UDC 57.044: 612.35 https://doi.org/10.15407/biotech12.01.066

INFLUENCE OF C_{60}-FULLERENE AQUEOUS COLLOID SOLUTION ON LIVER AND PANCREAS MORPHOLOGICAL STATE AND BLOOD AMINOTRANSFERASES OF RATS WITH EXPERIENCED ACUTE CHOLANGITIS

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Received 21.11.2018
Revised 08.12.2018
Accepted 14.01.2019

Aim of the work was to investigate the suspended C_{60}-fulleren effect on liver and pancreas state under intraperitoneal and intragastrial administration on rat experimental cholangitis model. Acute cholangitis was simulated by a single ingestion of α-naphthyl isothiocyanate — ANIT. C_{60}-fullerene aqueous colloid solution (C_{60}FAS, 0.15 mg/ml) was administered to animals at a volume containing C_{60}-fullerene at a dose of 0.5 mg/kg body weight in 24 and 48 h after ANIT administration. After 72 hof the experiment, the animals were euthanized. Blood serum ALT and AST activities were measured, the liver and pancreas states were analyzed by light-microscopy level. It was found that intragastrial and intraperitoneal administration of C_{60}FAS contributes to the correction of negative effects in the liver and pancreas caused by the induction of acute cholangitis. This was proved by the normalization of ALT activity, reduction of pancreatic parenchymal edema and liver fibrosis, and increased blood flow in these organs. Application of C_{60}FAS could improve the state of the liver and pancreas under acute cholangitis in rats.

Key words: C_{60}-fullerenes, acute cholangitis.

Primary sclerosing cholangitis (PSC) is a chronic cholestatic disease characterized by intrahepatic and extrahepatic bile ducts inflammation,obliteration and fibrosis [1], resulting in biliary cirrhosis, portal hypertension and hepatic failure. The etiology of PSC is unknown. Genetic predisposition (in particular hla b8, dr3, drw52) [2] and environmental factors, including bacterial and viral infections, are considered to be risk factors. Most often this pathology is associated with autoimmune diseases (primary biliary cirrhosis, rheumatoid arthritis, autoimmune hepatitis, systemic sclerosis, systemic lupus erythematosus, cystic fibrosis) and inflammatory bowel disease (70–80% of cases).

PSC-prognosis is extremely unfavorable due to following complications: portal hypertension accompanied by bleeding and functional renal insufficiency (hepatorenal syndrome), spontaneous bacterial peritonitis, chronic cholestasis accompanied by weakness, itching, steatorrhea, deficiency of fat-soluble vitamins (A, D, E and K) and osteoporosis. Specific complications of PSC include bacterial cholangitis (15–35%), cholelithiasis (25–56%), chronic pancreatitis (20–23%) and cholangiocarcinoma (6–18%). The survival rate of patients in the last case does not exceed 12 months [1].

PSC-therapy is symptomatic and includes medications for treatment itching and jaundice (choleretics, sorbents), antibiotics (for the treatment of infections), diet and vitamin supplements, immunosuppressants and anti-inflammatory drugs. The only effective method

