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Water-soluble pristine $C_{60}$ fullerene attenuates acetaminophen-induced liver injury
Water-soluble pristine $C_{60}$ fullerene attenuates acetaminophen-induced liver injury

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Abstract

**Introduction:** Oxidative stress has been suggested as the main trigger and pathological mechanism of toxic liver injury. Effects of powerful free radical scavenger $C_{60}$ fullerene on rat liver injury and liver cells (HepG2 line) were aimed to be discovered.

**Methods:** Acute liver injury (ALI) was simulated by single acetaminophen (APAP, 1000 mg/kg) administration, on a chronic CLI, by 4 weekly APAP administrations. Pristine $C_{60}$ fullerene aqueous colloid solution ($C_{60}$FAS; initial concentration 0.15 mg/mL) was administered per os or intraperitoneally at a dose of 0.5 mg/kg (ALI) or 0.25 mg/kg (CLI) daily for 2 or 28 days, respectively, after first APAP dose. Animals were sacrificed at 24th hour after the last dose. Biochemical markers of blood serum and liver autopsies were analyzed. EGFR expression in HepG2 cells after 48-hour incubation with $C_{60}$FAS was assessed.

**Results:** Increase of serum conjugated and unconjugated bilirubin (up to 1.4-3.7 times), ALT (by 31-37%), and AST (by 18%) in non-treated ALI and CLI rats were observed, suggesting the hepatitis (confirmed by histological analysis). Liver morphological state (ALI, CLI), ALT (ALI and CLI), bilirubin (CLI), α-amylase, and creatinine (ALI) were normalized with $C_{60}$FAS administration in both ways, which may indicate its protective impact on liver. However, unconjugated bilirubin sharply increased in ALI animals receiving $C_{60}$FAS (up to 12 times compared to control), suggesting the augmentation of bilirubin metabolism. Furthermore, $C_{60}$FAS inhibited EGFR expression in HepG2 cells in a dose-dependent manner.

**Conclusion:** $C_{60}$FAS could partially correct acute and chronic toxic liver injury, however, it could not normalize bilirubin metabolism after acute exposure.

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Introduction

Toxic liver injury includes a wide range of diseases associated with hepatotoxic effects of substances of various origins, which cause morphological changes in liver tissue and associated metabolic disorders. Hepatotoxic effects are inherent in some domestic and industrial chemicals, pesticides, alcohol, a number of medications, nutritional supplements, herbal products, and the like. Toxic hepatitis is an inflammatory disease of liver caused by above agents. It may develop within hours or days after exposure to toxicant. In other cases, the development of symptoms may last for months of regular exposure. The incidence of toxic hepatitis is 10-15 per 10000 in general population.1

Drug-induced liver injury (DILI) contributes the most to this category of diseases because of leading role of the liver in drug metabolism and biotransformation. The ability to cause liver damage is implicated in more than 900 medications and is the most common reason for drug withdrawal from the pharmaceutical market.2 DILI accounts for approximately 10% of all cases of acute hepatitis and approximately 2% of chronic liver disease and cirrhosis. It is responsible for over 50% of all cases of acute liver failure (ALF).3,4 Symptoms of drug-induced damage encompass almost all known liver lesions - both acute and chronic. Acute lesions may be cytotoxic (hepatocellular) or cholestatic. Chronic ones include hepatocellular,
cholestatic, vascular, or neoplastic manifestations.

The production of free radicals such as reactive oxygen and nitrogen species has been considered as an early event of drug hepatotoxicity and an indicator of hepatotoxic potential. A lot of drugs can cause oxidative stress in the liver, in particular the increase in the number of cellular prooxidants, lipid peroxidation, and the depletion of a pool of antioxidants. The oxidative stress induces irretrievable alteration of lipids, proteins, and DNA and therefore triggers hepatic damage. Moreover, it also modulates the signal pathways controlling normal biological functions. Since these pathways regulate genes transcription, protein expression, cell apoptosis, and hepatic stellate cell activation, oxidative stress is considered to be one of the pathological mechanisms leading to the initiation and progression of various liver diseases (chronic hepatitis, alcoholic liver disease, and non-alcoholic steatohepatitis (NASH) etc.). Moreover, systemic oxidative stress arising during liver disease can also cause damage of extra-hepatic organs, such as pancreatic and renal failure and even brain impairment.1,4,16

