform:paraffin (1:1) mixture at 37°C, and embedded in paraffin at 56°C. The section (5 μ) were sliced on a Mikrom HMM 355s microtome (Mikrom), mounted on slides, and dried in a thermostat at 37°C for 18 h. Non-stained microsections were analyzed in the dark field of an LSM 710 confocal fluorescent microscope (Carl Zeiss) at λ_{ex}=488 nm (blue-green laser) at ×200 and ×400. The images were recorded using the build-in video system of the microscope. LLG accumulation was evaluated using the following scale: "-", no fluorescence in 3 fields of view in all microsections of the group; "+/-", single fluorescent inclusions in 1-2 fields of view in 1-2 microsections of the group, which can be considered as an artefact; "+", single fluorescent inclusions in few cells or multiple inclusions in 1 cell in 2-3 fields of view in 2-3 microsections of the group; "++", single fluorescent inclusions in most cells or multiple inclusions in few (at least 2-3) cells in 3 fields of view in 2-3 microsections of the group; "+++", multiple fluorescent inclusions in most cells in 3 fields of view in all microsections of the group.

RESULTS

We analyzed 270 preparations from rats and mice of the control and treatment groups, 3 preparations of each organ in each animal group. LLG were registered in the yellow-orange band of the spectrum at λ=570-630 nm (this wavelength is typical of lipofuscin autofluorescence) [11]. Autofluorescence spectra of

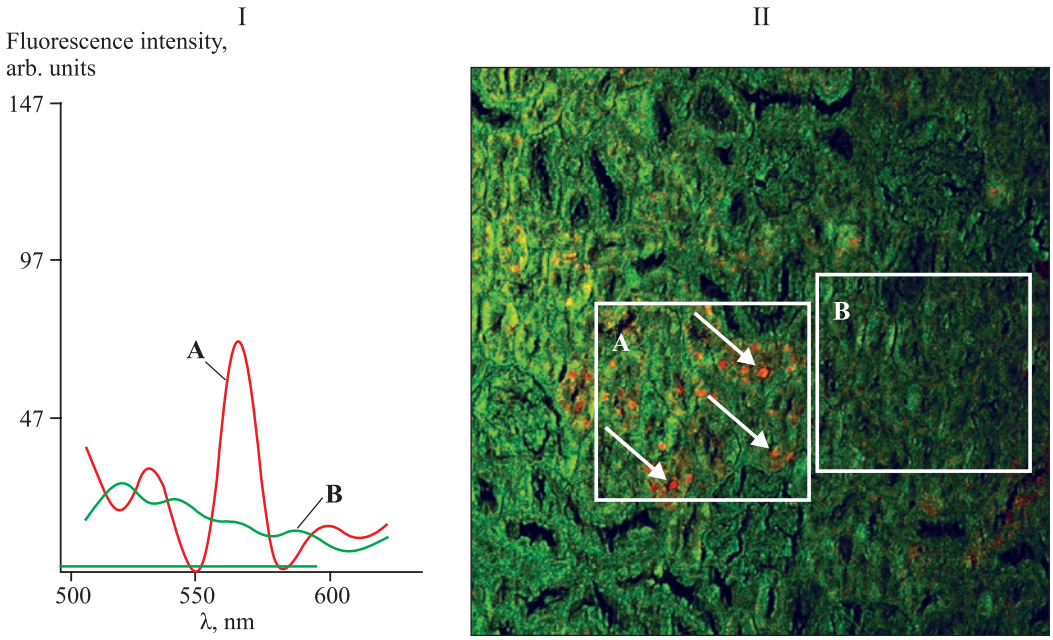

Fig. 1. Spectrum of lipofuscin fluorescence (I) from selected area (II) of rat kidney sample. A: area containing lipofuscin. B: area without visible lipofuscin accumulation. Red curve: spectrum of lipofuscin fluorescence; green curve: spectrum of fluorescence of the neutral area.