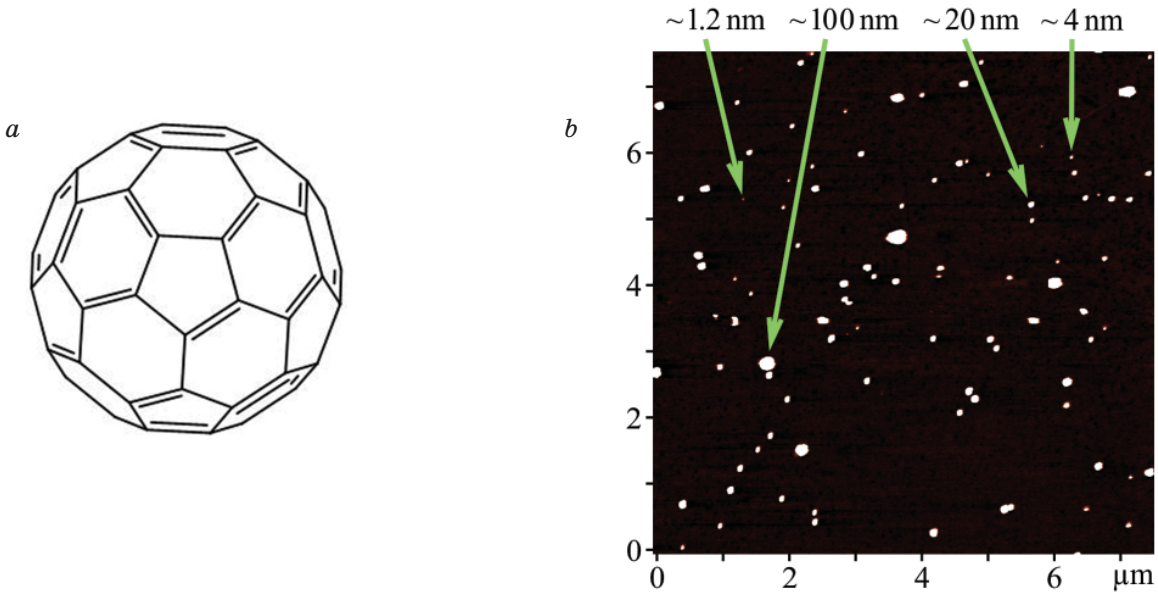

Fig. 1. **C_{60} fullerene (*a*), AFM image (tapping mode) of C_{60} fullerene particles on the mica surface: arrows indicates the height of individual nanoparticles (*b*)**

for PSC treating is liver transplantation, but PSC relapse after transplantation occurs in 15–20% of patients, and the 5-year survival rate is only 66–72% [3].

PSC, as any inflammatory disease, is accompanied with oxidative stress, increased levels of lipid peroxidation products and antioxidants deficiency. Therefore, the use of the latter is promising to correct at least the disease symptoms, remaining, however, beyond the attention of researchers. Numerous studies have shown the anti-inflammatory effects of natural antioxidants (vitamins, minor amino acids, polyunsaturated fatty acids, plant extracts) *in vitro*, but effectiveness of those in *in vivo* systems is highly questionable. On the other hand, artificial compounds, in particular fullerene, have clearly defined properties and are involved in a number of cellular processes [4–9], which determines their selective action and a more pronounced therapeutic potential. C_{60}-fullerene can effectively scavenge free radicals and thus act as antioxidants [10, 11], revealing anti-inflammatory properties [12, 13].They are non-toxic in *in vitro* and *in vivo* systems acting in physiological concentrations [6, 8, 14] and are capable to be accumulated in the liver [15]. The last makes them very attractive for direct impact on this organ. Thus, C_{60}-fullerene may be considered as potential therapeutics for effective prevention and treatment of liver diseases associated with oxidative stress.

Given the foregoing, the aim of the study was to investigate the morphofunctional state of the liver and pancreas in rats received suspended C_{60}-fullerene under α-naphthyl isothiocyanate-induced acute cholangitis condition.

Materials and Methods

The studywas conducted on 32 white outbred male rats with an average body mass 198 ± 10 g, which were kept under standard vivarium conditions. All experiments were conducted in compliance with bioethics principles, legislative norms and provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), General Ethical Principles for Experiments on Animals, adopted by the First National Bioethics Congress (Kyiv, 2001), and approved by an institutional review committee (protocol № 2 dated April 25, 2017, ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv).

Acute cholangitis was simulated by a single ingestion of α-naphthyl isothiocyanate (ANIT, Sigma, USA) at a dose of 100 mg/kg dissolved in sunflower oil (total volume of 0.1 ml). ANIT is a hepatotoxin which depending on dose and duration of administrationcauses liver damage, such as intrahepatic cholestasis, acute cholestatic hepatitis, sclerosing cholangitis, biliary fibrosis and cirrhosis [16]. The mechanism of action of

this toxicant is associated with a specific lesion of the epithelial cells of intralobular bile ducts. Cholangiocytes' injury causes bile ducts obstruction with detritus or cells excessive growth with subsequent sclerotic degeneration resulting in bile flow stop and periportal inflammation [16]. The systemic result is blood serum and urine bilirubin increase (mainly due to directbilirubin) and serum aminotransferases and alkaline phosphatase activity raise, which corresponds to the biochemical manifestations of acute and chronic sclerosing cholangitisin humans.

A highly stable pristine C_{60}-fullerene aqueous solution (C_{60}FAS) with purity of more than 99.95% has been prepared and characterized according to [17, 18]. Briefly, this method is based on transferring C_{60}-fullerene from organic solution into the aqueous phase by ultrasonic treatment. The maximal concentration of C_{60}-fullerene in water was 0.15 mg/ml. Themorphological state of C_{60}-fullerene in aqueous solution was monitored using atomic force microscopy and measurement of small-angle neutron scattering (Fig. 1) [19]. Concentrated C_{60}-FAS contained both single C_{60} moleculesand their nanoparticles (aggregates) with sizes of 1.2–100 nm which is in agreement with our previous results [17, 20].