A lot of studies demonstrate that agents having antioxidant activity also exhibit hepatoprotective properties.6,7 Some natural and nutritional antioxidants such as vitamins C and E, zinc, selenium, coenzyme Q10, methionine, alpha lipoic acid, N-acetylcysteine, and silymarin are undergoing the clinical trials in phases II/III and even IV.8-14 Some of them have been approved for treating patients with fatty liver disease and NASH in some countries.15,16 Thus, antioxidant therapy could be an effective approach for the prevention and treatment of liver disease.

An important area of research in modern medicine concerns nanomaterials as promising agents with a wide range of medical applications. Nanomaterials are currently used as carriers to deliver drugs and other substances to specific types of cells.17 Nanoparticles as part of chips with immobilized bioactive molecules are used in diagnostic techniques.18 Carbon, metal and synthetic nanomaterials are utilized in tissue engineering including organ transplantation and tissue regeneration.19 Carbon-based nanomaterials attract the attention as the most “biocompatible” ones. Among them fullerenes take one of the leading places.20

C_{60} fullerene (C_{60}) is almost a spherical molecule whose surface consists of 60 carbon atoms. The C_{60} spherical surface contains 20 hexagonal and 12 pentagonal structures. Bonds between adjacent hexagons are double, and those between hexagons and pentagons are single. Thus, hexa- and pentagon units are connected by a conjugated π-electron system.21 The diameter of the C_{60} molecule is 0.72 nm, which is close to that of polypeptides’ α-helix and the steroid molecules. Thus, the steric compatibility of C_{60} with biological structures such as receptor recognizing sites or enzyme active centers may be suggested. Non-modified (pristine) C_{60} is hydrophobic molecule and is able to be embedded into biological membranes and thus to penetrate into the cell.22 Therefore, C_{60} is discovered as a promising drug carrier due to its ability to be functionalized with different molecules and therapeutics on the one hand, and to penetrate the cell membrane on the other hand.23,24 It also possesses antiviral activity because of steric compatibility with hydrophobic cavity of viral enzymes.25 C_{60} also can be used as potential photosensitizer in photodynamic therapy due to its ability to generate reactive oxygen species (ROS) after photoradiation.26,27 It should be noted that double bonds in the C_{60} framework are electron deficient, which determines the electron-acceptor properties of the molecule and its ability to easily attach reagents containing unpaired electrons (i.e. free radicals).28 Due to this ability, C_{60} could act in biological systems as scavenger of free radicals, in particular hydroxyl and superoxide anions, which results in its anti-tumor, anti-inflammatory and hepatoprotective properties.22,29-34 In an aqueous solution, pristine C_{60} forms nanosize clusters.35,36 Additionally, water-soluble pristine C_{60} is non-toxic in in vitro and in vivo systems at least at low concentrations37,38 and can be accumulated in liver.39 Therefore, it is suggested to be a potential treatment of this organ.

In our previous studies we demonstrated that C_{60} realized anti-inflammatory and hepatoprotective effects on a model of acute colonic inflammation.32 We also showed the ability of C_{60} to improve liver biochemical parameters and histological state, particularly to diminish liver inflammation and fibrotic degeneration, under α-naphthylisothiocyanate-induced acute cholangitis.40 In addition, Halena et al39 revealed that C_{60} could prevent CCl{sub 4}-induced acute liver injury (ALI). However, they used the dose of C_{60} exceeded than that in the current experiment in 3 times. Some studies demonstrated the genotoxic and prooxidative effect of C_{60} realized in a dose-dependent manner,11,12 whereas another ones – at least no prooxidative and rather antioxidant effects.41 Thus, the possible effect of lower dose of C_{60} deserves to be investigated. Furthermore, all mentioned works described the impact of C_{60} under acute liver pathology. As the processes and mechanisms of development of chronic liver injury and liver adaptation differ from those of acute disease,42 the chronic action of the substance might differ from acute one. Moreover, if any substance was applied for a prolonged period, possible cumulative effect also should be taken into consideration.

