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BIOCOMPATIBILITY OF C_{60} FULLERENE WITH THE HEMOSTATIC SYSTEM

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Molecular compounds containing allotropic forms of carbon are intensively studied in various areas of science, such as nanobiotechnology, biomedicine, and pharmacology, for their practical application prospects. The most well-known carbon-based nanoparticles are the fullerene C_{60}, a spherical cage with distinctive physical, chemical, and biological characteristics (stability, biocompatibility, and antioxidant, antitumor, and photosensitizing properties). However, understanding the mechanisms by which these particles influence the body's regulatory and integral systems is necessary to prevent potential human health risks in clinical practice.

Aim. The impact of fullerene C_{60} on the cardiovascular system was examined, with particular focus on hemostasis, to assess its safety for biomedical applications.

Materials and Methodos. The study focused on the platelet, coagulation, anticoagulation, and fibrinolysis pathways of hemostasis.

Results. Research indicates that fullerene C_{60} at therapeutic doses of 0.1 μM and 1.0 μM does not affect the functional activity, shape, or granularity of human platelets during co-cultivation. When present, measurements such as PTT, APTT, thrombin activity, factor Xa, plasma protein C, and the overall hemostatic potential of blood plasma remain stable.

Conclusions. Fullerene C_{60} may be used in clinical settings as a bioinert carrier that interacts with blood at therapeutic doses without affecting or activating the hemostatic system.

Keywords: fullerene C_{60}, hemostasis, platelets, biocompatibility.

The discovery of fullerenes, carbon nanotubes, and graphene has sparked considerable interest among researchers across many scientific disciplines. Their nanoscale size and ability for diverse surface modifications make them essential materials in nanotechnology [1]. Fullerenes are spherical molecules with a 0.72 nm diameter, composed entirely of carbon atoms. They possess unique physical and chemical properties, exhibit a wide range of biological activities, and have been extensively researched over the past decade to broaden their practical applications [2, 3]. The chemical stability of fullerene structures and their low toxicity have facilitated the development of new technologies in medicinal chemistry, pharmacology, and toxicology. They also facilitate the creation of highly effective sorbents, antioxidants, and agents with antiviral and anticancer properties based on carbon nanomaterials [2].

Fullerene C_{60} is a distinctive organic compound first synthesized in 1985, representing the third pure carbon form after graphite and diamond. The molecule can be imagined as a spherical polyhedron composed of 20 hexagons and 12 pentagons, with carbon atoms at each of its 60 vertices. C_{60} and its

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derivatives are attractive new compounds for medical fields: anionic derivatives of fullerene have antioxidant properties, dicationic ones exhibit antiproliferative activity against various human cancer cell lines, including chemoresistant ones, and derivatives of the proline type inhibit the replication of the human immunodeficiency virus and hepatitis C virus, and can be utilized as multi-target drugs in the future [4].

Unmodified fullerene C_{60} exhibits notable physical and biological properties. For example, it functions as a photosensitizer, antioxidant, bioimaging agent, and can also act as a carrier for genes or drugs [3, 5]. Fullerenes can serve as photosensitizers for photodynamic inactivation (aPDI), offering a new method to counter drug resistance caused by the overuse of antibiotics. It is effective due to its high triplet-state yield, excellent photostability, broad antibacterial spectrum, and versatile functional activity [6]. The antioxidant mechanism involves bioactive, soluble carbon nanostructures that can bind six electrons, making them effective scavengers of reactive oxygen species (ROS). *In vivo* studies demonstrate that C_{60} improves blood parameters (hematological and biochemical) and liver health (histopathological analysis) in albino rats following high-toxic exposure to cyclophosphamide, a cytostatic anticancer chemotherapeutic agent [7].

It might have a combined antitumor effect with doxorubicin (Dox) by preventing Dox-induced oxidative damage that inhibits superoxide dismutase and glutathione peroxidase activities in the liver and heart of mice with Lewis lung carcinoma [8]. A colloidal solution of primary fullerene C_{60} (C_{60}FAS) also affects the balance of pro-oxidants and antioxidants in muscle tissue, aiding the recovery of contraction strength and extending the active working period of the rat's *triceps surae* muscle.

Additionally, fullerenes are among the pioneering classes of carbon-based nanoparticles for targeted drug delivery, as their specific geometry, size, surface characteristics, and spherical, apolar structure enable their use in lipid-like systems [9]. Fullerenes serve as intermediates in the construction of highly sensitive biosensor devices. For instance, C_{60} in organic solvents undergoes five reversible oxidation/reduction steps, enabling it to function as an electrophile or a nucleophile. The nanoparticle remains stable, and its spherical shape induces angular deformation, facilitating characteristic addition reactions to double bonds through a hybridization shift from sp(2) to sp(3) [10].

The use of fullerenes extends beyond clinical applications. Their antioxidant properties offer new opportunities to prevent and address contractile dysfunction in ischemia-damaged muscles, especially in athletes following intensive training sessions [11, 12].

The practical application of C_{60} in biomedical fields is often hindered by its limited water solubility [3]. Monopolar fullerenes, functionalized with hydrophilic groups, make them water-soluble while preserving the hydrophobic fullerene cage [13]. Surface-coated fullerene nanoparticles with cyclodextrin (C_{60}/CD) can generate active oxygen species under visible light, making them suitable for photodynamic tumor therapy. Water-soluble $C_{60}\ (OH)_{10}$CD nanoparticles help protect the liver by absorbing ROS [3].