Animals received C60FAS intraperitoneally or intragastrially in a dose equal to 0.5 mg / kg body weight C_{60}-fullerene in 24 and 48 h after ANIT administration. All manipulations with the animals in comparison groups were conducted similarly to the animals of the experimental ones, including the appropriate solutions administration. There were 4 experimental groups (n = 8): 1 — control; 2 —acute cholangitis; 3 — cholangitis and C_{60}FAS intraperitoneal administration; 4 — cholangitis and C_{60}FAS intragastrial administration. Since the effect of C_{60}FAS on healthy animals has been studied and described in our previous studies [9, 21], we did not include the description of groups of healthy animals receiving C_{60}FAS in the current manuscript.

In 72 h after the start of the experiment the animals were killed by inhalation of CO_2 and subsequent cervical dislocation.The blood for biochemical analysis was collected immediately after the sacrifice from the femoral vein, left for 20 min to form a clot and then centrifuged 8 min at 1000 g. Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined in blood serum using standard reagent kits (Filisit diagnosis, Ukraine) and expressed in μmol sodium pyruvate per ml of blood serum per h.

The fragments of liver and pancreas were harvested immediately after the sacrifice and fixed in Bouin mixture for 14 days for histological assay. Then, they were embedded into paraffin, sliced into 5 m sections, stained with hematoxylin and eosin [22] and examined under the light microscope (Olympus BX-41, OlympusEuropeGmbH, Japan). Liver centrilobular and periportal zones, exocrine and endocrine part of the pancreas were assessed separately.

Statistical processing of data was carried out using one-way analysis of variance (ANOVA) with the Tukey post hoc test [23]. The difference between compared groups was considered significant at $P \leq 0.05$.

Results and Discussion

The yellowness of the peritoneum and mucous membranes resulting from the accumulation of bilirubin in plasma blood and tissues, edema and liver granularity suggesting the micronodular fibrosis were observed atthe autopsies ofall animals from the cholangitis group (group 2). On liver micropreparations (Fig. 2, *B*) one can see fibrotically alteredportal tracts with surrounding cellular inflammatory infiltrate, sites with violation of the parenchymal limiting plate and portal-portal linking septs, suggesting the acute cholangitis. Atrophic and degenerative changes in the bile duct epithelium and somebile ducts replaced by fibrous cords (scars) were observed. Lymphoid follicles and fibrotic loci were detected in parenchyma. Also, thrombosis of some blood vessels including central veins, sinusoids dilation and a marked increase in the number of leukocytes in vessels were detected. Blood serum ALT and AST were higher than those in control group (by 81% and 75% respectively) (Fig. 3) indicating cytolysis of hepatocytes and cholestasis, which may be the result of bile ductsobstruction.

The pancreas of rats in this grouprevealed significant structural changes (Fig. 4, *B*): largeareas ofsecretory acinidestruction; dystrophic changes of acinar tissue; cellular and intercellular edema, which sometimes caused the destruction of both individual pancreatic cells and whole acini; cytoplasm zonation loss in pancreatic cellsfrom areas with destroyed structure. Observed changes could indicate a violation of

pancreaticexocrine parenchyma functional state and the development of acute pancreatitis. The gland microcirculatory also underwent changes: there was a stasis and sometimes expressed thrombosis of small blood vessels, sinusoid hemocapillaries dilation and sometimes microtrombosis. The pancreatic endocrine apparatus wasn't changedsignificantly,pancreatic endocrinocytes did not differ from control ones.

In rats received C60FAS (groups 3 and 4) the jaundice of mucous membranes and peritoneum was less pronounced compared to non-treated animals (group 2), but liver edema and micronodular fibrosis persisted (Figs. 2, *C*, *D*). The area of the liver fibrotically altered parenchyma was smaller compared to non-treated animals, portal-portal linking septsweren't detected. However, necrotic loci and loci of hypereosinophilic cells were observedin fibrotically altered sites. Also blood vessels overflow was markedly higher compared to control, sometimes even blood stasis occurred. Occasionally zones with dark inclusions were detected, which might be caused by C_{60} fullerene accumulation. It should be noted, that rats received C_{60}FAS intragastrially (group 4) demonstrated significantly less expressedsigns of periportal inflammation in comparison with those received C_{60}FASintraperitoneally (group 3). Manifestations of necrosis in fibrotically altered sites in this group also were less common and less frequent compared to group 3. ALT activity was restored to control values in rats received C_{60}FAS by both ways, although AST activity remained unchanged (Fig. 3), which mightindicate partial persistence of cholestasis. Thus, C_{60}FAS inhibited the symptoms of acute cholangitis but did not prevent hepatocyte cytolysis completely.