Thus, the purpose of this work was to study the effect of water-soluble biocompatible pristine C_{60} on the rat liver function under its acute and chronic toxic injury and on liver cells in vitro as well as to evaluate the ability of these nanoparticles to prevent extrahepatic complications.

Materials and Methods

Chemical reagents

Acetaminophen (Merck, Darmstadt, Germany) was
used for the simulation of liver injury. Reagent kits for detection of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total and direct (conjugated) bilirubin, creatinine, urea (Filisit diagnosis, Dnipro, Ukraine), alkaline phosphatase (ALP), α-amylase, triglycerides and total protein (Diagnosticum Zrt, Budapest, Hungary) were used in biochemical assays. Ethanol, acetic acid (Henan Bright Commercial Co., Zhengzhou, China), picric acid, formalin (Biopharma, Kyiv, Ukraine), paraffin, hematoxylin, eosin, orange G (Merck, Darmstadt, Germany) were used in histological assays. Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine (Merck, Darmstadt, Germany), and gentamicin (Biopharma, Kyiv, Ukraine) were used for cell culturing. Epidermal growth factor receptor (EGFR) monoclonal antibodies and reagent kit for immunohistochemical visualization (Dako, Santa Clara, CA, USA) were used in immunohistochemical assay. Au(111) films (SPI Supplies, West Chester, PA, USA) were used for scanning tunneling microscopy (STM). We used following equipment: light microscope Olympus BX-41 (Olympus Europa GmbH, Munich, Germany), spectrophotometer ULAB 101 (Ulab, Kyiv Ukraine), CO2 incubator (Memmert GmbH, Schwabach, Germany), STM (NT-MDT Spectrum instruments, Moscow, Russia), and Zetasizer Nano-ZS90 (Malvern, Worcestershire, UK).

Preparation and characterization of C60 fullerene aqueous colloid solution

A highly stable C60 fullerene aqueous colloid solution (C60FAS) was prepared according to the protocols described before.36 The initial concentration of C60 was 0.15 mg/mL.

Dynamic light scattering (DLS) and zeta potential measurements (ζ) were used for ascertaining the hydrodynamic size and electrokinetic potential of C60 particles in a prepared C60FAS. Measurements were conducted on Zetasizer Nano-ZS90 at 25°C. Obtained results were evaluated using the Smoluchowski approximation, which is known to be rigorously valid only for spherical-like particles.

Samples for STM analysis were obtained after deposition of C60 on an Au(111) surface by precipitation from the droplet of corresponding stock solutions. STM measurements were performed after complete evaporation of the solvents. The morphology of C60 particles in deposited films was monitored using STM technique. Typical values of the tunneling current and voltage were chosen within the ranges of 0.01–0.1 nA and 0.1–0.8 V, respectively.

Animals

Studies were conducted using 64 Wistar male rats with the initial body weight of 200±10 g (acute trial) and 130±10 g (chronic one), which were kept in vivarium of Taras Shevchenko National University of Kyiv at natural light on a standard diet with free access to drinking water. All experiments were conducted in compliance with bioethic 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.

Design of the studies

ALI was simulated by a single oral administration of APAP at a dose of 1000 mg/kg45 dissolved in sunflower oil (total volume 0.5 mL). In 5 and 24 hours after APAP dose, animals were administered with C60FAS in a volume corresponding to the amount of C60 0.5 mg/kg body weight (total received dose equal to 1 mg/kg) either intraperitoneally (i.p.) or per os (p.o.) (Table 1).