However, nanomaterials may pose health hazards, posing a significant challenge to their use in clinical settings [1, 14–16]. Moreover, research on the toxicity of nanomaterials significantly lags behind the rapid development of new functional nanoparticles [17]. Currently, reports indicate that some particles adversely affect biological systems and cellular compartments, including non-biodegradability, neurotoxicity, hepatotoxicity, nephrotoxicity, immunotoxicity, cardiotoxicity, genotoxic and epigenetic toxicity, dermatotoxicity, and carcinogenicity [14, 18]. In particular, fullerene C_{60} nanoparticles and their derivative, $C_{60}\ (OH)_{18}$, inhibited mitochondrial function and disrupted the structure and dynamics of the inner mitochondrial membrane [19]. Research on the interaction between the fullerene C_{60} and the β2-adrenergic receptor (β2AR) using molecular dynamics simulations showed a significant impact on the local structure of the β2AR [20]. Intravenous and intratracheal instillation of fullerene C_{60} in Fischer 344 rats resulted in its accumulation primarily in the liver (where the concentration was highest), as well as in the spleen, lungs, and kidneys, potentially leading to adverse effects [21].

Intratracheal administration of multilayered carbon nanotubes to C57Bl/6 mice resulted in a negative impact on the pulmonary and cardiovascular systems (moderate increases in IL-1β and IL-6 levels, an increase in blood neutrophil count, and a significant elevation in factor VIII levels) [22]. A similar administration of C_{60} fullerene nanoparticles to Balb/c mice led to impaired

lung mechanics and mitochondrial function, increased ROS levels, and decreased ATP production [23]. Low toxicity of the C_{60} fullerene aqueous solution against human embryonic kidney (HEK293) cells with an IC_{50} value of 500 µM and no toxic effect of C_{60} fullerene after intraperitoneal administration into mice in the dose range of 75–150 mg/kg with an LD_{50} value of 721 mg/kg was detected [24]. Introduction of carbon nanoparticles into the lungs of healthy volunteers caused systemic inflammation, driven by an increase in circulating neutrophils [25].

However, most published data pertain to individual toxicological phenomena, and the number of studies aimed at understanding the mechanisms of nanomaterial toxicity remains quite limited [15].

Therefore, assessing how fullerene C_{60} impacts the entire organism is crucial today, particularly the circulatory system, which is vital to overall health. As fullerene C_{60} can enter the bloodstream through infiltration or direct medical injection, understanding its interactions with the circulatory system remains a central focus in preclinical research. It is important to carefully evaluate whether biomaterials, such as C_{60}, can affect the functional activity of blood components involved in hemostasis [26]. Hemostasis involves a complex set of functional, morphological, and biochemical mechanisms that maintain blood in a liquid state, prevent and control bleeding, and preserve blood vessel integrity. Under normal conditions, blood remains liquid due to a balance of factors that regulate coagulation and inhibit clot formation. Disruption of this balance can result from various causes. Conversely, blood coagulation is a vital protective response when blood vessels are injured. In this context, we investigated how fullerene C_{60} nanoparticles influence coagulation, anticoagulation, fibrinolysis, and platelet function, all involved in hemostasis.

Materials and Methods

The highly stable C_{60} fullerene aqueous colloid solution (0.2 mM concentration, purity > 99.5%, nanoparticles average size 100 nm) was prepared by ultrasonication of toluene-dissolved C_{60} in the water phase, as described by U. Ritter et al. [27].

The effects of fullerene on platelet aggregation and activation were studied using flow cytometry and aggregometry. Platelet aggregation was examined in platelet-rich plasma (PRP) of human blood ($2–3\times10^5$ platelets/µL). Prior to the study, a screening assessment of donor platelet (de-identified blood samples) aggregation was conducted. If hyporeactivity was detected, the platelet-rich plasma was not used. Aggregometry was performed within the first three hours after blood collection on a photo-optical aggregometer "SOLAR AP2110" in accordance with [140]. In the cuvette, the aggregometry was performed by adding 430 µL of PRP and 20 µL of 0.025 M $CaCl_2$, and incubating *ex temporo* for 5 minutes at 37 °C. Platelet aggregation was initiated by adding one of the platelet aggregation inducers: ADP (at concentrations of 12.5 and 10 µM), collagen (2 µg/mL), and adrenaline (0.8 and 0.6 µg/mL). The aggregation process was recorded for 10 minutes in a mode that did not normalise the measurement results to the initial light transmission level. The degree of aggregation was assessed as the maximum light transmission of PRP after adding the aggregation inducer; the aggregation rate was the rate of change in light transmission of PRP after adding the inducer during the first 30 seconds; the aggregation time was the time to reach the maximum degree of aggregation. For collagen-induced aggregation, the lag phase duration was measured.

Platelet activation was examined using flow cytometry with the Coulter® EPICS XL™ Flow Cytometer ("Beckman Colter", USA). During analysis, two types of light scattering were measured: forward scatter, which indicates cell size, and side scatter, which reflects cytoplasmic density or platelet granularity [143]. One milliliter of washed human platelets, along with $CaCl_2$ to a final concentration of 1 µM, was placed into a flow cytometry tube. Fibrinogenase from *E. multisquamatis* toxin, prepared in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl at 0.007 mg/ml, was added to the platelet suspension. The mixture was incubated at 25 °C for 90 minutes. Changes in platelet activation, size, and granularity were monitored via alterations in direct and orthogonal light scattering [28].

Activation of prothrombin and factor X was determined using a prothrombin activator from the venom of the multi-headed snake Ekamulin and a factor X activator from Russell's viper venom RVV, respectively. For this, 0.01 ml of the tested plasma, 0.015 ml of S2238 (S2275 in the case of factor X), 0.01 ml of $CaCl_2$ solution (0.025 M), and 0.01 ml of Ekamulun (RVV in the case of factor X) were

mixed in 0.05 M Tris-HCl buffer at pH 7.4. The total volume of the reaction mixture was 0.25 ml.