Blood stasis in small vessels, wall thickening and edema of middle blood vessels were revealed in the pancreas of animals received C_{60}FAS (Figs. 4, *C*, *D*). The vast majority of exocrine pancreatic cells had a normal structure, although there were occasional sites with signs of acinus dystrophy and cells with cytoplasmic vacuolation. The cytoplasm of most cells was clearly delineated into basophilic and acidophilic zones, the nuclei had a rounded form, indicating the normal functional activity of the cells. The endocrine part of the pancreas did not undergo significant changes. However, significantly greater interacinar edemawas detected in animals received C_{60}FAS intraperitoneally compared to those received C_{60}FAS intragastrially. Summarizing, C_{60}FAS contributed to mitigate of pancreatic injury due to ANIT-induced acute cholangitis.

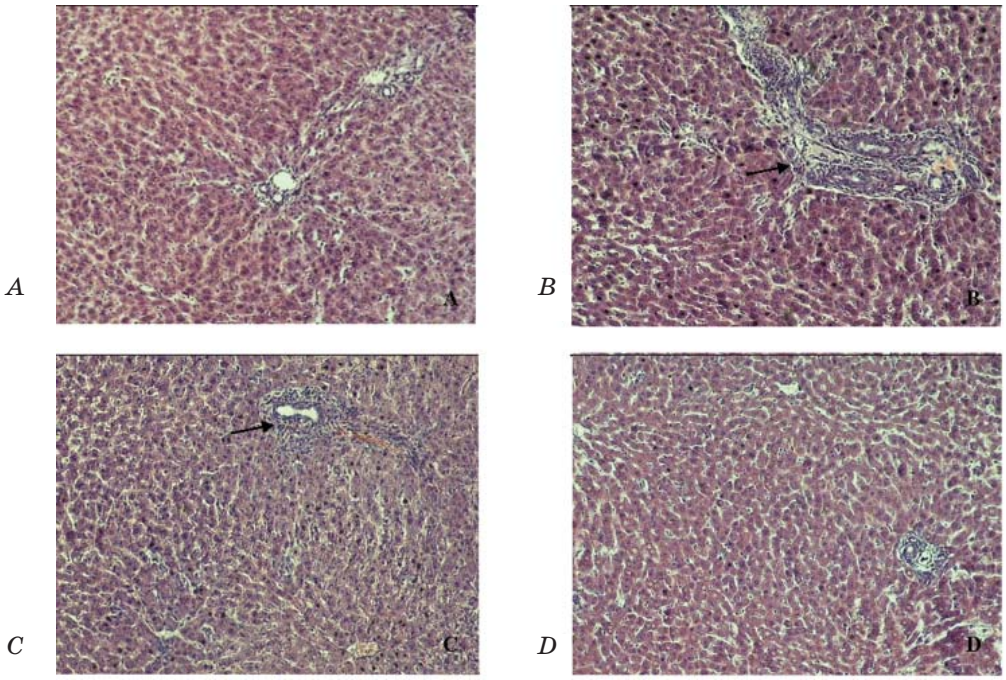

Fig. 2. **Microphotographs of liver of rats experienced acute cholangitis and treated with C60FAS: control (*A*), acutecholangitis (*B*), cholangitis+C_{60}FAS intraperitoneal (*C*) and intragastrial (*D*) administration. Hematoxylin-eosin staining, ×100 magnification; the arrows indicate fibrotically altered portal hepatic tracts with surrounding diffuse cell inflammatory infiltrate**