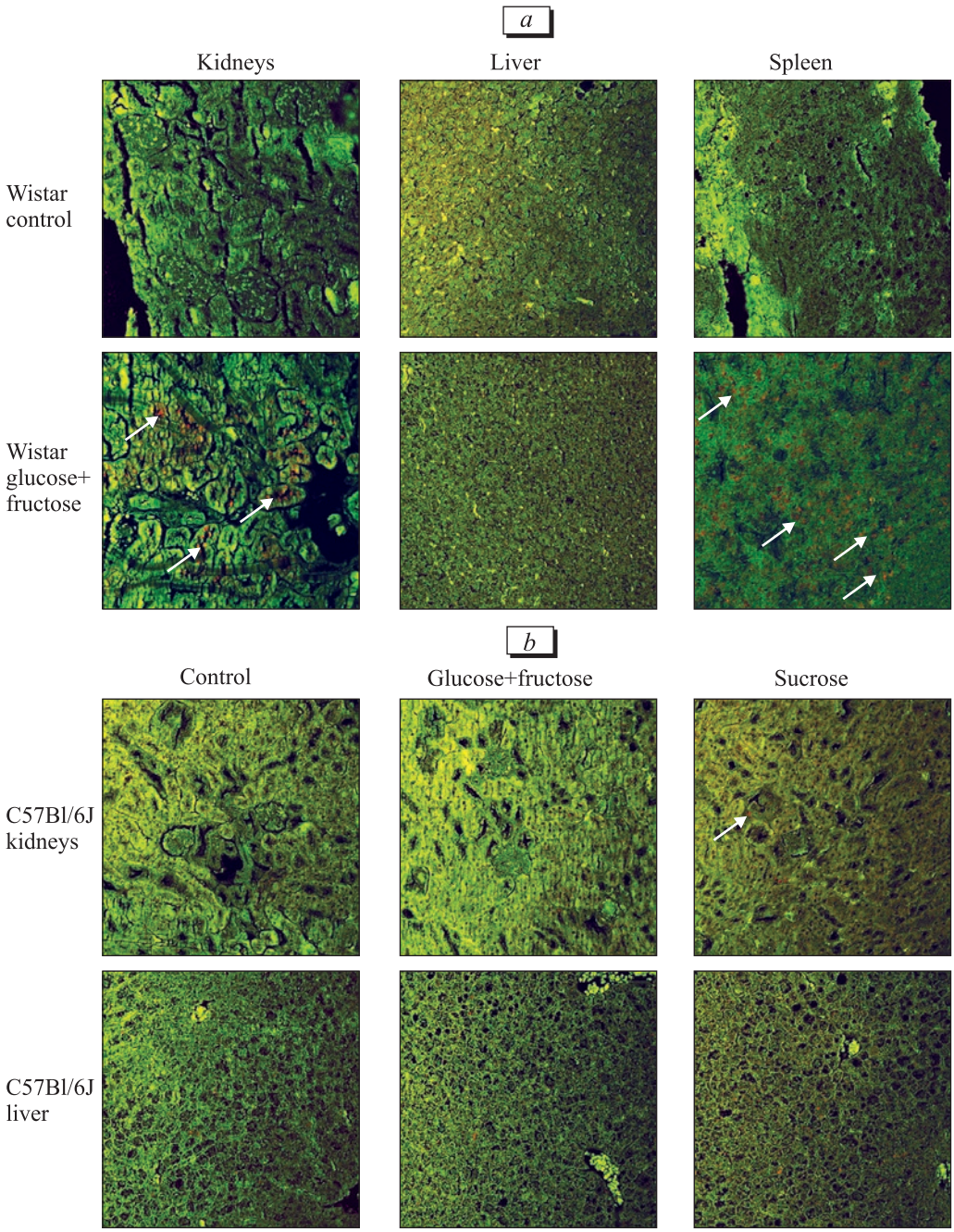

Fig. 2. Autofluorescence at λ_{ex}=488 nm of micropreparations from female Wistar rats and C57Bl/6J mice in experiment No. 1.

LLG in the kidneys of rats and mice are presented in Figure 1. LLG were detected as discrete dot or spherical structures with a diameter <2 μ evenly distributed in the cytoplasm and demonstrating no tropism to the nucleus or plasma membrane, which corresponded to typical lipofuscin accumulations [9].