To evaluate the impact of fullerene C_{60} on the anticoagulant system, the activation of protein C in blood plasma was assessed in its presence. Protein C activity was measured using the chromogenic substrate S2236 ("GE-Healthcare", USA). Specifically, 0.03 ml of the blood plasma sample was combined with 0.035 ml of S2236 and 0.05 ml of protein C activator ("Renam", Ukraine) in 0.05 M Tris-HCl buffer at pH 7.4. The total reaction volume was 0.21 ml. The substrate cleavage rate was monitored spectrophotometrically at 405–495 nm using a Multiscan EX reader ("Thermo Electron", Germany).

The covalent linkage was studied using an electrophoretic method. A series of microtubes was prepared with fibrinogen (2 mg/ml), $CaCl_2$ (0.025 M), and fibrin polymerization was induced with thrombin (0.5 NIH/ml). After 30, 60, 90, and 120 minutes, the reaction was stopped by adding a sample buffer containing 0.2% β-mercaptoethanol. The prepared samples were analyzed electrophoretically in 7% PAAG, according to Laemmli's method (1970), in the presence of SDS, on a vertical gel electrophoresis apparatus using 10% PAAG plates. (Laemmli SDS-polyacrylamide gel electrophoresis This protocol describes a denaturing polyacrylamide gel system utilizing sodium dodecyl sulfate (SDS) to separate protein molecules based on size, as first described by Laemmli (1970).

Protein zones were identified after staining with Coomassie R-250.

Samples for electrophoresis were prepared as follows: a protein solution at 1 mg/ml was mixed with a sample buffer containing 5% sucrose or glycerol, 2% SDS, and bromophenol until a color change was observed. The samples were heated to boiling. To restore disulfide bonds, the samples were pre-treated with 5% β-mercaptoethanol for 10 minutes. Separation was carried out at an electric current of 19 mA for the stacking gel and 36 mA for the resolving gel.

The gel for detecting protein-containing areas was stained in a staining solution (0.125% Coomassie G-250 in 25% isopropanol and 10% acetic acid) for 10 minutes. To remove residual dye, a 2–8% acetic acid solution was used. The method's resolution is 1 μg of protein.

To determine the molecular weight of proteins, a mixture of proteins with known molecular weights (Fermentas, European Union) was used: No. SM0671 (170; 130; 100; 72; 55; 40; 33; 24; 17 kDa) and No. SM0431 (116; 66.2; 45; 35; 25; 18.4; 14.4 kDa). For qualitative and quantitative assessment of protein composition in the samples, densitometry of SDS-PAGE gels was performed using the "TotalLab v2.0" software package.

Activation of prothrombin was carried out using an enzymatic blood clotting activator or thromboplastin. The enzymatic activity of the thrombin formed in the test was determined by the cleavage of the thrombin-specific chromogenic substrate S2238 H-D-Phe-Pip-Arg-pNA. For this, 10 μL of donor blood plasma was added to the incubation medium, along with 10 μL of 0.025 M $CaCl_2$, 10 μL of activator, 195 μL of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.13 M NaCl, and 25 μL of 2 mM chromogenic substrate S2238. The incubation was performed at 37 °C for 10 minutes. The amount of cleaved substrate was determined spectrophotometrically at a wavelength of 405 nm using a "Thermo Multiskan EX" microplate spectrophotometer (Finland) [29].

When determining the clotting time of blood plasma in the activated partial thromboplastin time (aPTT) test, 0.1 mL of the blood plasma being tested was placed into a glass conical test tube, and 0.1 ml of aPTT reagent was added. The mixture was heated for 3 minutes at 37°C. After this, 0.1 mL of a 0.025 M calcium chloride solution was added, and the blood plasma clotting time was measured by gently shaking the test tube in a water bath at 37 °C. The clotting time of donor blood plasma in the aPTT test was 5±45 seconds.

The overall hemostatic potential of blood plasma was determined spectrophotometrically ("Titertec Multiscan MC", Finland) by recording the light scattering of the fibrin clot formed in a spectrophotometric cuvette, into which 12 μlL of human blood plasma was sequentially added to 0.05 M HEPES buffer pH 7.4 with 0.15 M NaCl and 0.005 M $CaCl_2$ to a final concentration of 75 IU/mL. Measurements were taken at 350 nm. The clotting process was initiated by adding 5 μl of thromboplastin.

The research complies with international bioethical standards for conducting clinical trials and experimental studies (Protocol N4, 01.04.2016, Committee on Biosafety and Bioethics of the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine).

Results and Discussion

In the first stage of the research, we investigated the effect of fullerene C_{60} on platelet aggregation and granularity. Platelets are highly sensitive cells that are activated upon contact with foreign surfaces [30]. Studying the action of a therapeutic agent on platelets is a necessary condition for its clinical use. These blood cells are the main participants in the process of thrombus formation, as well as significantly influencing other links of haemocoagulation, providing activated phospholipid surfaces necessary for the realisation of plasma haemostasis processes, releasing several coagulation factors into the blood, modulating fibrinolysis, and disrupting haemodynamic constants both through transient vasoconstriction (caused by thromboxane A2 generation) and through the formation and release of mitogenic factors that promote hyperplasia of the vessel wall.