To induce chronic liver injury (CLI), animals were ingested with APAP weekly for 28 days at a dose corresponding to that in acute study. In 24 hours, animals were administered with C60FAS in a volume corresponding to the amount of C60 0.25 mg/kg body weight daily during 28 days (total received dose equal to 5 mg/kg) (Table 1). All manipulations with animals in comparison groups were conducted similarly to animals of experimental ones, including the administration of solvents.

As acute and chronic effects of C60FAS on healthy animals were previously investigated and described,32,39 we did not include the description of these groups in the manuscript. In 24 hours after the last dose, animals were euthanized by inhalation of CO2 and subsequent cervical dislocation.

Biochemical assays

The blood for biochemical analysis was collected immediately after the sacrifice from the femoral vein, left for 20 minutes to form a clot and then centrifuged for 10 minutes at 1000 g. ALT, AST, ALP and α-amylase enzymes, total and direct (conjugated) bilirubin, triglycerides, creatinine, urea and total protein as markers of liver, kidneys and pancreas functional states were determined in blood serum according to the protocols provided by the manufacturers.

Histological assay

The liver fragments were harvested immediately after the sacrifice and fixed in Bouin’s mixture for 14 days for histological assay. Then, they were embedded into paraffin, sliced into 5 μm sections, stained with hematoxylin-eosin-orange, and examined under the light microscope. States of liver centrilobular and periportal areas were estimated separately.

In vitro immunohistochemical assay

HepG2 cells were cultured under standard conditions

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(37°C, 5% CO₂, 95% humidity), in DMEM containing 10% FBS, 2 mM L-glutamine, and 40 mg/mL gentamicin. To evaluate the EGFR expression, the cells were seeded on coated glass in 6-well plates with 2×10⁴ cells/cm² density and used in 48 hours of cultivation. Cells were incubated in medium containing 10 or 100 μg of C₆₀ FAS per mL for 48 hours. Immunohistochemical assay was performed using primary EGFR monoclonal antibodies according to the protocol provided by the manufacturer. In addition, specimens were stained with hematoxylin for nuclei visualization. The number of immunopositive cells was counted in 10 random microscopic fields of view for each sample using a standard scale of measurement (object-micrometer) at the same magnification and calculated as a percentage of the total cell number taken for 100%. At least 200 cells were counted.

### Statistical analysis

The Gaussian distribution of the data was estimated using Shapiro-Wilk test. As the distribution was normal, the statistical analysis of data was performed using one-way analysis of variance (ANOVA) with the Tukey post hoc test. The difference was considered statistically significant at $P<0.05$.

### Results

#### DLS and STM studies

To fully understand the bioactivity of prepared water-soluble C₆₀ particles, we examined their size distribution profile and electrokinetic potential ($ζ$ potential) value with DLS technique. In C₆₀ FAS, C₆₀ have the hydrodynamic diameter of 65 nm. Tested C₆₀ nanoparticles demonstrated a slightly negative average surface charge ($ζ=-13.70$ mV), indicating a further tendency for their aggregation. Finally, a studied nanoparticle system with polydispersity index value 0.25 was indicated that has moderately disperse distribution.

STM image of particles deposited on Au(111) surface from C₆₀ FAS is presented in Fig. 1. The height of particles was estimated along the cross-section line (Z-profile) marked by yellow (Fig. 1 top). As one can see, C₆₀ FAS in addition to single C₆₀ molecules (~0.72 nm) also contains nanoparticles (C₆₀ aggregates) with a height more than 1.2 nm (Fig. 1 bottom).

#### Acute liver injury

The liver of ALI animals demonstrated no visible lesions. However, the blood vessels overflow and sinusoid hemocapillars elevation was observed at light microscopy level. Signs of hepatocyte necrosis, protein and lipid dystrophy were also detected, as well as the ground-glass hepatocytes occurrence, lymphocytic infiltration and fibrosis of portal tracts (Fig. 2, Table 2). Observed features have been suggested to be typical for the acute hepatitis.⁴⁶

Under acute APAP exposure, ALT, AST, and unconjugated (indirect) bilirubin increased by 37%, 18% and 185%, respectively, as well as conjugated (direct) bilirubin and ALP which increased by 34% and 64%, respectively (Table 1).