Experiment No. 1. The effects of various high-carbohydrate diets (additions of glucose, fructose, and their equimolar mixture, or sucrose) on LLG accumulation in the organs of rats and mice standard lines were studied. LLG accumulation was not observed in the liver and kidneys of control rats and mice (Fig. 2). Among animals receiving sugars, the least pronounced signs of LLG accumulation in the liver were found in mice receiving fructose (Table 1). Significant accumulation of LLG was observed in the kidneys of rats receiving 30% glucose or the mixture of glucose and fructose. In all mice receiving sugars (except for glucose; Fig. 2), intensive accumulation of LLG was found in the kidneys. In all cases, LLG were located in the cytoplasm of epithelial cells of distal and proximal convoluted tubules. In the spleen (in this experiment

studied only in rats), single fluorescent LLG inclusions were seen in the red pulp of all group animals, but diet containing glucose and sucrose led to significant accumulation of LLG (Table 1).

Experiment No. 2. The effects of 30% fructose on LLG accumulation in the organs of mice and rats depending on the sex and line were studied. The signs of LLG presence were not observed or were minimal in the liver of rats and mice of all groups. On the contrary, significant accumulation of LLG was registered in the spleen of female and male rats and mice, which apparently did not depend on animal line, sex, or diet. The most pronounced changes in LLG content were found in the kidneys of female ICR-1 mice: this parameter significantly increased under the effect of high-fructose ration (Fig. 3). LLG accumulation in the kidneys of rats of both lines was pronounced in both control and treatment groups, a tendency to enhanced

TABLE 1. Design of the Experiment and the Results of Analysis of LLG in the Organs of Rats and Mice

No. of the experiment	Species	Line	Sex	Initial body weight ($M\pm m$), g	Age at the end of the study, months	Composition of drinking liquid	LLG accumulation in organs		
							kidney	liver	spleen
No. 1	Rat	Wistar	Female	146±3	4	Water	–	–	+
	Rat	Wistar	Female	146±3	4	30% glucose	++	+/–	+++
	Rat	Wistar	Female	146±3	4	30% fructose	–	–	+
	Rat	Wistar	Female	146±3	4	15% fructose, 15% glucose	+++	+/–	++
	Rat	Wistar	Female	146±3	4	30% sucrose	+/–	–	+++
No. 1	Mice	C57Bl/6J	Female	16.3±0.4	4	Water	–	–	n/s
	Mice	C57Bl/6J	Female	16.3±0.4	4	30% glucose	–	–	n/s
	Mice	C57Bl/6J	Female	16.3±0.4	4	30% fructose	+++	+	n/s
	Mice	C57Bl/6J	Female	16.3±0.4	4	15% fructose, 15% glucose	+++	–	n/s
	Mice	C57Bl/6J	Female	16.3±0.4	4	30% sucrose	+++	–	n/s
No. 2	Rat	Wistar	Female	256±8	6.5	Water	++	–	+++
	Rat	Wistar	Female	256±8	6.5	30% fructose	++	–	n/s
	Rat	Wistar	Male	359±12	6.5	Water	+++	–	++
	Rat	Wistar	Male	359±12	6.5	30% fructose	+++	+	+++
No. 2	Rat	Dark Aguti	Female	145±3	6.5	Water	++	+	+++
	Rat	Dark Aguti	Female	145±3	6.5	30% fructose	+++	+	n/s
	Rat	Dark Aguti	Male	215±4	6.5	Water	+++	+	+++
	Rat	Dark Aguti	Male	215±4	6.5	30% fructose	+++	+	+++
No. 2	Mice	ICR-1	Female	33.1±1	4	Water	+/–	–	+++
	Mice	ICR-1	Female	33.1±1	4	30% fructose	+++	–	++
No. 2	Mice	BALB/c	Female	22.7±0.5	4	Water	+/–	–	++
	Mice	BALB/c	Female	22.7±0.5	4	30% fructose	+/–	–	++
No. 2	Mice	C57Bl/6J	Male	25.7±0.7	4	Water	+	+	n/s
	Mice	C57Bl/6J	Male	25.7±0.7	4	30% fructose	++	+	+++
No. 3	Mice	DBA/2J	Female	25.1±0.5	2	Water	–	+/–	+
	Mice	DBA/2J	Female	24.3±0.5	2	30% sucrose	++	–	+/–
No. 3	Mice	ICR-1	Female	39.3±2.1	2	Water	–	+/–	+
	Mice	ICR-1	Female	38.5±2.1	2	30% sucrose	++	–	+/–
No. 3	Mice	DBCB	Female	24.4±0.8	2	Water	+	–	+/–
	Mice	DBCB	Female	24.9±0.8	2	30% sucrose	+++	+/–	–

Note. n/s: not studied.