During thrombus formation, platelets trigger glycoprotein activation, phospholipase activation, phospholipid exchange, secondary mediator formation, protein phosphorylation, arachidonic acid metabolism, actin-myosin interaction, and Na^+/H^+ exchange, and then induce adhesion (the initial step of hemostasis), secretion, and aggregation. Both endogenous and exogenous substances cause platelet aggregation: thrombin, collagen, ADP, arachidonic acid, thromboxane A2, prostaglandins G2 and H2, serotonin, adrenaline, platelet-activating factor, and others. Adhesion and aggregation lead to the development of the secretion reaction – a specific Ca^{2+}-dependent secretory process in which platelets release several substances into the extracellular space. The secretion reaction is induced by ADP, adrenaline, subendothelial connective tissue, and thrombin. Initially, the contents of dense granules are released: ADP, serotonin, and Ca^{2+}. For the release of the contents of α-granules (platelet factor 4, β-thromboglobulin, platelet growth factor, von Willebrand factor, fibrinogen, and fibronectin), more intense platelet stimulation is required. Liposomal granules containing acidic hydrolases are released only in the presence of collagen or thrombin [31–34].

The study on the impact of fullerene C_{60} on ADP-induced platelet aggregation found no effect on the extent or rate of aggregation within the concentration range suggested for clinical use (Fig. 1). A minor reduction in aggregation was observed at a C_{60} concentration of 10 µM, which is 10 times the highest therapeutic dose.

The study of platelet shape and granulation in the presence of fullerene C_{60}, using flow cytometry, showed that even after a 2-hour incubation, the cell structure did not change (Fig. 2).

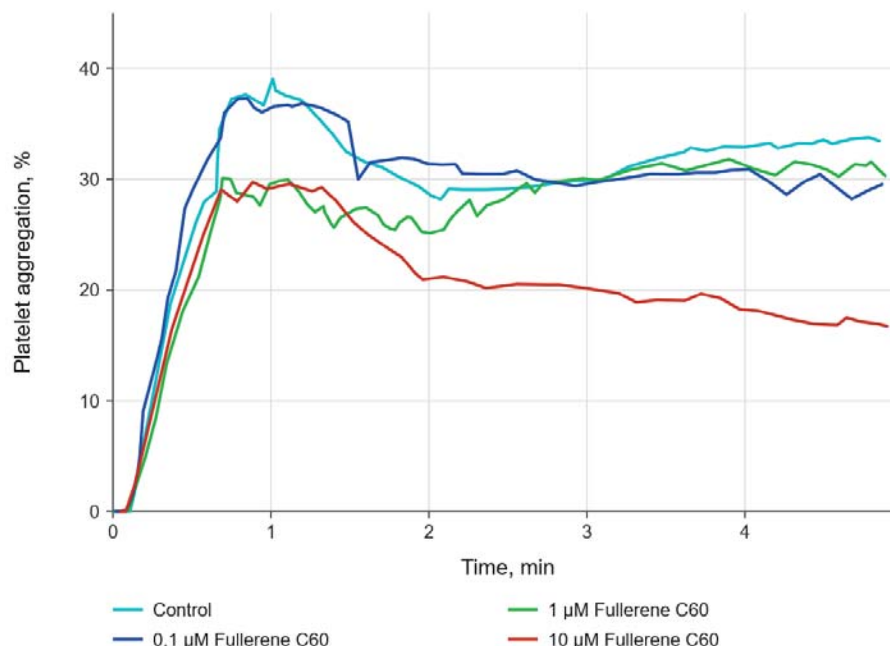

Fig. 1. **Typical ADP-induced aggregation curves of human platelet-rich plasma in the presence of C_{60} fullerene**