### Table 1. Experimental groups

| Experimental groups | Study, duration | Acute, 48 h | Chronic, 28 days |
|---------------------|----------------|------------|-----------------|
| Control, n=8        |                | -          | -               |
| APAP, n=8           | 1000 mg/kg once| -          | 1000 mg/kg 4 times weekly |
| APAP+C₆₀ FAS i.p., n=8 | 0.5 mg/kg 2 times (in 5 and 24 h after APAP) i.p. | 0.25 mg/kg daily during 28 days i.p. |
| APAP+C₆₀ FAS p.o., n=8 | 0.5 mg/kg 2 times (in 5 and 24 h after APAP) p.o. | 0.25 mg/kg daily during 28 days p.o. |

Note. APAP: acetaminophen, C₆₀ FAS: C₆₀ fullerene aqueous colloid solution, i.p.: intraperitoneally, p.o.: per os.
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14% respectively ($P>0.1$), suggesting the damage of hepatocytes. The level of creatinine also tended up by 15% ($P>0.1$), which might indicate renal dysfunction. Additionally, triglycerides level was diminished by 48%, maybe due to the impairment of their absorption from the gastrointestinal tract and/or a violation of their synthesis in liver and, consequently, insufficient entering into the blood. The level of total protein was unchanged. The serum α-amylase was significantly lower (23%) compared to control, which is a typical phenomenon of acute and chronic hepatitis (Fig. 3).46

Blood vessels and hemocapillars dilation, signs of hemostasis, and thrombosis were observed in ALI rats receiving C60FAS by both ways. However, hepatocytes protein dystrophy was weaker compared to APAP group and lipid dystrophy was not detected. Moreover, less number of necrotic cells was found. In addition, areas of fibrosis and lymphocytic infiltration surrounding portal tracts were diminished under C60FAS i.p. (Fig. 2, Table 2).

In C60FAS-received ALI rats, either i.p. and p.o. ALT rates closed to control values, indicating reduction of hepatocyte cytolysis. At the same time, AST rates remained elevated (by 21% compared to control), the unconjugated bilirubin even more increased (up to 12 and 8 times compared to control and up to 4 and 3 times compared to APAP group for C60FAS i.p. and p.o., respectively), which might indicate a bilirubin metabolism disorder. An increase of unconjugated bilirubin fraction without significant changes in conjugated one may be the consequence of increased bilirubin production (due to hemolysis) or a violation of its hepatic absorption and/or conjugation. C60 has been shown to have weak hemolytic properties,47,48 which might be the reason of observed changes. On the other hand, it has been established that the increase of unconjugated bilirubin may also occur due to liver function disorders.49 Hyperbilirubinemia may follow as increased bilirubin production, as abnormalities of any stage of its metabolism, including capture from the blood, intracellular conjugation and biliary excretion. Unconjugated bilirubin elevation is often caused by drugs affecting any stage of its metabolism. Usually, the effect of those on the uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase) catalyzing the conversion of unconjugated bilirubin into conjugated one by conjugation with glucuronic acid is taken into account. Although, according to Shipelin et al.,51 C60 does not affect the activity of this enzyme, it can affect another bilirubin metabolic pathways, in particular, its transport within the hepatocyte via glutathione-S-transferase. Recently, we have shown that C60 could inhibit its activity.32

C60FAS when administered by both ways also caused reduction of serum triglycerides and normalized α-amylase and creatinine, indicating the pancreatic and renal function restore (Fig. 3). Serum triglycerides decrease might be an indirect consequence of C60 antioxidant properties; Halim et al.31 showed that the therapeutic use of antioxidants leads to triglycerides accumulation in the