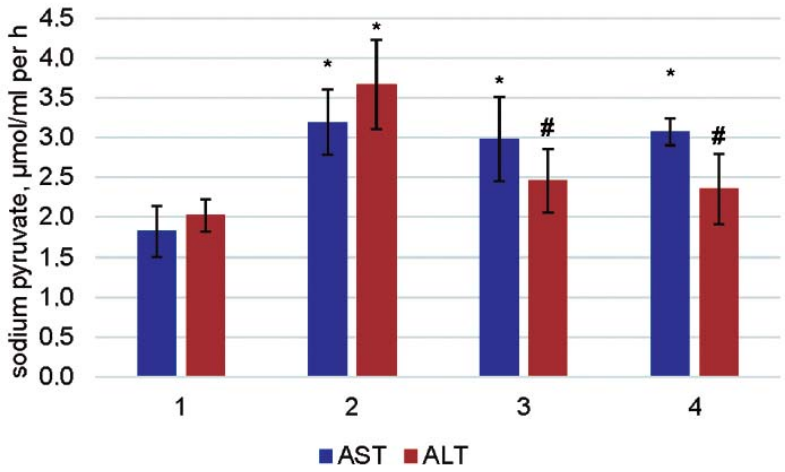

Fig. 3. **Blood serum ALT and AST activities of rats experienced acute cholangitis and treated with C_{60}FAS:** control (*1*); acute cholangitis (*2*); cholangitis+C_{60}FAS intraperitoneal (*3*) and intragastrial (*4*) administration;
*$P \leq 0.05$ compared to control, #$P \leq 0.05$ compared to acute cholangitis group

One of the mechanisms involved in the pathogenesis of PSC is an abnormal immune response, which leads to the activation of auto-reactive T and B lymphocytes and the subsequent production of numerous inflammatory mediators [24]. As a result, the liver undergoes significant destructive impacts of, in particular, excessive amounts of bile acids (having detergent properties), toxins, inflammatory mediators, autoimmune complexes. This eventis the cause of oxidative stress development [25]. Thus, patients with PSC demonstrate significantly increased lipid and protein peroxidation, cholesterol auto-oxidation and downregulation of antioxidant defense system [26–28]. In addition, ANIT-induced liver damage in rats (a valid model of human sclerosing cholangitis) is accompanied by oxidative stressdevelopment and is widely used to study hepatoprotective efficacy of different compounds including ones possessing antioxidant properties [29–31].

Biliary pathology is the most common cause of acute pancreatitisand exacerbation of chronic one. The main mechanism of biliary pancreatitis is bile influx into the pancreatic duct (due to the pressure difference in common bile duct and pancreatic duct under the biliary hypertension) with subsequent interactionof bile with pancreatic enzymes and bacteria. Hence, the release of bound bile acids and the activation of pancreatic enzymes immediately in the pancreatic duct systemoccur. As a result, its protective barrier is damaged and the parenchyma of the gland is affected. Based on clinical and pathological studies the association of PSC with pancreatic disease was proven [32], and the joint autoimmune component of both diseases was noted [33].

C_{60}-fullerene is a molecular form of carbon having a spheroidal structure. Due to the presence of π-conjugated double bonds between hexa- and pentagonal structures on the surface, C_{60}-fullerene is capable to scavenge reactive oxygen species (ROS) effectively [4, 11, 14]. Its unique properties include strong electron acceptor activityand high polarization and hydrophobic ability, which enables them to effectively bind free radicals not only in the extracellular space, but also penetrate into the cell [5, 10, 34]. Hence, C_{60}-fullerene are capable not only to scavenge free radicals directly [10], but also to be involved in the regulation of intracellular signaling pathways associated with ROS overproduction [4, 11]. An important feature of C_{60}-fullerene is its relatively low toxicity [11], non-immunogenicity [4] and the ability to be accumulated in liver [6, 15], which makes this compound attractive for selective impacton liver and treatment of this organ's diseases associated with oxidative stress. In addition, the ability of C_{60}-fullerene to suppress colonic inflammation under systemic and topical application and to correct its systemic effects [9], to prevent the development of toxic hepatitis [12] and to realize antitumor properties [10] were demonstrated in many studies.