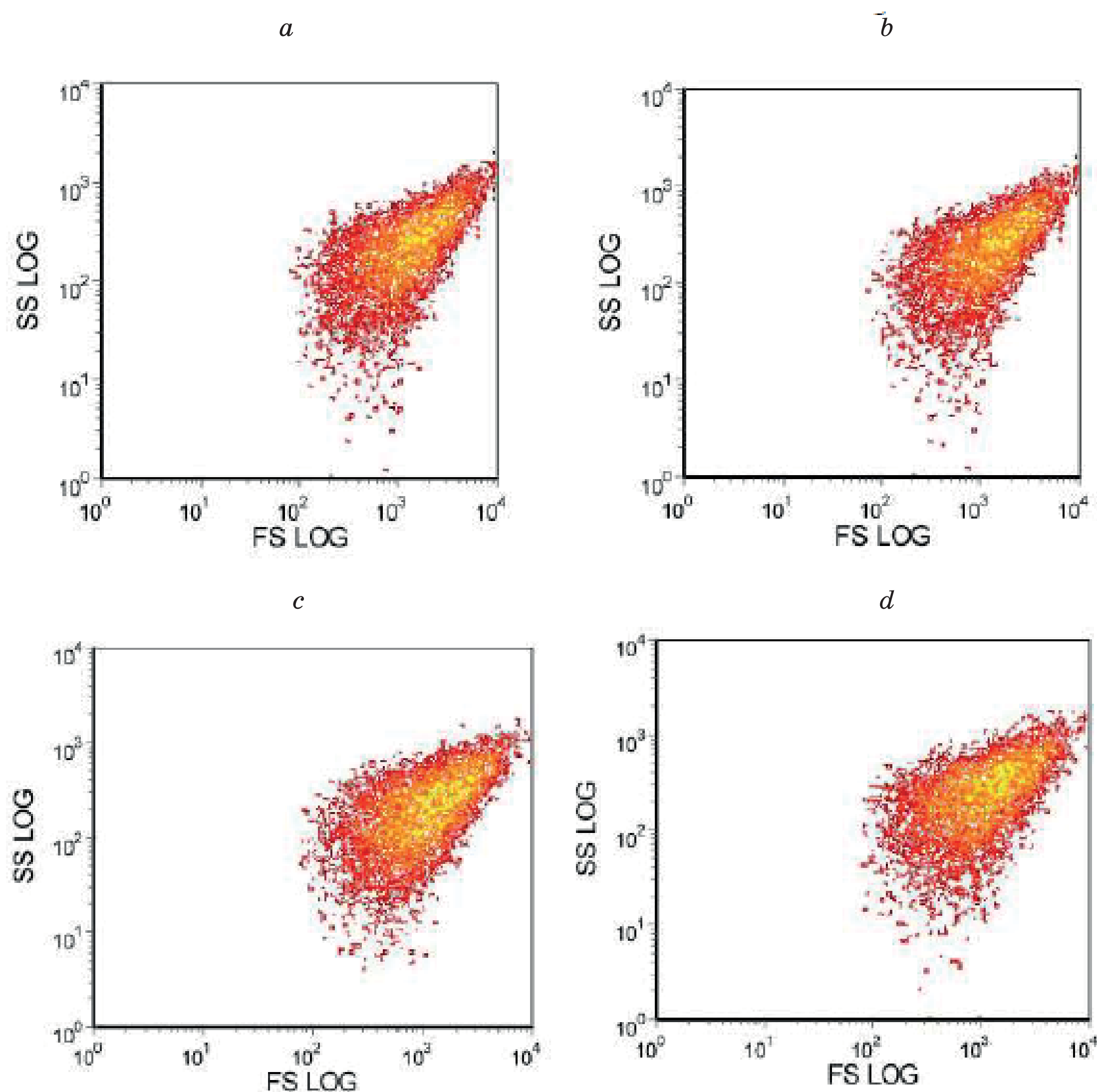

Fig. 2. **Distribution of platelets by size (FS) and granularity (SS) in the presence of 10 µM fullerene C_{60} (*b*) and in control (*a*) after 1 hour (*c*), after 2 hours (*d*) from the start of incubation**

The obtained data indicate no changes in the functional activity and structure of platelets; C_{60} has neither anti- nor pro-aggregant properties and does not affect the platelet component of hemostasis.

To evaluate how fullerene C_{60} influences the blood coagulation cascade, the activation of key proenzymes, prothrombin, and factor X, was measured. These are vitamin K-dependent glycoproteins present in human blood as inactive precursors. Prothrombin (inactive factor II) is converted into thrombin (active factor IIa) through the action of factor X, prothrombinase, and other coagulation factors V, VII, and IV during phase II of hemostasis (contact-kinin cascade activation). In the intrinsic pathway, prothrombinase formation involves plasma factors XII, XI, IX, VIII, X, the kallikrein-kinin system, and platelets. This leads to the formation of a factor Xa and V complex on the phospholipid surface (platelet factor 3) in the presence of Ca^{2+}, which functions as prothrombinase to convert prothrombin into thrombin. In the extrinsic pathway, tissue factor (factor III), expressed on cell surfaces after tissue injury, interacts with factor VIIa and calcium to form a complex that can convert factor X into factor Xa [33, 34].

Our studies have shown that C_{60} fullerene does not affect the activation of prothrombin or factor X by ekamuline or RVV, respectively (Fig. 3, *a*, *b*).

The study of the effect of fullerene C_{60} on fibrin polymerization in blood plasma was conducted using PTT and aPTT tests. As a positive control, calixarene C-145

was used, which exhibits strong anti-polymerization activity. Both compounds were taken at concentrations recommended for therapeutic use. PTT – the time required for fibrin clot formation in plasma upon addition of thrombin. It depends solely on the concentration of fibrinogen (indirectly reflecting its level) and the activity of thrombin inhibitors (ATIII, heparin, paraprothrombin), and characterizes the third phase of blood clotting (fibrin formation), as well as the state of natural and pathological anticoagulants. aPTT — one of the most valuable general tests for characterising the blood coagulation system, which detects purely plasma defects of the intrinsic activation system of factor X in the first phase of blood clotting (prothrombinase formation) [33, 34].

It was shown that fullerene C_{60} did not possess the ability to modify the coagulation time of human blood plasma in PTT and APTT tests (Fig. 4). At the same time, prolongation of coagulation time was observed under the influence of calixarene C-145, which may