![Fig. 2. Microphotographs of the liver for ALI rats received C60FAS: 1 – control; 2 – APAP; 3 – APAP+C60FAS i.p.; 4 – APAP+C60FAS p.o.; A and B - centrilobular and perportal areas, respectively. Hematoxylin-eosin-orange staining, ×400, scale 100 μm. Sinusoids and blood vessels dilation (2 A, 3 A, 4 A), lymphocytic infiltration of portal tracts (2 B, 4 B) and surrounding tissue (2 B), hepatocyte necrosis (2 A).](image)

### Table 2. Pathological changes of liver of ALI rats receiving C60FAS

| Pathological changes                  | Control | APAP | APAP+C60FAS i.p. | APAP+C60FAS p.o. |
|--------------------------------------|---------|------|-----------------|-----------------|
| Protein dystrophy of hepatocytes     | -       | ++   | +               | +               |
| Lipid dystrophy of hepatocytes       | -       | ++   | +               | -               |
| Ground-glass hepatocytes             | -       | ++   | +               | +               |
| Necrotic hepatocytes                 | -       | +++  | +               | +               |
| Connective tissue accumulation       | -       | ++   | ++              | ++              |
| Lymphocytes accumulation             | +       | +++  | ++              | +++             |
| Blood vessel dilation                | -       | ++   | ++              | ++              |

*Note. APAP: acetaminophen, C60FAS: C60 fullerene aqueous colloid solution, i.p.: intraperitoneally, p.o.: per os.; trait intensity: “-“ – not observed, “+“: single or slight, “++“: moderate, “+++“: strong.*
liver and, consequently, to decline their levels in blood serum.

**Chronic liver injury**

The liver of CLI rats was spotty, had dilated large blood vessels, suggesting the macroscopic features of hepatitis. Pathological changes were aggravated compared to acute ones: blood vessels and sinusoid hemocapillars dilation, thrombosis and hemostasis, hepatocytes protein and lipid dystrophy, necrotic and apoptotic cells, a lot of ground-glass hepatocytes, portal tracts' fibrosis and lymphocytic infiltration were observed (Fig. 4, Table 3).

![Fig. 3](image-url) Serum biochemical markers of liver, pancreas and kidney functional states for ALI rats received C₆₀ FAS normalized against control: 1 – control; 2 – APAP; 3 – APAP+C₆₀ FAS i.p.; 4 – APAP+C₆₀ FAS p.o.; *P<0.05 compared to control, #P<0.05 compared to APAP.

![Fig. 4](image-url) Microphotographs of the liver for CLI rats received C₆₀ FAS: 1 – control; 2 – APAP; 3 – APAP+C₆₀ FAS i.p.; 4 – APAP+C₆₀ FAS p.o.; A and B - centrilobular and periportal areas, respectively. Hematoxylin-eosin-orange staining, ×400, scale 100 µm. Sinusoids and blood vessels dilatation and overflow (2 A, 4 A), lymphocytic infiltration of portal tracts and surrounding tissue (2 B), hepatocyte necrosis (2 A)
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ALT activity increased (by 31%), as well as conjugated and unconjugated bilirubin (up to 2 and 3.7 times, respectively), indicating chronic hepatitis development. Creatinine and α-amylase tended up (by 23% and 25%, respectively, P<0.1), suggesting the kidney and pancreas function impairment. At the same time, urea decreased by 22% (Fig. 5), while other parameters were unchanged. Creatinine and α-amylase increased significantly (by 37% and 34%, respectively), which might indicate renal and pancreatic dysfunction deterioration compared to non-treated CLI animals. Necrotic and apoptotic foci were local, fibrosis and lymphocytic infiltration of portal tracts decreased significantly. However, signs of hepatocytes dystrophy still occurred (Fig. 4, Table 3). ALT, unconjugated bilirubin, and urea restored to control values, but conjugated bilirubin remained elevated. Moreover, creatinine and α-amylase increased significantly (by 37% and 34%, respectively), which might indicate renal and pancreatic dysfunction deterioration compared to non-treated CLI animals. Serum triglycerides tended up (by 17%, p>0.1) and ALP – declined (by 50%) (Fig. 5).