One of the leading roles in the development of autoimmune inflammation belongs to oxidative stress. The last is accompanied by a violation of the pro-and antioxidant balance and overproduction of ROS and reactive

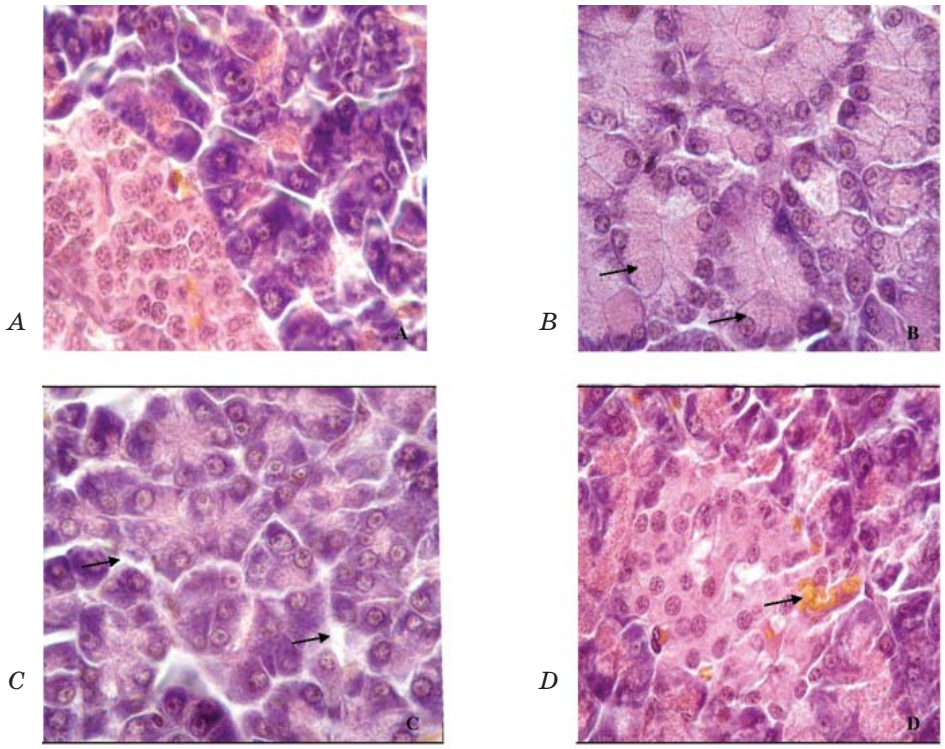

Fig. 4. **Microphotographs of pancreasof rats experienced acute cholangitis and treated with C_{60}FAS:** control (*A*); acutecholangitis (*B*);cholangitis+C_{60}FAS intraperitoneal (*C*) and intragastrial (*D*) administration. Hematoxylin-eosin staining, ×400 magnification; the arrows indicate acinar cellsedema andcytoplasm zonation loss (*B*), interacinar edema (*C*), blood vessels overflow (*D*)

nitrogen species. The consequence is the initiation of cell phagocytosis and apoptosis leading to tissue damage and intracellular autoantigensrelease, which in turn results in the production of autoantibodies. In addition, the oxidative modification of macromolecules, in particular DNA, in apoptotic cellsleads to the formation of new epitopes, which causes the production of a wide range of polyspecific autoantibodies and the escalation of the autoimmune response process [34, 35].

As mentioned above, most researchers suggest the autoimmune cause of PSC [36, 37], therefore it is logical and reasonable to assume the efficacy of antioxidant use for correction at least the diseasesymptoms. Therefore, we suppose that C_{60}FAS suppressed the symptoms of acute cholangitis and pancreatitis precisely because of its antioxidant properties. Indeed, the only drug approved for the treatment of PSC–ursodeoxycholic acid–possesses antioxidant properties and normalizes the content of reduced glutathione in liver tissue and blood serum by activating of γ-glutamylcysteinesynthase [38], which in turn can cause a positive systemic effect on the organism and particularly on the pancreas. In addition, in our previous studies we demonstrated the ability of C_{60}-fullerene to suppress lipid and protein peroxidation and to upregulate the antioxidant enzymes in liver under an inflammatory process in the organism [9].

It should be noted that intragastrial administration of C_{60}-fullerene in a dose of 10 mg/kg body weight causes an increase ofCYP 2B1 enzyme activity in liver, reduces serum blood uric acid, increases serum blood urea and enhances the small intestinal wallpermeability for macromolecules [39]. This can testify the occurrence of some local and systemic toxic effects of the chemical in case of its ingestion. This fact might be an explanation of more expressed positive effect of C_{60}FAS intraperitoneal administration on liver and pancreascompared to intragastrialone. In addition, incomplete absorption of C_{60}-fullerene in the gastrointestinal tract might occur, and, correspondingly, a lower dose of the compound enters into the systemic circulation. This assumption may be confirmed by the data of low absorption of C_{60}-fullerene from the gastrointestinal tract [36] and by information of the dose-dependent mannerof C_{60}-fullerene hepatoprotective activity when realized due to their antioxidant properties [14].