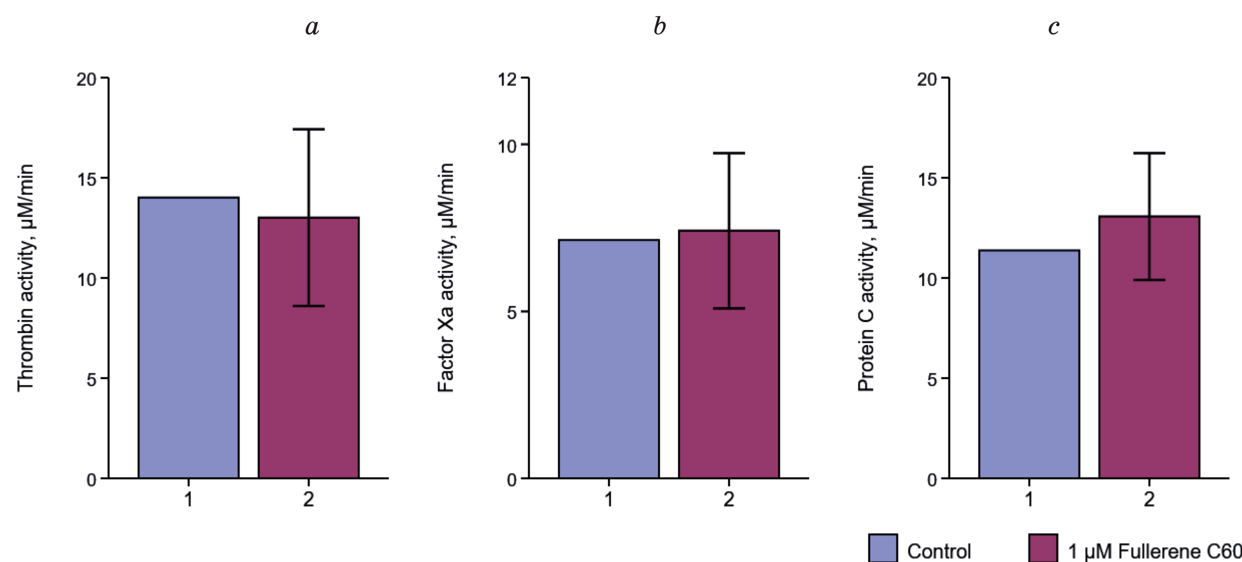

Fig. 3. **Thrombin activity (*a*), factor Xa (*b*), and protein C (*c*) in the absence (*1*) and presence of 1 μM (*2*) C_{60}**

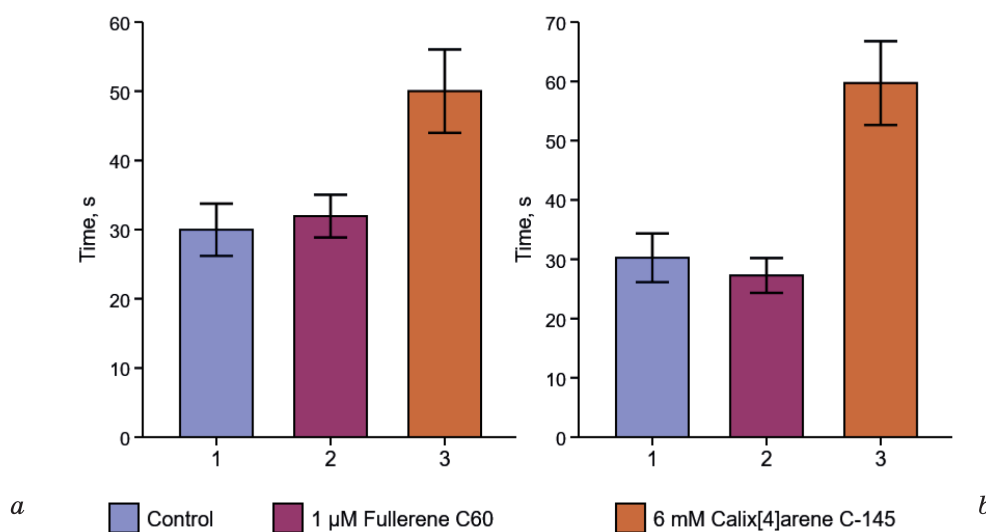

Fig. 4. **Time of clot formation in thrombin time tests (*a*) and activated partial thromboplastin time (*b*) in the presence of C_{60} fullerene (3 μM) and calix[4]arene C-145 (6 mM)**

indicate hypocoagulation in the presence of this substance.

We have also studied one of the important processes involved in fibril formation, namely, covalent stitching (stabilization). The stabilization of fibrils in the presence of Ca^{2+} ions is carried out by factor XIIIa through covalent stitching of the γ- and α-chains of neighboring molecules.

Factor XIII (fibrin-stabilizing factor, fibrinase) belongs to the β2-glycoproteins, is present in the vascular wall, platelets, erythrocytes, kidneys, lungs, muscles, and placenta, and characterizes the third phase of blood coagulation (fibrin formation). When the vessel wall is damaged, Factor XIII participates in the process of platelet aggregation and adhesion; in plasma, it exists as a proenzyme bound to fibrinogen, which, under the influence of thrombin, is converted into the active form XIIIa. During fibrin clot formation, it ensures the formation of cross-linked fibrin. Clots formed in the presence of fibrinase are very slowly lysed. However, when its activity decreases, the clots rapidly break down, even if the fibrinolytic activity of the blood is normal. A decrease or increase in fibrinase activity is considered a risk factor for hemorrhagic or thrombotic events [33]. Our studies showed that the rate of covalent cross-linking of fibrin γ-chains did not depend on the presence of C_{60} fullerene (Fig. 4), meaning that the activity of factor XIIIa remained unchanged and the final stage of forming a stable clot was not affected.

To examine how fullerene C_{60} affects the anticoagulant system, we measured the activation of a key physiological anticoagulant, protein C, in blood plasma by detecting its activation product. Protein C is a vitamin K-dependent glycoprotein, synthesized by the liver in the form of an inactive precursor, which is converted into an active form under the influence of the thrombin-thrombomodulin complex. Activated protein C is an enzyme that selectively inactivates factors Va and VIIIa by hydrolysis in the presence of Ca^{2+}, phospholipids, and its cofactor, protein S, thereby preventing the conversion of prothrombin to thrombin. A deficiency of protein C, which can be quantitative (Type I, characterized by low protein concentration) or qualitative (Type II, in which the protein is present but inactive or less active), is associated with a high risk of thrombosis, especially venous thrombosis and pulmonary embolism in young people, and thromboembolic diseases in the elderly. An increased activity of this indicator generally has no diagnostic significance [31]. As shown in Fig. 3, *c*, the fullerene C_{60} did not affect the activation of protein C, indicating that the functional activity of this component of the anticoagulant system remained unchanged.