Under C₆₀FAS p.o. action, hepatic dystrophy was lesser compared to non-treated CLI animals, lymphocytic infiltration and fibrosis of portal tracts also were diminished. However, blood vessels and hemocapillars remained dilated and overflown, with no thrombosis compared to non-treated CLI animals. Necrotic and apoptotic foci were local, fibrosis and lymphocytic infiltration of portal tracts decreased significantly. However, signs of hepatocytes dystrophy still occurred (Fig. 4, Table 3). ALT, unconjugated bilirubin, and urea restored to control values, but conjugated bilirubin even more increased (up to 6 times compared to control). Creatinine and urea were maintained at the level of APAP group. Serum α-amylase and triglycerides also increased (by 35% and 87%, respectively) in these animals like under C₆₀FAS i.p. application (Fig. 5).

EGFR expression

C₆₀FAS inhibited the expression of EGFR in HepG2 cells after 48 hours incubation in a dose-dependent manner (Fig. 6): there were 41±2% immunopositive cells in control and only 25.3±1.3% and 12.3±0.6% in C₆₀FAS 10 μg/mL and C₆₀FAS 100 μg/mL groups, respectively.

Discussion

APAP is one of the most widely used analgesic and antipyretic medications. It is safe and effective if used at recommended doses, while overdose can lead to hepatotoxicity and ALF. APAP-induced hepatotoxicity is the most common cause of ALF. APAP is metabolized in liver, predominantly via UDP-glucuronosyltransferase and sulfotransferase, to non-toxic urine-excreted compounds. However, 5-9% of APAP is metabolized by cytochromes P450, preferably CYP 2E1, to the highly reactive metabolite N-acytely-p-benzoquinone imine (NAPQI). Usually, NAPQI rapidly conjugates with glutathione, but APAP overdose depletes hepatic glutathione pool, an excessive amount of NAPQI covalently binds to sulfhydryl groups of cellular proteins, in particular mitochondrial ones, which ultimately result in mitochondrial dysfunction and oxidative stress, and lead to hepatocytes necrosis. Generally, biotransformation of certain xenobiotics by the enzymes of CYP family is accompanied by the production of short-lived highly reactive metabolites, such as electrophiles, nucleophiles, free radicals, and redox-active reactants. These chemical species can covalently bind cellular proteins and lipids, and in case of overproduction cause damage of cellular organelles, including mitochondria, and lead to development of oxidative stress, endoplasmic reticulum stress, hepatocytes apoptosis and necrosis. These events have been suggested the background of liver toxic injury.

The assessment of antioxidants effect under toxic liver injury, in particular DILI, viral and non-viral hepatitis, alcoholic liver disease and NASH were aimed to be discovered in numerous in vitro and in vivo studies. The therapeutic efficacy of vitamins C and E, silymarin, N-acetylcysteine, glycyrrhizin and ursodeoxycholic acids, plant extracts and antioxidant mixtures were
demonstrated in combination therapy and separately. Nevertheless, their clinical effectiveness still remains unproved. The only drug approved in clinical practice for DILI treatment is N-acetylcysteine - APAP specific antidote. However, this remedy is less effective in case of non-APAP ALF. In the setting of DILI with autoimmune features and hypersensitivity reactions, corticosteroids have been used predominantly. It should be noticed that all the abovementioned non-hormonal agents possess antioxidant properties which contribute to their therapeutic activity. These chemicals can affect the signaling pathways associated with the production and/or neutralization of free radicals and proinflammatory cytokines and, moreover, can directly scavenge free radicals. Pristine \( \text{C}_{60} \) is one of the most powerful free radical scavengers. One \( \text{C}_{60} \) molecule is able to bind up to 34 radicals depending on their size. Therefore, we suggest that therapeutic effects of \( \text{C}_{60} \) might be realized through its strong antioxidant properties. Indeed, the ability of \( \text{C}_{60} \) to inhibit lipid peroxidation and to stimulate antioxidant defense enzymes under \( \text{CCl}_4 \)-induced ALI were shown. We also proved this ability of \( \text{C}_{60} \) on model

**Fig. 5.** Serum biochemical markers of liver, pancreas and kidney functional states for CLI rats received \( \text{C}_{60} \) normalized against control: 1 – control; 2 – APAP; 3 – APAP+\( \text{C}_{60} \) i.p.; 4 – APAP+\( \text{C}_{60} \) p.o.; *P<0.05 compared to control, **P<0.05 compared to APAP.