Consequently, C_{60}FAS when administered intragastrally or intraperitoneally could correct the negative effects of ANIT-induced acute cholangitis on liver and pancreas. The prove is the normalization of blood serum ALT and diminishing of pancreatic parenchyma's edema and liver fibrosis.

The authors would like to acknowledge Uwe Ritter and Peter Scharff (Technical University of Illmenau, Germany) for kindly provided samples of C_{60}FAS and their characterization, and prof. Yu.I. Prylutskyy (Taras Shevchenko National University of Kyiv, Ukraine) for the help in preparing the manuscript.

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ВПЛИВ ВОДНОГО КОЛОЇДНОГО РОЗЧИНУ C_{60}-ФУЛЕРЕНУ НА МОРФОЛОГІЧНИЙ СТАН ПЕЧІНКИ, ПІДШЛУНКОВОЇ ЗАЛОЗИ ТА АМІНОТРАНСФЕРАЗНУ АКТИВНІСТЬ СИРОВАТКИ КРОВІ ЩУРІВ ЗА ГОСТРОГО ХОЛАНГІТУ

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Метою роботи було дослідити вплив C_{60}-фулерену на морфологічний стан печінки та підшлункової залози за інтраперитонеального та інтрагастрального введення на моделі експериментального холангіту щурів. Гострий холангіт відтворювали одноразовим інтрагастральним введенням α-нафтил-ізотіоціанату — ANIT. Водний колоїдний розчин C_{60}-фулерену (C_{60}FAS, 0,15 мг/мл) вводили тваринам в об'ємі, еквівалентному кількості C_{60}-фулерену на 0,5 мг/кг маси тіла, через 24 та 48 год після введення ANIT. Через 72 год після початку досліду тварин піддавали евтаназії. У крові тварин вимірювали рівень АЛТ та АСТ. Печінку і підшлункову залозу аналізували на світлооптичному рівні. Встановлено, що інтрагастральне та інтраперитонеальне введення C_{60}FAS сприяє корекції негативних ефектів у печінці та підшлунковій залозі, спричинених індукцією гострого холангіту. Свідченням цього є нормалізація рівня активності АЛТ, зменшення набряку паренхіми підшлункової залози і фіброзу печінки, а також посилення кровонаповнення цих органів. Застосування C_{60}FAS позитивно впливає на морфологічний стан печінки і підшлункової залози у щурів з індукованим гострим холангітом.

Ключові слова: С60-фулерен, гострий холангіт.

ВЛИЯНИЕ ВОДНОГО КОЛЛОИДНОГО РАСТВОРА C_{60}-ФУЛЛЕРЕНА НА МОРФОЛОГИЧЕСКОЕ СОСТОЯНИЕ ПЕЧЕНИ, ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ И АМИНОТРАНСФЕРАЗНУЮ АКТИВНОСТЬ СЫВОРОТКИ КРОВИ КРЫС ПРИ ОСТРОМ ХОЛАНГИТЕ

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Целью работы было исследовать влияние C_{60}-фуллерена на морфологическое состояние печени и поджелудочной железы при интраперитонеальном и интрагастральном введении на модели экспериментального холангита крыс. Острый холангит воспроизводили однократным интрагастральным введением α-нафтилизотиоцианата — ANIT. Водный коллоидный раствор C_{60}-фуллерена (C_{60}FAS, 0,15 мг/мл) вводили животным в объеме, эквивалентном количеству C_{60}-фуллерена на 0,5 мг/кг массы тела, через 24 и 48 ч после введения ANIT. Через 72 ч после начала опыта животных подвергали эвтаназии путем цервикальной дислокации после анестезии. В крови животных измеряли уровень АЛТ и АСТ. Печень и поджелудочную железу анализировали на светооптическом уровне. Установлено, что интрагастральное и интраперитонеальное введение C_{60}FAS способствует коррекции негативных эффектов в печени и поджелудочной железе, вызванных индукцией острого холангита. Свидетельством этого является нормализация уровня активности АЛТ, уменьшение отека паренхимы поджелудочной железы и фиброза печени, а также усиление кровенаполнения этих органов. Применение C_{60}FAS положительно влияет на морфологическое состояние печени и поджелудочной железы у крыс с индуцированным острым холангитом.

Ключевые слова: С60-фуллерен, острый холангит.