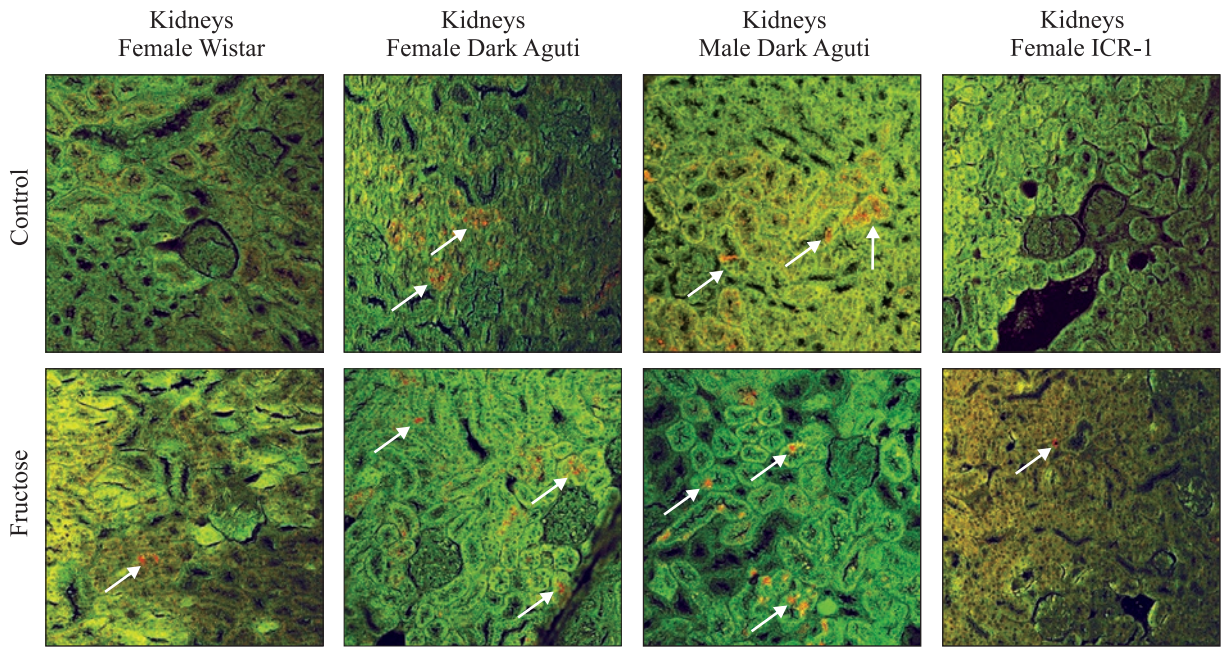

Fig. 3. Autofluorescence at λ_{ex}=488 nm of micropreparations of the kidneys of rats and mice in the experiment No. 2.

LLC accumulation was found in males Wistar rats in comparison with females (Table 1). The differences from in the results of experiments 1 can be related to older age of rats in experiment No. 2 (6.5 months *vs*. 4 months in experiment 1).

Both experiments showed that addition of 30% fructose to the ration did not significantly affect LLG accumulation, except for the kidneys of outbred ICR-1 mice.

Experiment No. 3. The effects of sucrose-enriched diets on LLG accumulation in organs of mice of different lines were studied. The age of animals in this experiment was lower than in experiments Nos. 1 and 2. No significant accumulation of LLG in the liver and spleen of mice of all groups was observed (Table 1). In the kidneys, consumption of 30% sucrose led to significant increase in the number of fluorescent inclusions in all three mouse lines.