To assess the direct effect of fullerene C_{60} on the formation and lysis of a fibrin clot, we determined the overall hemostatic potential of the serum, which is a modern approach for defining the delicate balance between the blood coagulation and fibrinolysis systems. The method is based on analyzing the curve of light absorption by the clot, reflecting the processes of formation and its destruction in blood plasma in the presence of thrombin (which activates the blood coagulation system) and tissue plasminogen activator (t-PA – converts plasminogen into plasmin, which causes the breakdown of the fibrin clot). As a result, the area under the light absorption curve (measured in optical units per second) varies

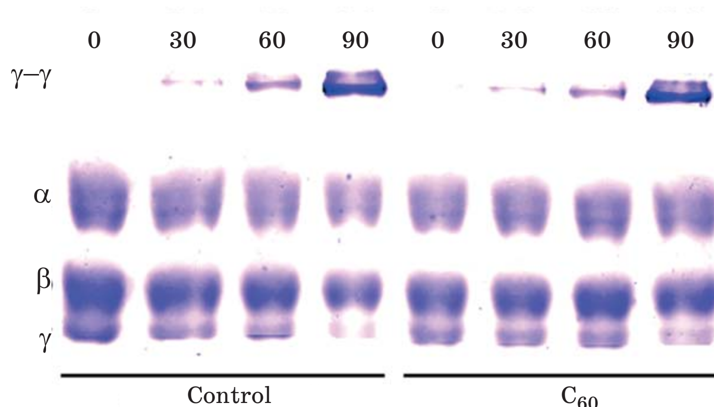

Fig. 5. **Electrophoretogram of fibrin preparations (0), prepared after 30, 60, and 90 minutes following fibrin formation in the presence of C_{60} fullerene and in its absence (Control)**

depending on the concentration of coagulation factors, anticoagulants, and fibrinolytic components [35, 36].

It has been shown that fullerene C_{60} does not affect the formation of a fibrin clot and does not significantly modify the process of its breakdown (Fig. 6), indicating the absence of disturbances in the coagulation balance.

Thus, fullerene C_{60} is a potential bioinert carrier that can contact blood at therapeutic doses without inhibitory or activating effects on hemostasis.

Discussing the results presents certain difficulties, as the literature on the biological safety of fullerenes and their derivatives is quite contradictory. This is due to differences in the design and conditions of the conducted experiments — the type of biological object, concentration, method of administration, storage conditions, whether the experiments were *in vitro* or *in vivo*, the duration of observation; as well as the interpretation of results, which can be difficult to compare due to the presence of incompatible and hard-to-control factors, such as particle size in fullerene dispersions, types of carriers used (surfactants, plant oils, polysaccharides, solvents with their own toxic effects) [37].

Unlike the inertia of fullerene C_{60} registered in our studies, research using both experimental and computational methods dedicated to the mechanisms of modulation of thrombin activity by nine types of C_{60} nanoparticles with different chemical properties has shown that nanoparticles with high hydrophobicity and acidity of the hydrogen bond donor or basicity of the acceptor act as competitive inhibitors of thrombin, that is, they have an anticoagulant effect [17]. Other studies involving prolonged cultivation (7–50 days) of water-soluble fullerene (C_{60} (OH)24) with platelets have shown that C_{60} (OH)24 itself does not induce platelet aggregation but promotes ADP-induced aggregation and acts as a competitive inhibitor of ADP receptor antagonists. The authors conclude that the impact of nanomaterials may increase the risk of atherothrombotic diseases, as nanomaterials entering the bloodstream can cause vascular damage, thereby contributing to atherosclerosis [38]. Such differences in research results may be explained by the fact that fullerenes in water are capable of forming colloidal solutions, in which individual molecules aggregate into quite large, negatively charged hydrated clusters, whose size influences their biological activity, which is associated with the special properties of water-based spherical shells of the compounds [1, 37]. The effect of water-soluble derivatives of fullerenes, in which substituents are located all over the surface of the fullerene cage, specifically polyhydroxylated derivatives of fullerene C_{60} — fullerenols, on the hemostasis system was studied. Small fullerenols (small size fullerenol — average diameter 1.13±0.32 nm, with a negative surface charge) influenced both the intrinsic and extrinsic coagulation pathways — prolongation of activated partial thromboplastin time and prothrombin time was observed. Inhibition of the activities of activated factor X (Xa) and thrombin was confirmed in vitro using AutoDock Vina (an open-source molecular

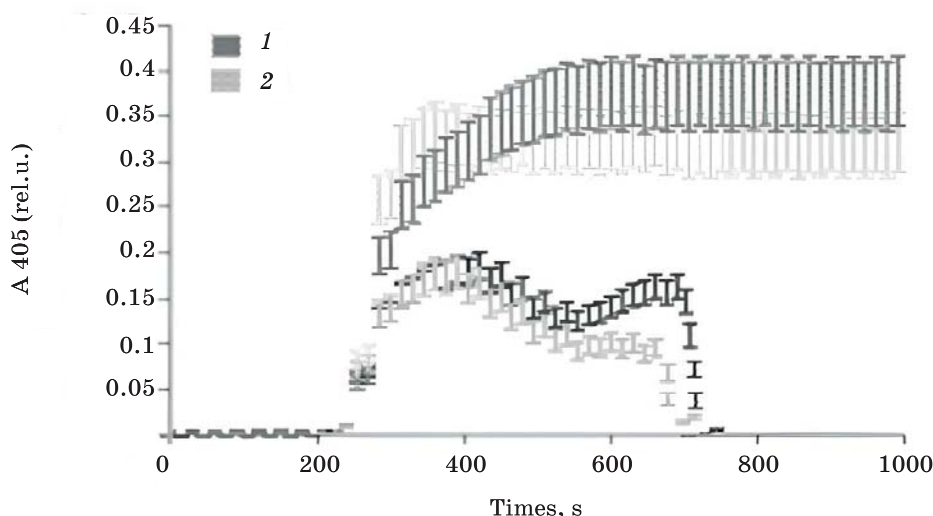

Fig. 6. **Overall haemostatic potential of blood plasma (*1*) in the presence of 1 μM fullerene C_{60} (*2*)**

docking program). The results indicate that fullerenols occupied the active sites of Xa and thrombin, thereby blocking their activity [39].