**Fig. 6.** EGFR expression in HepG2 cells after 48 h incubation with \( \text{C}_{60} \): 1 – control; 2 – \( \text{C}_{60} \) 10 µg/mL; 3 – \( \text{C}_{60} \) 100 µg/mL. Antibodies V9 clon (IS630, Dako, USA) and hematoxylin staining, ×400, scale 100 µm
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We have shown that C60 could suppress EGFR expression in HepG2 cells. EGFR is known to be involved in liver regeneration. Its upregulation is tightly related to liver fibrogenesis and fibrosis and cirrhosis development, and its downregulation attenuates the fibrotic lesions. Therefore, mitigation of peripoortal fibrosis under C60-FAS chronic exposure might be through inhibition of EGFR expression by that. Additionally, the rapid downregulation of EGFR under acute APAP-induced liver injury diminishes liver damage and inhibits cell necrosis possibly by another mechanism. Hence, it was shown that immediately after APAP application (within 1-4 h) EGFR is overexpressed and upregulated and translocated in mitochondria, and the intensity of these events correlates with the rate of liver necrotic lesions. Moreover, the administration of EGFR inhibitor in this period reduces and even eliminates completely APAP toxic effects on liver. Authors explained this phenomenon by the fact that EGFR activation is involved in apoptosis through its ability to phosphorylate "death receptor" (CD95) and stabilize the death-inducing signaling complex. Accordingly, inhibition of EGFR results in inhibition of apoptosis. Therefore, we might suggest that inhibition of EGFR could contribute to hepatoprotective effect of C60.

Some studies revealed that C60 could realize prooxidative effects in biological systems and cause oxidative DNA damage. However, these effects strongly depend on C60 nanoparticles size and dozing. The C60 system we have used in the current study did not demonstrate any toxic or another effects which could be interpreted as prooxidative ones. Nevertheless, possible accumulation of C60 in tissues and organs especially after prolonged exposure should not be neglected. Therefore, local high amount of C60 in some organs, for instance in pancreas, might occur. The last could cause observed negative effects like renal and pancreatic hypofunction.

Conclusion

We concluded that C60-FAS application reduces the hepatocytes cytolysis and restores the liver morphological state, but affects the bilirubin metabolism through APAP-induced ALI. C60-FAS also contributes to the recovery of renal and pancreatic functions.

Under chronic APAP exposure, C60-FAS attenuates the dystrophic processes in hepatocytes and manifestations of apoptosis and necrosis, as well as normalizing the values of most liver functional biochemical markers. However, renal and pancreatic dysfunction features also occur, suggesting the possible cumulative effects of C60. Therapeutic effects of C60 might be realized through its ability to scavenge free radicals and affect the expression of EGFR.

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Conflicts of interest

Authors declare no conflicts of interest.

Ethical statement

All experiments with animals use 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.

Authors’ contribution

The work presented here was carried out in collaboration between all authors. YP and UR prepared and characterized C60-FAS. HK and ND performed in vivo experiments, biochemical study and statistical analysis. OL performed histological study. TH performed in vitro experiment and immunohistochemical study. YP and VR coordinated the experimental work and analyzed data. HK wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Research Highlights

What is the current knowledge?

- Toxic liver injury is accompanied by oxidative stress and has no universal and effective treatment.

What is new here?

- Water-soluble pristine C60 fullerenes as the powerful free radical scavengers reduce the local and systemic manifestations of toxic liver injury in rats.
- C60 fullerenes inhibit EGFR expression in HepG2 cells.
C60 fullerene attenuates liver toxic injury

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