It can be concluded that the addition of simple carbohydrates to the rations of rats and mice affects LLG accumulation in the cells of internal organs, particularly the kidneys. Experiment No. 1 showed significant accumulation of LLG in the nephrons of female Wistar rats receiving 30% sucrose solution or equivalent mixture of glucose and fructose instead of drinking water. Similar pattern was observed in female ICR-1 and partially C57Bl/6J mice receiving fructose in the experiment No. 2, as well as in younger female ICR-1, DBA/2J, and tetrahybride DBCB mice in the experiment No. 3. In female and male Wistar and Dark Agouti rats of older age (experiment No. 2), LLG content in the kidneys was high even in control groups, due to which changes in this parameter under the effects of fructose-rich ration cannot be evaluated.

High content of LLG was also found in the spleen of rats and mice. In two cases, LLG accumulation under the effect of carbohydrate consumption (experiment No. 1, female Wistar rats receiving 30% glucose and 30% fructose) was revealed. LLG content in the liver of rats and mice was low in all cases, or it was not detected due to low method sensitivity.

The origin and biological role of LLG deposits in cells of animals of various taxonomic groups (from mollusks and insects till mammals) is a subject of intensive scientific discussion. Initially, lipofuscin deposits were attributed exclusively to old postmitotic (non-dividing) cells, such as neurons, retinal cells, and muscle filaments and this substance was even named "yellow pigment of aging" [3]. It was thought that lipofuscin deposit are released from the cells during mitosis [3]. However, further studies showed that hepatocytes, spleen cells, testicle cells, macrophages, and epithelial cells of nephrons (podocytes) can also accumulate this substance [1]. It was demonstrated that kidneys accumulate the maximal amount of lipofuscin [11]. Transmission electron and light microscopy detected lipofuscin granules in the cytoplasm, which did not allow to identify certain cell organelles generating it. According to modern views, lipofuscin granules are degraded phagolysosomes [9], though some authors attribute lipofuscin generation to functioning of the Golgi complex, peroxisomes, and mitochondria. Lipofuscin consists of oxidized proteins (up to 30-70% according to different sources) and lipids (20-50%), as

well as trace amounts of sugars and metal ions (particularly, Fe, Cu, Zn, Al, Mn, and Ca) [1,8]. There are data that LLG fluorophores are retinoid molecules covalently bound to the protein-lipid complex [1].

Even though the connection between lipofuscin and aging is clear, little is known about the role of the substance in this process. Lipofuscin is mostly considered as the terminal product of abnormal degradation of proteins and lipid membranes in lysosomes and autophagosomes [8]. Lipofuscin itself can be cytotoxic due to the presence of Fe^{2+} and Cu^{2+} ions, inducers of Fenton-type peroxidation reactions, in its surface layer and its ability to inhibit proteosomes. On another hand, lipofuscin is considered as a storage compound in the cells, the processes of metabolism and renovation of which are impaired during aging [1].

Lipofuscin accumulation in the body is sensitive to alimentary, pharmacological, and pathological factors. Enhanced accumulation of lipofuscin in the liver of rats receiving fructose-rich diet was reported [12]. Its accumulation, in particular in the kidneys, is also promoted by high-fat diet [14], vitamin E insufficiency [6], exhaustion of reduced glutathione pool in the tissues [7], and inhibition of lysosomal proteolysis [10]. Lipofuscin accumulation can be prevented by taurine [12], vitamin E [5], and bioflavonoid Silubin [2].

The molecular mechanisms of lipofuscin accumulation under the influence of high-carbohydrate rations or hyperglycemia can be related to peroxisome activation, which was shown in experiments with exogenous peroxisome proliferators [13]. Similar processes can develop in cells of mice and rats with MetS due to enhanced expression of receptors binding peroxisome proliferators (PPARα and PPARγ) [4].

Thus, our data confirm the suggestion that excessive consumption of carbohydrates can lead to accelerated aging of cells, particularly nephron epitheliocytes that experience overload when filtrating excessive amounts of metabolites due to increased proportion of simple carbohydrates in the ration. Among these sugars, sucrose (and the mixture of glucose and fructose imitating sucrose) seems to have the most pronounced effects. LLG accumulation in kidney cells in laboratory animals can be considered as an informative marker that allows tracing the dynamics of the pathological process in *in vivo* modeled MetS.

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