It is known that platelets have a double phospholipid membrane with receptor glycoproteins that interact with adhesion and aggregation stimulators [40]. A fullerene molecule is characterized by high lipophilicity, which determines its ability to interact with biological membranes, affecting their structure and mechanical properties and altering the catalytic activity of membrane enzymes [37]. Perhaps this ability is related to the decrease in platelet aggregation we observed at a high C_{60} dose (10 μM).

There is also data on the beneficial effect of carbon-based nanoparticles on the platelet component of hemostasis. Studies examining the effects of various nanoparticles of different origins (functionalized chitosan, anionic dendrimers, metallic and non-metallic nanoparticles) have shown that single-walled carbon nanotubes, functionalized with polymeric nanoparticles, promote platelet survival and improve platelet function [41].

The data we obtained regarding the absence of the effect of fullerene C_{60} are indirectly confirmed by the results of *in vivo* studies by other authors. Thus, intraperitoneal administration of C_{60} suspension (2.5 g/kg) to intact mice did not cause death or behavioral disturbances over 8 weeks [42]. In rats that received a high-toxic dose of cyclophosphamide (200 mg/kg, single intraperitoneal injection), therapy with C_{60} (4 mg/kg, orally daily for 10 days) resulted in the normalization of platelet, leucocyte, and erythrocyte counts, improvement in hemoglobin and hematocrit levels, as well as a hepatoprotective effect [43]. Intravenous or intrarectal administration of a colloidal C_{60} solution (0.5 mg/kg) to rats that had undergone acute colitis showed the restoration of the mucous membrane of the large intestine, the integrity of the epithelial barrier, an increase in antioxidant protection (glutathione peroxidase activity), a reduction in signs of anaemia, and an increase in the number of platelets [44]. The mentioned studies indicate a positive effect of fullerene C_{60} in simulated pathologies and the absence of its negative impact at the used doses on a living organism.

Conclusions

1. At therapeutic doses of 0.1 μM and 1.0 μM, fullerene C_{60} shows no impact on markers of platelet function, coagulation, anticoagulant activity, or fibrinolysis, indicating it can be regarded as a bioinert carrier.
2. Fullerene C_{60} does not alter the functional activity, shape, or granularity of human platelets during co-culture.
3. Fullerene C_{60} does not affect PTT, aPTT, thrombin activity, factor Xa levels, the stabilization of the covalent fibrin network, or protein C activity in plasma from nearly healthy donors.

Author Contributions

V.O. Chernyshenko — conceptualization, methodology, validation, formal analysis, investigation, data curation, writing — original draft preparation, writing — review and editing, visualization; O.P. Matyshevska — writing — review and editing, data curation; Yu.D. Vinnichuk — writing — review and editing; A.Yu. Labyntsev — writing— review and editing; O.E. Lugovska — formal analysis. All authors contributed to the manuscript's revision and read and approved the submitted version.

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Conflict of Interest

The authors declare no conflict of interest.

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БІОСУМІСНІСТЬ ФУЛЕРЕНУ C_{60} З СИСТЕМОЮ ГЕМОСТАЗУ

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Молекулярні сполуки, що належать до алотропних форм вуглецю, інтенсивно досліджуються у різних галузях науки, зокрема нанобіотехнології, біомедицині, фармакології, що відкриває перспективи для їх практичного застосування. Найбільш відомим з вуглецевих наночасток із замкнутою сферичною структурою є фулерен C_{60}, який має унікальні фізико-хімічні та біологічні властивості (стабільність, біосумісність, а також антиоксидантні, протипухлинні, фотосенсибілізаційні властивості). Проте розуміння механізмів впливу цих часток на регуляторні/інтегральні системи організму необхідне для виключення потенційної небезпеки для здоров'я при їх клінічному застосуванні.

Мета. З'ясувати вплив фулерену C_{60} на систему кровообігу, зокрема на гемостаз, з метою розуміння безпеки його використання в біомедичних галузях.

Матеріали й методи. Досліджені тромбоцитарна, коагуляційна, антикоугаляційна та фібринолітична ланки гемостазу.

Результати. Показано, що фулерен C_{60} у терапевтичних дозах 0,1 мкМ та 1,0 мкМ не впливає на функціональну активність, форму, а також гранулярність тромбоцитів людини при сумісному культивуванні. У його присутності не змінюються показники ТЧ, АЧТЧ, активність тромбіну, фактору Ха, протеїну С плазми крові, а також не зазнає впливу загальний гемостатичний потенціал плазми крові.

Висновки. Фулерен C_{60} може розглядатися для клінічного застосування як потенційний біоінертний носій, здатний контактувати з кров'ю у терапевтичних дозах без інгібіторного чи активаційного ефекту на систему гемостазу.

Ключові слова: фулерен C_{60}, гемостаз, тромбоцити, біосумісність.

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